

## Supporting Information

### **Polygemcitabine nanogel with accelerated drug activation for cancer therapy**

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## MATERIALS AND METHODS

**Materials.** Gemcitabine (**Ge**), monophosphate (dFdCMP), diphosphate (dFdCDP), and triphosphate (dFdCTP) derivatives of gemcitabine were purchased from Wuhu Huaren Science and Technology Co., Ltd and used as received. Chlorotrimethylsilane, benzoyl chloride, 4-dimethylaminopyridine, triethylamine, 4,4'-dimethoxytritylchloride, diisopropylethylamine, 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite were purchased from Sigma-Aldrich. Solvents, including acetonitrile (MeCN), dichloromethane (DCM), pyridine (Py), 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. 6-(3',6'-dipivaloylfluoresceinyl-6-carboxamido)-hexyl-1-O-(2-cyanoethyl)-(N,N-diisopropyl)-phosphoramidite (5'-Fluorescein Phosphoramidite), and 1-[3-(4-monomethoxytrityloxy)propyl]-1'-[3-[(2-cyanoethyl)-(N,N-diisopropyl)phosphoramidityl]propyl]-3,3,3',3'-tetramethyl-4,5-benzindodicarbocyanine iodide (Cyanine 5.5 Phosphoramidite) were purchased from Glen research, Sterling, VA. Roswell Park Memorial Institute (RPMI)-1640 medium and fetal bovine serum (FBS) were purchased from PAA Laboratories GmbH. Alexa fluor<sup>®</sup> 488 Annexin V/dead cell apoptosis assay kit was 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. Gemcitabine is denoted as **Ge** in the sequences.

**Table S1.** The detailed information of DNA sequences

Name	Detailed sequence information
ss-Ge <sub>10</sub>	5'-GeGeGe GeGeGe GeGeGe Ge-3'
FAM-Ge <sub>10</sub>	5'-FAM-GeGeGe GeGeGe GeGeGe Ge-3'
Cy5.5-Ge <sub>10</sub>	5'-Cy5.5-GeGeGe GeGeGe GeGeGe Ge-3'

## Measurements.

**Nuclear magnetic resonance (NMR).**  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were recorded on Bruker AVANCEIII 400 spectrometer with dimethylsulfoxide- $d_6$  (DMSO- $d_6$ ) as a solvent ( $^1\text{H}$  at 400 MHz,  $^{13}\text{C}$  at 100 MHz).  $^{31}\text{P}$  NMR spectroscopy was recorded on Bruker AVANCE NEO at 700MHz using DMSO- $d_6$  as a solvent.

**Mass spectra (MS).** Mass spectra of  $\text{Ge}_{10}$  was obtained using Thermo Finnigan LCQ Deca-plus ion-trap mass spectrometer through electron spray ionization (Thermo Electron, San Jose, USA).

**Ultraviolet-visible absorption (UV-vis).** The UV-vis absorption of the sample solution 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<sup>-1</sup>.

**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

### Preparation of the 5'-O-DMT-N<sub>4</sub>-benzoyl-2',2'-difluoro-2'-deoxycytidine 3'-CE phosphoramidite (Ge phosphoramidite)

Ge phosphoramidite was synthesized according to the literature<sup>1</sup> and the synthetic route was shown in Figure S2. Briefly, gemcitabine (**1**) and chlorotrimethylsilane were mixed in anhydrous pyridine at 0 °C, and then reacted with benzoyl chloride to get N<sub>4</sub>-benzoyl-2'-deoxy-2',2'-difluorocytidine (**2**). Thereafter, the obtained product (**2**) was dissolved in anhydrous pyridine and reacted with 4-dimethylaminopyridine,

triethylamine, and 4,4'-dimethoxytritylchloride for overnight at ambient temperature to get 5'-O-(4,4'-dimethoxytrityl)-N<sub>4</sub>-benzoyl-2'-deoxy-2',2'-difluorocytidine (**3**). Finally, compound **3**, diisopropylethylamine, and 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite was dissolved in anhydrous tetrahydrofuran and reacted for 3 h to produce **Ge** phosphoramidite (**4**), which was further purified by silica gel flash column chromatography.

### **Synthesis of Ge<sub>10</sub> single strand**

**Ge<sub>10</sub>** single strand was synthesized on an ABI 3400 DNA/RNA synthesizer (Applied Biosystems, Foster City, CA, USA) using a default coupling protocol. The obtained oligonucleotides were purified by reverse-phase high performance liquid chromatography (HPLC) using a Varian Microsorb C18 column (10  $\mu$ m, 300  $\times$  10 mm).

### **Formation of Ge<sub>10</sub> nanogels, FAM-labeled Ge<sub>10</sub> nanogels, and Cy5.5-labeled Ge<sub>10</sub> nanogels**

2  $\mu$ M **Ge<sub>10</sub>** single strands were added to the Eppendorf (EP) tubes with a buffer solution containing 1  $\times$  tris-acetic-EDTA-Mg<sup>2+</sup> (TAE/Mg<sup>2+</sup>) buffer. Subsequently, the solution was slowly cooled from 95  $^{\circ}$ C to room temperature over 24 h to form the desired **Ge<sub>10</sub>** nanogels. Besides, 2  $\mu$ M FAM-labeled **Ge<sub>10</sub>** single strands were slowly cooled from 70  $^{\circ}$ C to room temperature over 24 h to obtain FAM-labeled **Ge<sub>10</sub>** nanogels. Similarly, Cy5.5-labeled **Ge<sub>10</sub>** single strands follow the same procedure to prepare Cy5.5-labeled **Ge<sub>10</sub>** nanogels.

In principle, the self-assembly of **Ge<sub>10</sub>** nanogel is controlled by the free-energy change ( $\Delta G$ ), which is determined by the following equation:  $\Delta G = \Delta H - T\Delta S$ . The growth of nanogel is majorly driven by the decrease of enthalpy (negative  $\Delta H$ ) due to the formation of multitude of hydrogen bonds and  $\pi$ - $\pi$  stacking. However, the growth of nanogel will also result in the decrease of entropy (negative  $\Delta S$ ) since more ordered structures are formed. Therefore, the growth of **Ge<sub>10</sub>** nanogel will end up at an appropriate size to reach the thermodynamic equilibrium.

In cellular experiments, the final concentration of **Ge<sub>10</sub>** nanogel was switched to 4

$\mu\text{M}$  to obtain higher fluorescence signals in cellular uptake studies, such as flow cytometry and confocal laser scanning microscopy experiments. Briefly,  $2\ \mu\text{M}\ \text{Ge}_{10}$  nanogel was prepared by annealing ss- $\text{Ge}_{10}$  from  $95\ ^\circ\text{C}$  to room temperature. Thereafter,  $2\ \mu\text{M}\ \text{Ge}_{10}$  nanogel was concentrated using an Amicon Ultra-15 ultrafiltration device (Millipore, MWCO 3 kDa) to the final concentration of  $4\ \mu\text{M}$ . To demonstrate that the hydrodynamic diameter of  $\text{Ge}_{10}$  nanogel at  $4\ \mu\text{M}$  was similar to that assembled at  $2\ \mu\text{M}$ , DLS and AFM were performed as well. As shown in Figure S9, the average hydrodynamic diameter ( $D_h$ ) of  $\text{Ge}_{10}$  nanogel at  $4\ \mu\text{M}$  is approximate  $53\ \text{nm}$ , which is comparable with the  $D_h$  of  $\text{Ge}_{10}$  nanogel at  $2\ \mu\text{M}$  ( $40\ \text{nm}$ , Figure 1a). In addition, AFM images indicate that  $\text{Ge}_{10}$  nanogel at  $4\ \mu\text{M}$  has an average diameter of  $\sim 37\ \text{nm}$ , which is slightly larger than that of  $\text{Ge}_{10}$  nanogel at  $2\ \mu\text{M}$  ( $24\ \text{nm}$ , Figure 1b). Therefore, the hydrodynamic diameter of  $\text{Ge}_{10}$  nanogel at  $4\ \mu\text{M}$  is similar to that at  $2\ \mu\text{M}$ .

### **AFM imaging**

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

### **Circular dichroism (CD) spectroscopy**

All CD spectra were collected on a JASCO PS-150J CD spectrometer. The CD spectra were recorded at  $25\ ^\circ\text{C}$  over a spectral window of  $210\text{-}320\ \text{nm}$ , with data sampling of  $0.1\ \text{nm}$  at a scan speed of  $200\ \text{nm}\ \text{min}^{-1}$ . Spectra were demonstrated by averaging three successive acquisitions.

### **Cell culture**

A549 cells (a human lung adenocarcinoma cell line) were purchased from Chinese Academy of Sciences Institute of Cell Resource Center (Shanghai, China). A549 cells were cultured in RPMI-1640 medium containing 10% FBS and antibiotics

(50 units mL<sup>-1</sup> penicillin and 50 units mL<sup>-1</sup> streptomycin) at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub>.

### **Cellular uptake studies of Ge<sub>10</sub> nanogels**

Confocal laser scanning microscopy (CLSM) and flow cytometry were used to characterize the cell uptake behaviors of Ge<sub>10</sub> nanogels. For CLSM studies, A549 cells were seeded in 24-well culture plates at a density of  $3.0 \times 10^4$  cells per well and cultured for 24 h. Then the medium was replaced with medium containing FAM-labeled Ge<sub>10</sub> nanogels at a concentration of 4 μM in OptiMEM (Life Technologies, Inc.) for 24 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, A549 cells were seeded in 24-well culture plates at a density of  $5.0 \times 10^4$  cells per well and cultured for 24 h. Then the medium was replaced with medium containing FAM-labeled Ge<sub>10</sub> nanogels at a concentration of 4 μM in OptiMEM for 24 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 Ge<sub>10</sub> nanogels were quantified using BD LSRFortessa™ flow cytometry system.

### **Cell apoptosis assay**

A549 cells were seeded in 24-well plates at a density of  $5.0 \times 10^4$  cells per well and cultured for 24 h. Then the A549 cells were treated with Ge<sub>10</sub> nanogels and free Ge at the same concentration (equivalent Ge, 40 μM) for 48 h. The untreated A549 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 LSRFortessa™ flow cytometry system. Finally, the cell populations were analyzed with the FlowJo software.

### **Detection of intracellular metabolic dFdCMP, dFdCDP, dFdCTP**

A549 cells were seeded in 10 cm dishes at a density of  $140 \times 10^4$  cells per dish and cultured for 24 h. Then the A549 cells were treated with **Ge**<sub>10</sub> nanogels and free **Ge** at the same concentration (equivalent **Ge**, 40  $\mu$ M) for 48 h, respectively. The untreated A549 cells were used as control. Then, the culture medium was removed and the cells were washed for three times with cold PBS. After incubation, the treated and untreated cells were harvested, counted and resuspended in 1 mL of ice-cold 50% methanol containing 5-fluoro-2'-deoxyuridine-5'-triphosphate (5F-dUTP) as internal quality control standards. Thereafter, the cell suspensions were placed in thermostatic ice bath at 0 °C and sonicated for 5 min using ultrasonic cell disrupter with alternative cycles of 20 s pulses after every 10 s intervals for 10 min using ultrasonicator probe (Vibra cell 750). After sonication, the extracts were centrifuged (15000 rpm for 15 min at 4 °C) to remove cell debris, precipitated protein, and nucleic acids. Afterwards, the supernatant was collected, evaporated, and lyophilized. Then the resultant pellet was resuspended in 100  $\mu$ L methanol:water (v/v, 1:1) and analyzed by Waters Acquity Ultra Performance LC system equipped with a Triple Quadrupole Mass Spectrometer (UPLC-3QMS, ACQUITY UPLC&SCIEX SelexION Triple Quad 5500 System).

### **Tumor implantation**

All male Balb/c nude mice (4-week-old) were purchased from Chinese Academy of Sciences (Shanghai, China). Animal studies were conducted in accordance with the guidelines for the care and use of laboratory animals proved by the Animal Ethics Committee of Shanghai Jiao Tong University. A549 cells were injected subcutaneously in flanks of male Balb/c nude mice with 0.2 mL of cell suspension containing  $2 \times 10^6$  cells. The tumors were allowed to grow for a mean volume of 50 mm<sup>3</sup> and 250 mm<sup>3</sup> for antitumor studies and *in vivo* optical imaging experiments, respectively.

### ***In vivo* optical imaging and *ex vivo* biodistribution studies**

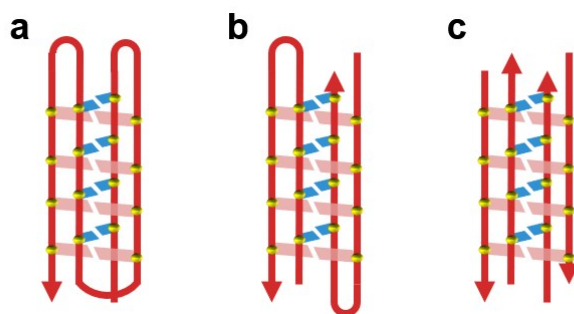
*In vivo* optical imaging was performed and analyzed using an IVIS Lumina II *in vivo* imaging system (Caliper Life Sciences, USA) with 650 nm excitation wavelength and 700 nm emission wavelength. The tumor bearing nude mice were anesthetized with isoflurane. Successive images were taken at  $t = 0.5, 1, 2, 3, 4,$  and 6 h after intravenous injection of Cy5.5-labeled  $\text{Ge}_{10}$  nanogels and free Cy5.5. For the *ex vivo* biodistribution study, the tumor bearing nude mice were injected with Cy5.5-labeled  $\text{Ge}_{10}$  nanogels and free Cy5.5 through the tail vein. Major organs and tumor tissues were carefully harvested at 3 h post-injection. All samples were rinsed with saline and immediately imaged using an IVIS Lumina II *in vivo* imaging system.

### ***In vivo* antitumor evaluation**

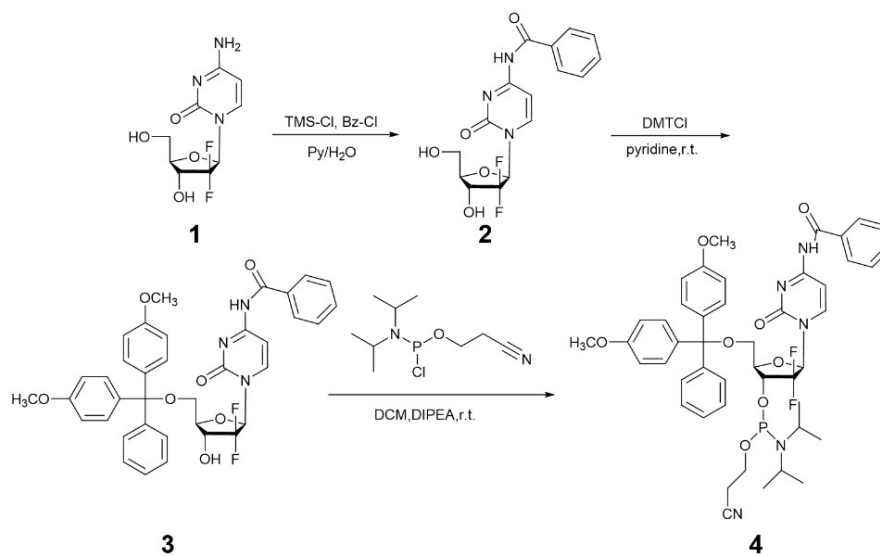
The subcutaneous tumor-bearing nude mice were randomly divided into three cohorts of five mice each.  $\text{Ge}_{10}$  nanogels and free  $\text{Ge}$  (a dose of  $25 \text{ mg} \cdot \text{kg}^{-1}$ , 200  $\mu\text{L}$ ) were intravenously injected via the tail vein every three days for 21 days. The control group only received an equal amount of PBS injection. The length (a) and width (b) of the tumors were measured by using the digital caliper every 3 days and calculated according to the formula:  $V (\text{mm}^3) = 1/2 \times a (\text{mm}) \times b (\text{mm}) \times b (\text{mm})$ . The body weight of mice was measured using an electronic balance at the time of each treatment. Mice were sacrificed on day 21. Their major tissues were taken and sectioned into around 3 mm slices and fixed in 4% paraformaldehyde overnight for hematoxylin and eosin (H&E) staining, immunohistochemical analysis, and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assays, which were conducted following the same procedure in previous literature.<sup>2</sup>



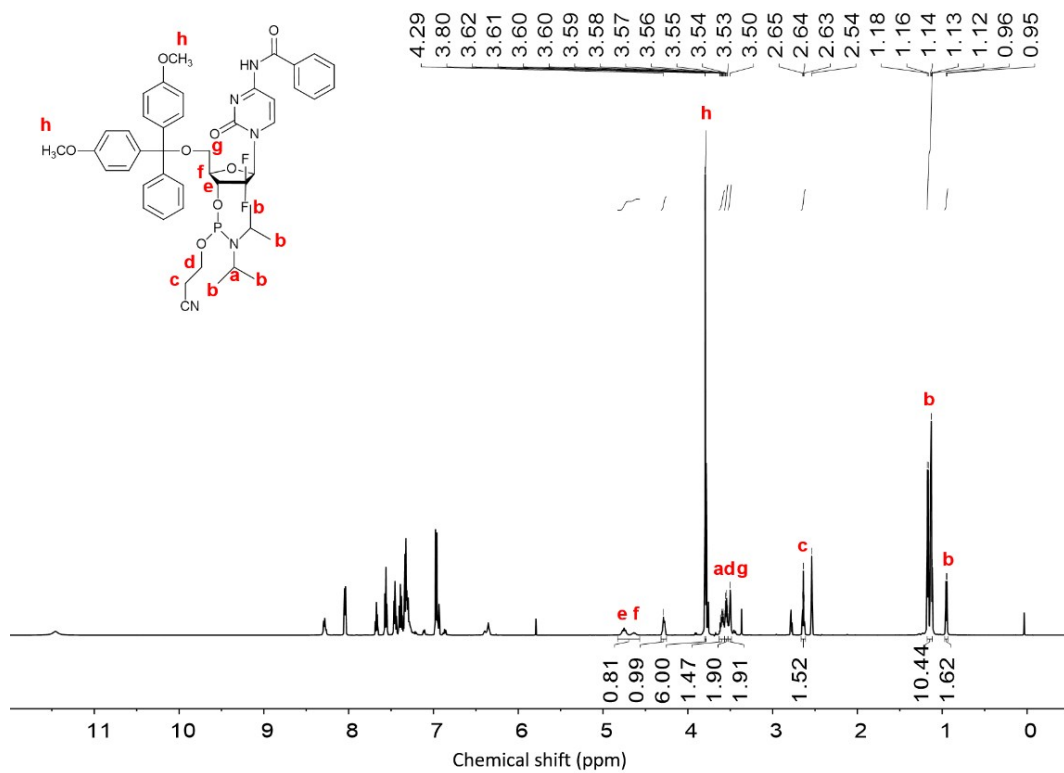
## Supplementary Figures



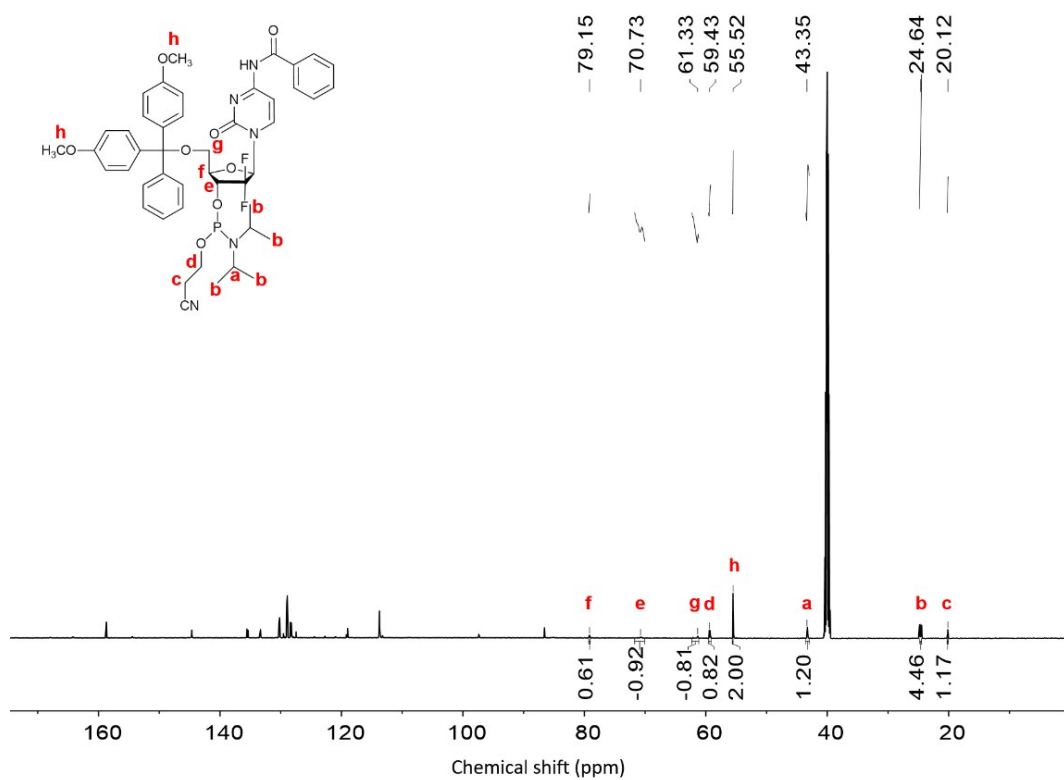
**Figure S1.** Schematic illustration of i-motif conformations assembled by cytosine-rich DNA sequences in acidic environment. (a) intramolecular i-motif structure; (b) dimer i-motif structure; (c) tetramer i-motif structure.



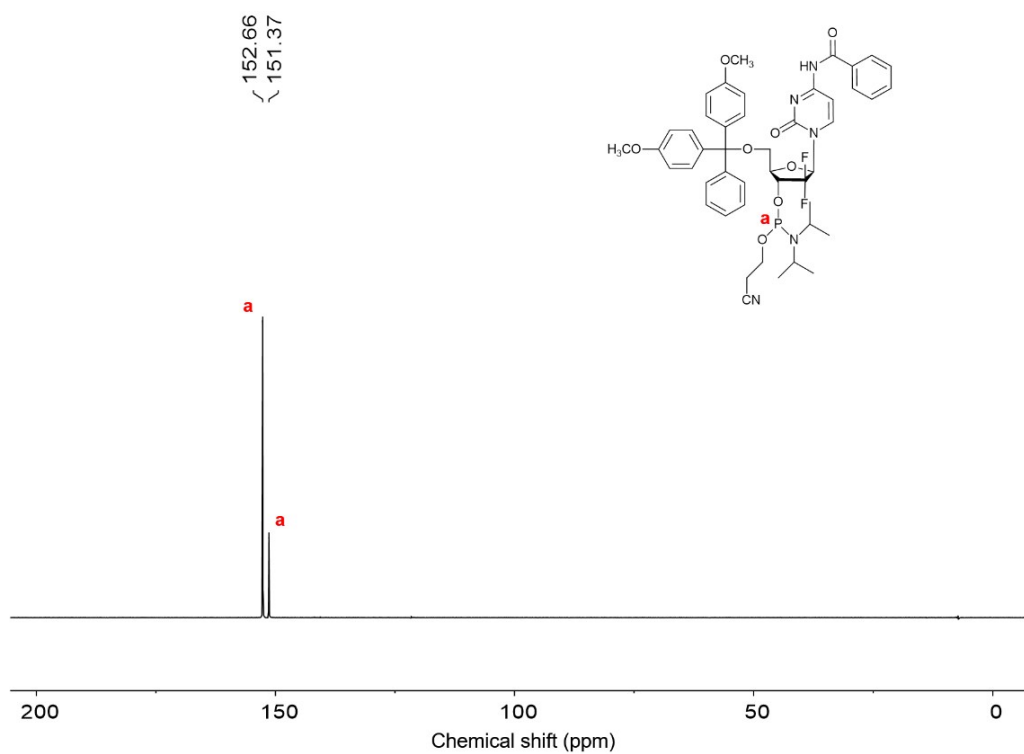
**Figure S2.** The synthetic route of Ge phosphoramidite.



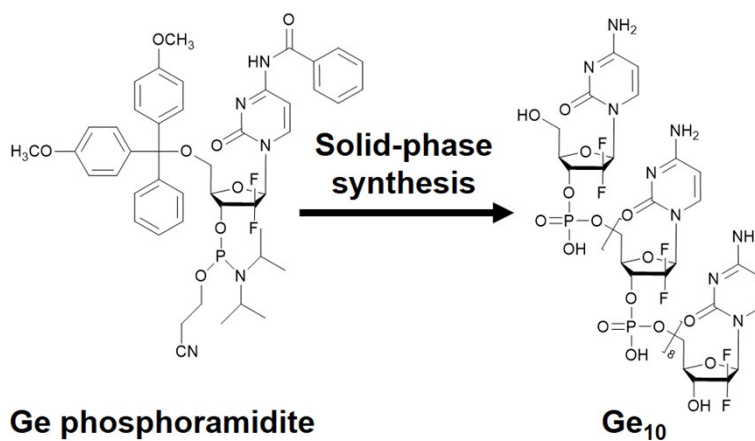
**Figure S3.**  $^1\text{H}$  NMR spectrum of Ge phosphoramidite in  $\text{DMSO-}d_6$ .



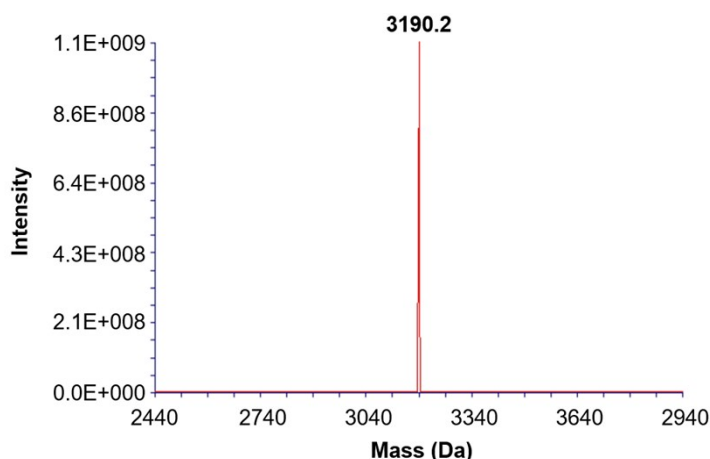
**Figure S4.**  $^{13}\text{C}$  NMR spectrum of Ge phosphoramidite in  $\text{DMSO-}d_6$ .



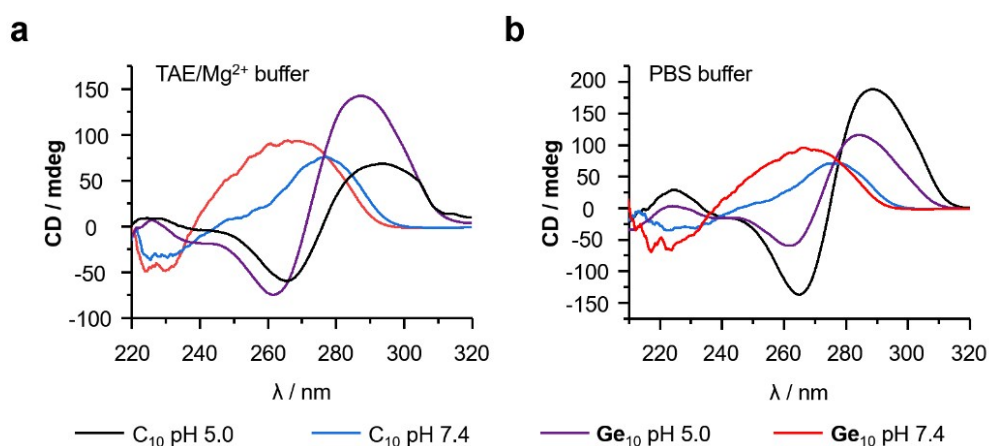
**Figure S5.**  $^{31}\text{P}$  NMR spectrum of **Ge** phosphoramidite in  $\text{DMSO-}d_6$ . Note that there are two sharp peaks which can be attributed to two diastereomers of **Ge** phosphoramidite, indicating the successful synthesis of **Ge** phosphoramidite.



**Figure S6.** Solid-phase synthesis of single-stranded **Ge**<sub>10</sub> (ss-**Ge**<sub>10</sub>) polyprodrug, using **Ge** phosphoramidite as the monomer.



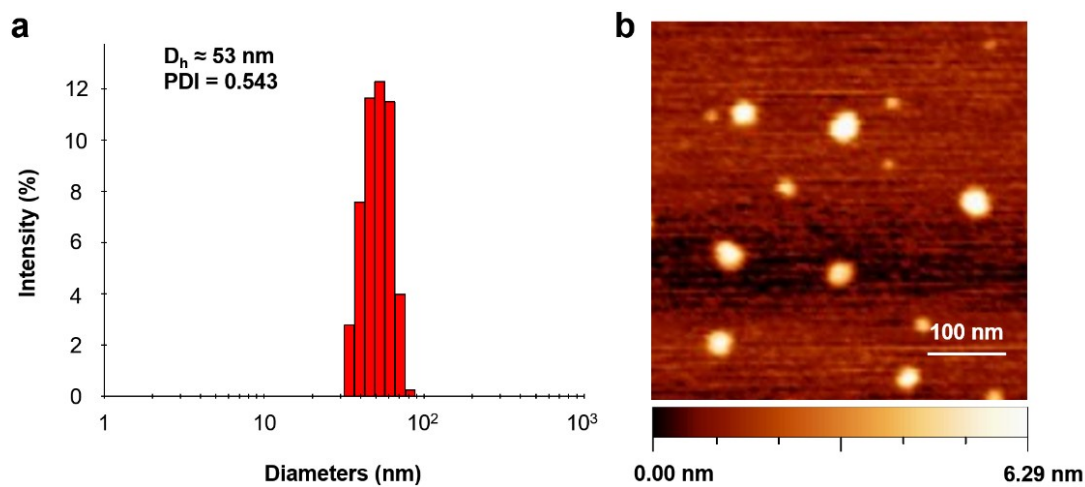
**Figure S7.** Mass spectrum of ss- $\text{Ge}_{10}$ . The target mass of ss- $\text{Ge}_{10}$  was 3190.2, which agrees well with the calculated value 3189.7.



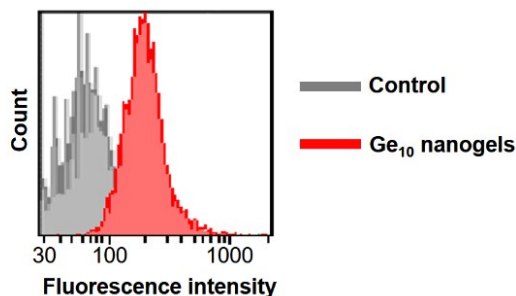
**Figure S8.** CD spectra of  $\text{Ge}_{10}$  assemblies and  $\text{C}_{10}$  assemblies at pH 5.0, 7.4, in TAE/ $\text{Mg}^{2+}$  and PBS buffer, respectively.

As an analogue of deoxycytidine,  $\text{Ge}_{10}$  strands have a similar chemical structure with deoxycytidine oligomer (especially the same base structure). Therefore,  $\text{Ge}_{10}$  strands can also form i-motif structure in acidic environment, which was demonstrated by the CD spectroscopy (Figure S8). For example, at pH 5.0 in both TAE/ $\text{Mg}^{2+}$  and PBS buffer,  $\text{Ge}_{10}$  strands show a positive peak near 290 nm, a crossover at around 270 nm, and a negative peak near 260 nm, indicating a typical i-motif conformation.<sup>3</sup> In this work, we found that  $\text{Ge}_{10}$  strands could also assemble into nanogel at neutral pH (the pH condition that used for in vitro and in vivo experiments). However, as shown in Figure S8, there is no i-motif structure in  $\text{Ge}_{10}$  assemblies at pH 7.4 in either PBS buffer or TAE/ $\text{Mg}^{2+}$  buffer, which is similar to that of  $\text{C}_{10}$  assemblies. We speculate that the

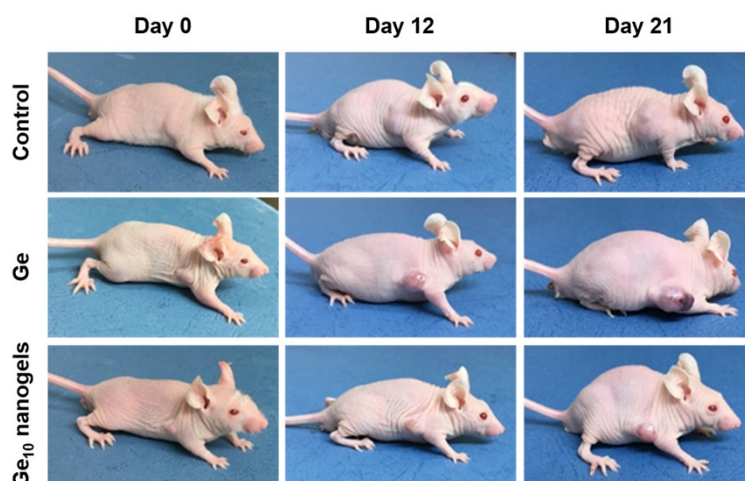
$\text{Ge}_{10}$  strands may still hybridize together through a multitude of hydrogen bonds and  $\pi$ - $\pi$  stacking, resulting in the formation of nanogel with random coil structure.



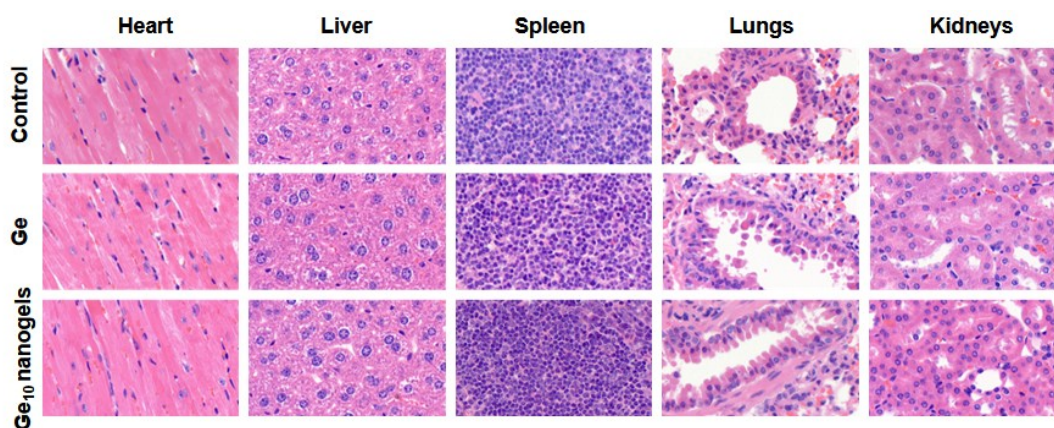
**Figure S9.** Characterizations of  $\text{Ge}_{10}$  nanogel after concentrating to  $4 \mu\text{M}$ . (a) Hydrodynamic diameter of  $\text{Ge}_{10}$  nanogel at  $4 \mu\text{M}$  in buffered solution as determined by DLS; (b) Representative AFM image of  $\text{Ge}_{10}$  nanogel at  $4 \mu\text{M}$ , scale bar, 200 nm.



**Figure S10.** The cellular uptake of  $\text{Ge}_{10}$  nanogels determined by flow cytometry assay. Cells were incubated with FAM-labeled  $\text{Ge}_{10}$  nanogels at an equivalent FAM concentration of  $4 \mu\text{M}$  for 24 h, respectively.



**Figure S11.** Photographs of the tumor-bearing nude mice during the treatment.



**Figure S12.** Microscopic images of H&E-stained sections of the major organs.

## References

- [1] F. C. Richardson, K. K. Richardson, J. S. Kroin, L. W. Hertel, *Nucleic Acids Res.* **1992**, *20*, 1763.
- [2] Q. Mou, Y. Ma, G. Pan, B. Xue, D. Yan, C. Zhang, X. Zhu, *Angew. Chem., Int. Ed.* **2017**, *129*, 12702.
- [3] H. Liu, Y. Xu, F. Li, Y. Yang, W. Wang, Y. Song, D. Liu, *Angew. Chem., Int. Ed.*, **2007**, *46*, 2515.