Supporting Information

Self-assembly of DNA Nanogels with Endogenous MicroRNA Toehold Self-regulating Switches for Targeted Gene Regulation Therapy

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Experimental Procedures

Materials: All oligonucleotides were synthesized and HPLC-purified by Sangon Biotechnology Co., Ltd. (Shanghai, China), and the sequences are listed in Table S1. Cetyltrimethylammonium chloride (CTAC) (25 wt % in H2O); triethanolamine (TEA); Tetraethyl orthosilicate (TEOS); 1-octadecene ammonium nitrate (NH4NO3); ethanol solution; Acrylamide, 3-(trimethoxysilyl) propyl methacrylate (TMSPMA); ammonium persulfate (APS) and N, N, N, N- tetramethylethylenediamine (TEMED) were all bought from Shanghai Macklin Biochemical Co.,Ltd. Tris-EDTA buffer solution (TE, pH 8.0). Gemcitabine hydrochloride (GEM) was purchased from Sigma Finland. Solutions involved in microRNA-related experiments was prepared with DEPC-treated water (RNase-free) (Sigma, Switzerland) to minimize the degradation of microRNAs.

Synthesis MSN and MSN-TMSPMA: MSN was synthesized as previously reported.[1] briefly, 24 ml of (25 wt %) CTAC solution and 0.18 g of TEA was added to 36 ml of water and stirred gently at 60 °C for 1 h in a 100-mL round bottom flask, then 20 mL of (20 v/v %) TEOS in 1-octadecene was carefully added to the water-CTAC-TEA solution and kept at 60 °C in an oil bath under a magnetic stirring. A standard Teflon-coated stirring bar with a length of 2 cm was employed, and the stirring rate was set to be ~ 150 rpm. The reaction was then kept at a constant temperature with continuous stirring for 24 h. Then, the upper phase, 1-octadecene solution was completely removed and the products was collected by

centrifugation and washed for several times with ethanol to remove the residual reactants. Then, the collected products were extracted with a 0.6 wt % ammonium nitrate (NH_4NO_3) ethanol solution at 60.0 °C for 6 h twice to remove the template. TMSPMA modified MSN (MSN-TMSPMA) was obtained by stirring 6 mL TMSPMA with 0.2 g MSNs in 50 mL methanol for 5 hours. Then the MSN-TMSPMA was centrifuged and washed several times with methanol.

Preparation of GEM loaded MSN: 40mg GEM and 20mg MSN-TMSPMA was dissolved in 6 mL of water, after stirring at 10ml beaker overnight, the GEM loaded MSN (GEM@MSN) was obtained after two rounds of centrifugation at 16000 rpm and washed with water.

Preparing DNA hydrogel: DNA hydrogel was synthesized as previously reported.[2] Two oligonucleotides modified with acrydite (strand-A, and strand-B) at the 5′-end were separately dissolved in 50 μL Tris-EDTA buffer solution (TE, pH 8.0) containing 4% acrylamide and pretreated in vacuum desiccator as stock solutions (5 mM). The oligonucleotide stock solutions were then placed in the vacuum oven at 37 ℃ for 5 min. 0.01 g ammonium persulfate APS and 5 μL TEMED were separately added in 100 μL deionization water to prepared fresh APS solution and TEMED solution. 5 μL APS solution and 10 μL TEMED solution were added to both stock solutions and then placed in the water bath at 37 ℃ for 10 min to initiate the polymerization. Polymer strands A and B (P-A and P-B) were mixed with 3 μL DNA adhesive (100 mM). The mixed solution was incubated at 70 ℃ for 15 min and then cooled down to 37 ℃. After hybridization, the sequences were cross-linked to form the DNA hydrogel. The bulk DNA hydrogel was prepared and used to test the sensitivity of miRNA-21. We first grafted strand A and strand B to PAA. Subsequently, adhesion strands were added to facilitate the growth of hydrogel in situ under 70℃ water bath, and the total volume is 100uL. For the hydrogel miRNA sensitivity experiment, 20uL miRNA-21 strands were added to the hydrogel, and after strongly shaking for 48 hours, we found that the hydrogel was gradually degraded.

Immobilization of hybrid DNA hydrogel on the surface of Gem-loaded MSN (GEM@MSN@DNA): Different concentrations of two oligonucleotides modified with acrydite (strand-A, and strand- B) at the 5′-end were separately dissolved into 50 μL Tris-EDTA buffer solution (TE, pH 8.0) containing 4% acrylamide and pretreated in vacuum desiccator as stock solutions (10 μM, 50 μM, 100 μM, 150 μM, 200 μM, 250 μM for each strand). The oligonucleotide stock solutions were then placed in the vacuum oven at 37 ℃ for 5 min. Then, 200 μg MSN was dispersed by using 50 μL PBS and the medium prepared upper mentioned. 0.01 g ammonium persulfate APS and 5 μL TEMED were separately added in 100 μL deionization water to prepared fresh APS solution and TEMED solution. 5 μL APS solution and 10 μL TEMED solution were added to both stock solutions and then placed in the vacuum oven at 37 ℃ for 10 min to initiate the polymerization. DNA adhesive strand were mixed with previous solution separately to achieve the final concentration at 200 μM. The mixed solution was incubated at 60 ℃ for 15 min and then cooled down to 37 ℃. After hybridization, the sequences were cross-linked to form the DNA hydrogel on the surface of NPs.

Native polyacrylamide gel electrophoresis (PAGE): The prepared different samples at 37 °C were loaded into 20% native PAGE and run at a constant voltage of 100V for 1.5 h in 1×TBE buffer. The Ultra-Low Range DNA Ladder (range from 10 bp to 300 bp) was purchased from ThermoFisher. After staining with GelRed or DAPI for 5 min, photos were obtained on a GelDoc Go Gel Imaging System (Bio-Rad) with an excitation wavelength of 365 nm.

Characterization of the nanoparticles: Particle size of MSN-TMSPMA; DNA; GEM@MSN; GEM@MSN@DNA NPs were performed using dynamic light scattering with Zetasizer Nano ZS. For each measurement, the sample (1.0 mL) was put in a disposable polystyrene cuvette. The nanocarrier surface ζ-potential was measured with Zetasizer Nano ZS by using disposable folded capillary cells. Both the size and ζ-potential were recorded as the average of three measurements. The TEM samples were

prepared by using a tweezer to hold the carbon-coated copper grids and soak it in the particle solution, then remove it and dry it in the air prior to imaging.

Gem•Hcl Loading (Gem@MSN@DNA): 10mg of Gem•Hcl and 10mg MSN nanoparticles were blended within 20 mL Milli-Q water and stirred vigorously on a magnetic stirrer. After 24 h of drug loading, the Gem@MSN NPs were centrifuged at 10000 g and washed with water three times. The Gem loading degree (LD) and loading efficiency (LE) were calculated according to the following Equations (1) and (2):

$$LD(\%) = \frac{entrapped\ drug}{weight\ of\ nanoparticles\ +\ entrapped\ drug} \times 100\% \quad (1)$$

$$LE\ (\%) = \frac{total\ input\ of\ drug - amount\ of\ drug\ in\ the\ supernatant}{total\ input\ of\ drug} \quad (2)$$

In vitro drug release: MicroRNA miR-21 solutions of different concentrations (0, 5, 10 μM) was prepared by diluting microRNA stock solution (100 μM) with DEPC-treated water. GEM@MSN@DNA was dispersed in 1.0 mL of miR-21 solutions and then, it was kept in solutions and gently shaken at 37 °C. At selected time intervals, the solution was centrifuged at 16000rpm, then the 0.9ml supernatant was withdrawn and analyzed by fluorescence spectrum, and 0.9ml fresh medium was returned to the original solution, after sonicated until dispersed well then put it back into the shaker. For DNA releasing experiment, DNA was labeled with Cy3 fluorescence and observed in the UV spectrometer at the maximum absorption peak of 553nm. DNA adhesion chain release experiments were conducted with nanoparticles at different concentrations of miRNA-21. At different time points, we centrifuged the nanoparticles to the bottom, while we measured the fluorescence intensity of Cy3 in the supernatant at each specific time point. The release volume of the nanoparticles was 1ml, and 900 microliters of the supernatant were removed after each centrifugation and fresh release medium was added again.

Cell culture and maintenance: Pancreatic cancer cell line PANC-1 and pancreatic islet B epithelial Min6 cells was grown in RMPI 1640 medium with 10% FBS and 5 μg/mL insulin at 37 °C. Cells was passaged 2–3 times a week once they reached 90–100% confluency. Human pancreatic cancer cell line PANC-1 cells and pancreatic islet B epithelial Min6 cells were purchased from Beijing Baiou Bowei Biotechnology Co., Ltd.

Cellular uptake study: Cells were incubated in 6 well plates (1×10^5 cells per well) overnight. When the cells are attached, solutions of FITC@MSN@DNA NPs were utilized to replace the cell growth media. All the group were keeping the concentration of FITC@MSN@DNA NPs at 2 μg/mL at 37 °C. After incubated for 8 h and 16h, cells were collected by trypsin and washed with PBS. The acquisition of cellular uptake was determined by a flow cytometer BD LSRFortessa (BD Biosciences) with FITC channel, the results were analyzed by Flowjo_V10 The gate was defined for live cells only; 10000 cells were recorded per sample.

Fluorescence in situ hybridization (FISH) detection: The fluorescence in situ hybridization (FISH) kit was purchased from Guangzhou exons biological technology co., LTD. The probe was diluted with hybridization buffer and denatured at 85℃ for 2 minutes. Then the probe was used for hybridization with 4% paraformaldehyde fixed cells for 72 h. The cells were labeled with DAPI (Amax = 358, Emax = 461). The florescence of probe was FITC green light (Amax = 496, Emax = 524)

Cytotoxicity assay: The drug efficacy in cancerous and healthy cells was determined by a WST-1 cell viability assay. PANC-1 cancer cells and Min6 cells were incubated overnight in a 96-well-plate (3000 cells per well) in cell growth media at 37 °C with 5% CO_2. The following day, the cell growth media was replaced with fresh media containing a different concentration of MSN, GEM, GEM@MSN, MSN@DNA, Gem@MSN@DNA NPs and incubated for 48 h. Free Gem stock solutions was prepared in DMSO and nanoparticles GEM@MSN@DNA was suspended in medium. All the dilutions for the cell

viability assay were prepared in cell growth media. After incubated with free drug or nanoparticles, 10 μL of WST-1 reagent was added to each well and the cells was incubated for 2 h at 37 °C with 5% CO_2. After incubation, the absorbance was measured by a Varioskan Flash Multimode Reader (Thermo Scientific Inc., Waltham, MA, USA) at 440 nm.

Calcein/PI Live/Dead Cell Assay: The cells were seeded in a cell culture dish for 24h. The cell death staining experiments were divided into five groups, including MSN, Gem, Gem@MSN, MSN@DNA and Gem@MSN@DNA groups. The concentration of MSN for each group was 20 μg/mL. After culturing for 24 hours, the culture medium was aspirated, the cells were washed with PBS once. 250μL Calcein AM/PI detection working solution was added to each well and incubated for 30 mins at 37°C in the dark. After the incubation, the staining effect was observed under a fluorescence microscope. (Calcein AM is green fluorescence, Ex/Em=494/517nm; PI is red fluorescence, Ex/Em=535/617nm)

Annexin V-FITC cell apoptosis detection: The cells were digested by trypsin solution and aspirated until the adherent cells can be blown down by gentle pipetting. Then the cells were transferred into a centrifuge tube, centrifuged at 1000g for 5 minutes and the supernatant was discarded. Subsequently, the cells were gently resuspended with PBS and counted. 50,000-100,000 resuspended cells were taken and centrifuged at 1000g for 5 minutes. 195 μL Annexin V-FITC binding solution was added to gently resuspend the cells. 10 μL propidium iodide staining solution Annexin V-FITC was then add and mixed gently. After incubated for 10-20 minutes at room temperature (20-25°C) in the dark, the cell then placed in an ice bath. Cell apoptosis was determined by a flow cytometer BD LSRFortessa (BD Biosciences), the results were analyzed by Flowjo_V10; 10000 cells were recorded per sample.

Western blot assay: After the cells were incubated with different groups of nanomaterials for 48 hours, the cells were lysed with RIPA lysis buffer, and centrifuged at 12000 rpm for 10 minutes, and the supernatant was taken for quantification; cell proteins were separated by SDS-PAGE gradient gel and

transferred to PVDF membrane. After adding protein loading buffer, mix well and fully denature the protein sample at 98°C for 10 minutes. Incubate the membrane with primary antibodies against PTEN and PDCD4 (Abcam, Cambridge, UK) (1:1000) at 4°C overnight. Then incubate the membrane with the secondary antibody at 37°C for 1 hour. Use GelDoc Go Gel Imaging System (Bio-RAD) to evaluate protein bands. Software ImageJ was used for fluorescence quantitative analysis

Localization of NPs by confocal microscopy: The localization of FITC@MSN@DNA NPs in PANC-1 and Min6 cells were determined by confocal microscopy. Cells were grown on Confocal Dishes (15 ×104 cells per Dish, VWR® 35 mm Confocal Dish, Sterile) overnight. After 1 h, 8 h and 16h, the cells were washed twice with PBS, fixed with 4% PFA for 5 minutes, and then the samples were stained with DAPI for 10 minutes. Images were made by using a confocal microscope (Plan-Apochromat 100×/1.40 Oil DIC), an oil lens, and a Zen 2010 software setting.

Dynamic Study on the Unwinding Process of Nanoparticles in Cells: MSN@DNA NPs were first stained with DAPI, and after centrifugation and washing, DAPI-labeled MSN@DNA NPs were obtained for endocytosis experiments in PANC-1 and Min6 cells through confocal microscopy. Confocal fluorescence images were analyzed with software Image J[3].

Statistics analysis: The Student t-test unpair was employed for comparison between two groups. $P < 0.05$ was considered statistically significant.

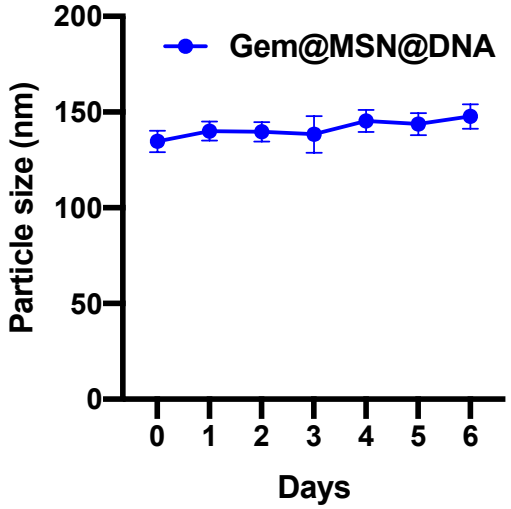

Figure S1. The stability of Gem@MSN@DNA NPs under PBS buffer for one week.

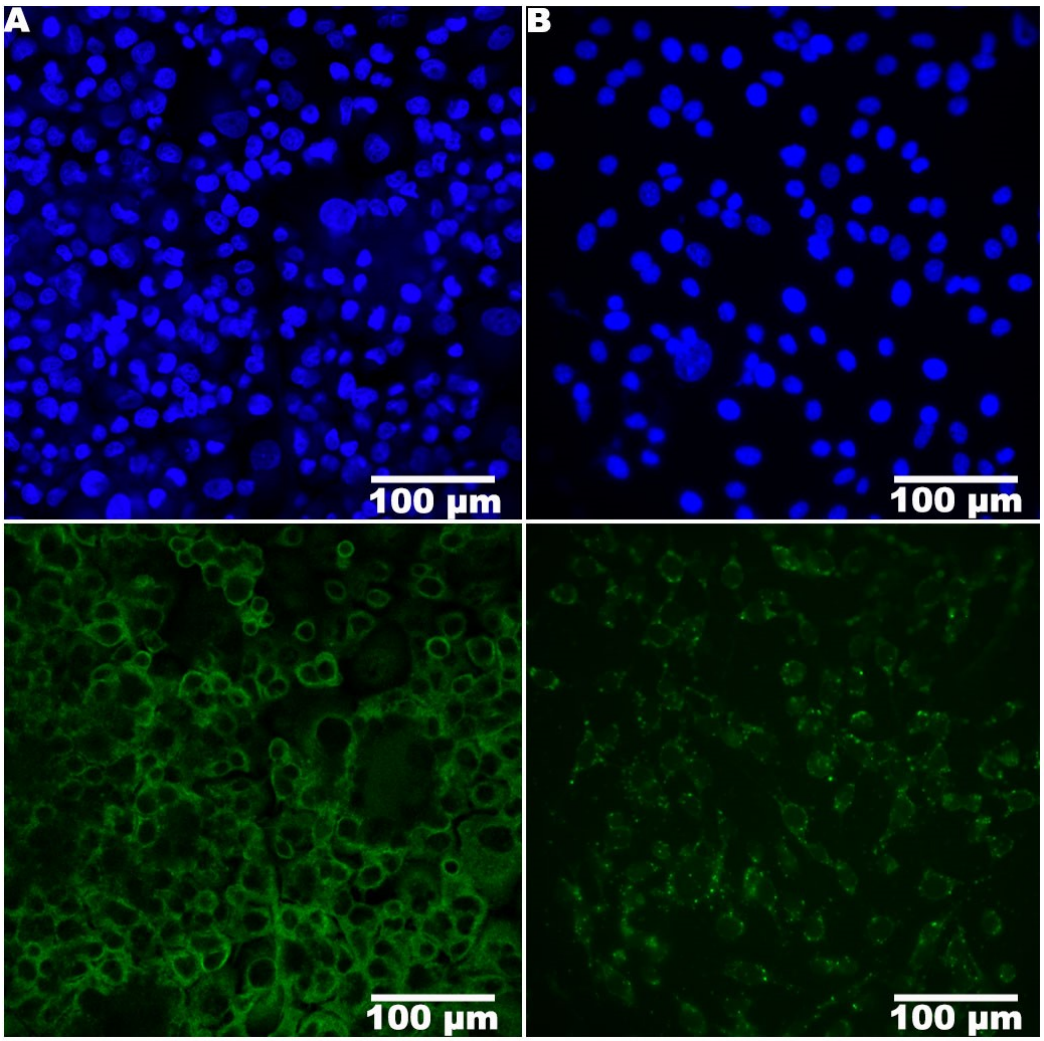

Figure S2. The content of miR-21 in different cells. A.PANC-1 cells, B min6 cells.

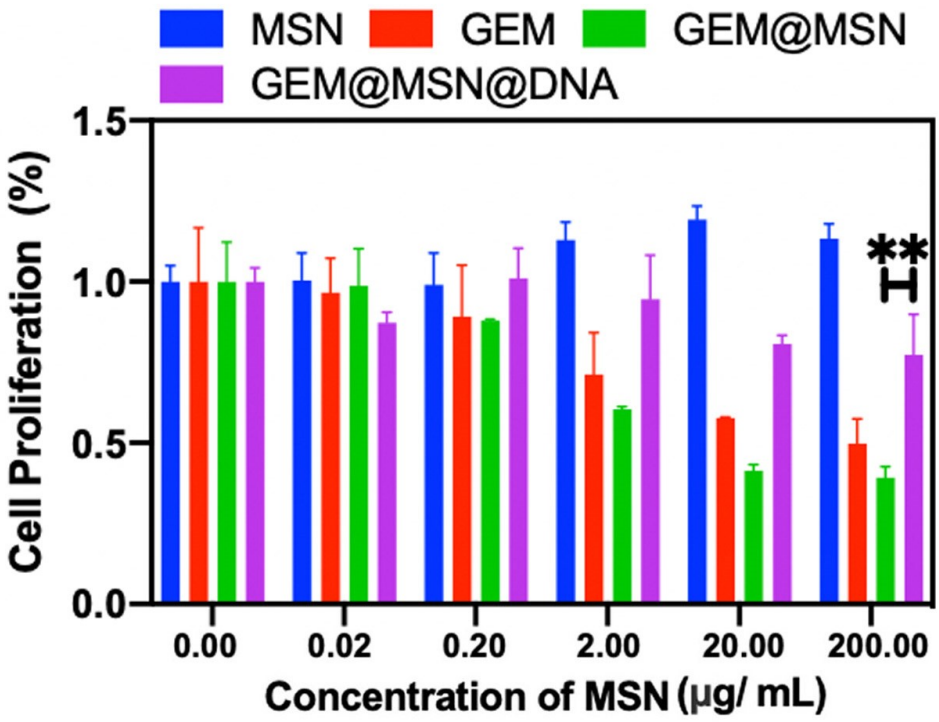

Figure S3. Toxicity evaluation of drugs and DNA hydrogel based NPs for Min6 cells.

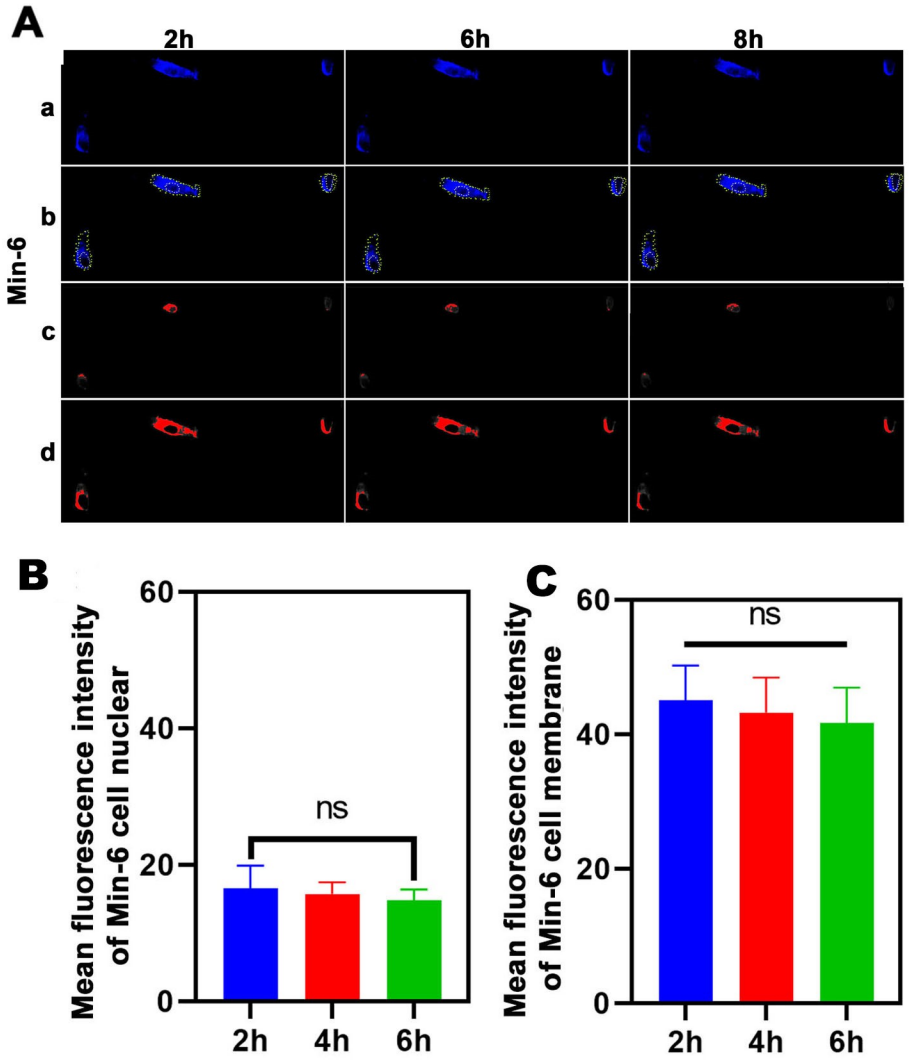

Figure S4. Quantitative analysis of intracellular DAPI fluorescence intensity with software Image J.

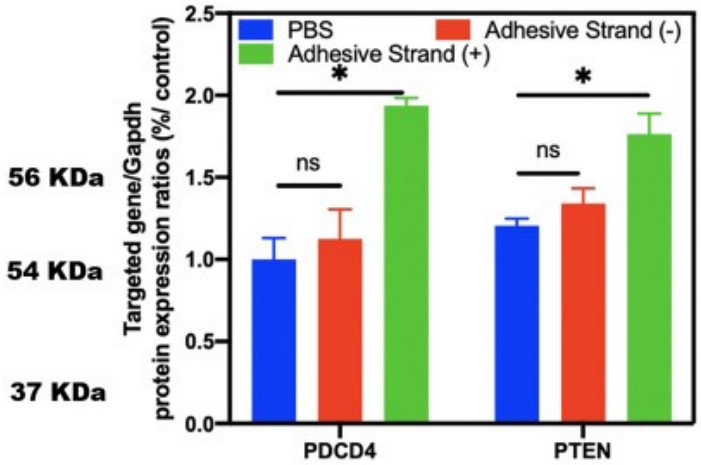

Figure S5. Quantitative analysis of the expression of PTEN and PDCD4 protein

Table S1. Sequences (5'-3') of DNA and microRNA used in this work. "*" means phosphorothioate

Strand-A	acrydite-AAAATGTTGATATAT
Strand-B	acrydite-AAATATCAGACTGA
DNA adhesive	A*T*A*T*A*T*C*A*A*C*A*T*C*A*G*T*C*T*G*A*T*A*A*G*C*T*A
5`Cy3 labeled DNA adhesive	Cy3-ATATATCAACATCAGTCTGATAAGCTA
MiR-21	UAGCUUAUCAGACUGAUGUUGA

Table S2. Drug loading content and encapsulation efficiency of nanoparticles.

	Loading Capacity	Loading Efficiency
Gem	22.95 ± 6.3 %	67.5 ± 8.21