

## Electronic Supplementary Information (ESI)

### DNA Tetrahedron-Based Nanogels for siRNA Delivery and Gene Silencing

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## 1. Materials and Methods

### Materials

All Cy5 labeled siRNAs, sense-siEGFP, sense-siEGFP linker, antisense-siEGFP, antisense-siEGFP linker, and four single strand oligonucleotides were purchased from Bei Jing Zixi Bio Tech Co., Ltd (Beijing, China). DNA marker (25-500 bp), RNase A, RNase H, Diethyl pyrocarbonate (DEPC) water, Lipofectamine 2000 (Lipo2000) and nuclear dye Hoechst were purchased from Sangon Biotech Co., Ltd (Shanghai, China). Dulbecco's modified eagle medium (DMEM), Opti-MEM medium, lysis buffer containing 1 × protease, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), LysoTracker DND-26 (green), methyl- $\beta$ -cyclodextrin (Me- $\beta$ -CD), sucrose, wortmannin, trizol buffer and phosphatase inhibitor were purchased from Beyotime (Shanghai, China). The BCA protein assay kit, 4-20% precast gradient gel and  $\beta$ -actin were provided by Wuhan Service biotechnology (Wuhan, China). EGFP and secondary antibodies were purchased from Abcam (Cambridge, United Kingdom). cDNA and the Stepone Plus Real-Time PCR system were provided by Thermo Fisher (Waltham, USA). FastStart Universal SYBR Green Master was provided by Roche (Basel, Switzerland). All other reagents and consumable items were purchased from Tansoole Co., Ltd (Shanghai, China).

**Table S1. Sequences of component strands of DNA tetrahedron and tetrahedron with tails (tailed-TET)**

DNA	Sequences (5' to 3')
Tetra A-L	<b>TTG ACC TGT GAA</b> TT ACA TTC CTA AGT CTG AAA CAT TAC AGC TTG CTA CAC GAG AAG AGC CGC CAT AGT A
Tetra B-L	<b>TTG ACC TGT GAA</b> TT TAT CAC CAG GCA GTTGAC AGT GTA GCA AGC TGT AAT AGA TGC GAG GGT CCA ATA C
Tetra C-L	<b>TTG ACC TGT GAA</b> TT TCA ACT GCC TGG TGA TAA AAC GAC ACT ACG TGG GAA TCT ACT ATG GCG GCT CTT C
Tetra D-L	<b>TTG ACC TGT GAA</b> TT TTC AGA CTT AGG AAT GTG CTT CCC ACG TAG TGT CGT TTG TAT TGG ACC CTC GCA T
Tetra A	ACA TTC CTA AGT CTG AAA CAT TAC AGC TTG CTA CAC GAG AAG AGC CGC CAT AGT A

<b>Tetra B</b>	<b>TAT CAC CAG GCA GTTGAC AGT GTA GCA AGC TGT AAT AGA TGC GAG GGT CCA ATA C</b>
<b>Tetra C</b>	<b>TCA ACT GCC TGG TGA TAA AAC GAC ACT ACG TGG GAA TCT ACT ATG GCG GCT CTT C</b>
<b>Tetra D</b>	<b>TTC AGA CTT AGG AAT GTG CTT CCC ACG TAG TGT CGT TTG TAT TGG ACC CTC GCA T</b>

**Notes:**

1. Red sequences are sticky ends in tetrahedron for hybridization with siRNA linker;
2. Tetra A (B, C, D)-L were employed to self-assemble into tailed-TET and then form nanogel with siRNAs embedded. Tetra A-L and Tetra B, C and D were applied to self-assemble into tetrahedron, only the sticky end of Tetra A-L hybridized with siRNA linker.

**Table S2. Sequences of siRNA and siRNA-linker**

<b>siRNA</b>	<b>Sequences (5' to 3')</b>
<b>sense-siEGFP linker</b>	<b>rUrUrCrArCrArGrGrUrCrArArGrArCrGrUrArArArCr GrGrCrCrArCrArArG rUdTdC</b>
<b>antisense-siEGFP linker</b>	<b>rUrUrCrArCrArGrGrUrCrArArArCrUrUrGrUrGrGrCr CrGrUrUrUrArCrGrUrCdGdC</b>
<b>sense-siEGFP</b>	<b>rGrArCrGrUrArArArCrGrGrCrCrArCrArArGrUdTdC</b>
<b>antisense-siEGFP</b>	<b>rArCrUrUrGrUrGrGrCrCrGrUrUrUrArCrGrUrCdGdC</b>

**Notes:**

1. Red sequences are sticky ends in siRNA linkers for hybridization with tailed-TET;
2. The Cy5 fluorophores are labeled at the 3' terminus on both sense and antisense of siRNA linkers (siRNA-L) for cellular uptake study.
3. siRNA-L without dye were used to assemble with tailed-TET to form TET-nanogel, which was further used for characterizations and gene silencing study.
4. Double-stranded siEGFP (naked-siEGFP) assembled by sense-siEGFP and antisense-siEGFP was used as either negative or positive control when transfecting the cells without or with Lipo2000.

### **Synthesis of tailed DNA tetrahedron**

The sequences of component strands for both TET and tailed-TET assemblies are designed based on previously-reported work.<sup>1</sup> For TET without tails, all four component strands (Tetra A, Tetra B, Tetra C and Tetra D) contain 55 nucleotides (nt) with the sequences listed in Table S1. To further hybridize with the siRNA linker, a 12-base overhang with an identical sequence that is complementary to the sticky ends of the siRNA linker (Table S2) was further introduced at the 5' terminus of each TET component strand using 2 T as a spacer, termed as Tetra A-L, Tetra B-L, Tetra C-L and Tetra D-L (Table S1). To form the tailed-TET, Tetra A-L, Tetra B-L, Tetra C-L and Tetra D-L (69 nt) were solved in DEPC water and used according to the designated molecular ratio. Here we assembled at equimolar ratio and the final concentration of each strand was 5  $\mu$ M, all component strands were combined in  $1 \times$  TAE/Mg<sup>2+</sup> buffer ((40 mM Tris, 2 mM EDTA•2Na•2H<sub>2</sub>O, 20 mM acetic acid, 12.5 mM (CH<sub>3</sub>COO)<sub>2</sub>Mg•4H<sub>2</sub>O, pH =8.2, adjusted by acetic acid). The solution was heated to 95 °C for 5 min and then quickly cooled down to 4 °C to form the desired TET nanostructure with four tails. Meanwhile, TET without any sticky-ends (assembled by Tetra A, Tetra B, Tetra C, Tetra D) and with only one sticky-end (assembled by Tetra A-L, Tetra B, Tetra C, Tetra D) was assembled as control groups for the gel electrophoresis (Fig. S1).

### **Synthesis of double sticky-end siEGFP linkers, single sticky-end siEGFP linkers, and siEGFP**

Similar to our previous work,<sup>2</sup> the siRNA linker used to crosslink tailed-TET is made up of two parts: a central effective double-stranded oligonucleotide and two nonfunctional single-stranded segments serving as sticky ends to hybridize with overhangs at each vertex of the tetrahedron. To obtain the double sticky-end siEGFP linker, sense-siEGFP linker and antisense-siEGFP linker strands were first solved in DEPC water followed by mixed at equimolar ratio in  $1 \times$  TAE/Mg<sup>2+</sup> buffer and heated to 85 °C for 5 min, hierarchy annealing (75 °C for 30 min, 65 °C for 30 min, 55 °C for 30min, 45 °C for 30 min) to room temperature and stored at 4 °C for future use. The concentration of each strand was 20  $\mu$ M. Similarly, single sticky-end siEGFP were assembled by sense-siEGFP linker and antisense-siEGFP in the same way. siEGFP was assembled by hybridization of sense-siEGFP and antisense-siEGFP strands.

### **Synthesis of TET-nanogel**

To determine a proper ratio for nanogel construction, herein 5  $\mu$ M tailed-TET and 20  $\mu$ M siRNA linker stock solutions were mixed in  $1 \times$  TAE/Mg<sup>2+</sup> buffer with a ratio range from 2:1 to 1:4 (tailed-TET: siRNA linkers) and incubated for 1 h at 25 °C, in which the final TET concentration was adjusted to 3  $\mu$ M. By tuning the ratio of tailed-TET and siRNA linker, the formation of nanogel was systematically investigated. Tailed-TET to siRNA linker with varied ratios of 1:1.2, 1:1.4, 1:1.6, 1:1.8, 1:2.0 and 1:2.2, 1:2.4, 1:2.6 and 1:2.8 respectively were tested for the synthesis of TET-nanogels. As shown in Figure S2, when the ratio is far from the 1:2, although large assemblies can be formed, leftover of component motifs (tailed-TET or siRNA linker) appeared on the gel images.

Instead, ratios of 1:1.4, 1:1.6, 1:1.8 and 1:2.0 could form pure nanogel. Meanwhile, to obtain nanogel with extra TET outside the structure and protect the functional siRNA, the ratio of 1:1.8 was chosen to synthesize the final used TET-nanogel, which was further employed for subsequent characterizations and cellular experiments.

### **Synthesis of TET@siEGFP**

5  $\mu\text{M}$  tailed-TET and 20  $\mu\text{M}$  single sticky-end siEGFP linkers were mixed in  $1 \times \text{TAE}/\text{Mg}^{2+}$  buffer with the ratio of 1:4 and incubated for 1 h at 25  $^{\circ}\text{C}$  to form TET with siRNA loading outside (TET@siEGFP), in which the TET concentration was 2.5  $\mu\text{M}$ . TET@siEGFP was employed as a control for gene silencing experiment.

### **Native polyacrylamide gel electrophoresis (PAGE) analysis**

PAGE is a technique widely used to separate biological macromolecules, including nucleic acids, according to their electrophoretic mobility. 40% acrylamide (19:1, acrylamide/bisacrylamide) solution was added in  $1 \times \text{TAE}/\text{Mg}^{2+}$  buffer, 75  $\mu\text{L}$  ammonium persulfate and 7.5  $\mu\text{L}$  tetramethyl ethylenediamine (TEMED) were used as initiating agent and accelerator separately. Then each sample (20  $\mu\text{L}$  of 5  $\mu\text{M}$  tailed-TET, 20  $\mu\text{M}$  siRNA linker, and 3  $\mu\text{M}$  TET-nanogel, respectively) was mixed with an equivalent amount of loading dye and characterized at 4  $^{\circ}\text{C}$  (100 V, constant voltage) in  $1 \times \text{TAE}/\text{Mg}^{2+}$  buffer. After electrophoresis, the gels were immersed into ethidium bromide (EB) for staining, visualized by UV illumination (254 nm), and photographed by a digital camera.

### **Agarose gel electrophoresis**

0.3 g agarose was put in 60 mL  $1 \times \text{TAE}/\text{Mg}^{2+}$  buffer, and heated for 1 min 30 s, then add water to offset the volume loss. After freezing, each DNA sample (10  $\mu\text{L}$  of 5  $\mu\text{M}$  tailed-TET, 20  $\mu\text{M}$  siRNA linker, and 3  $\mu\text{M}$  TET-nanogel, respectively) was mixed with loading dye (2  $\mu\text{L}$ ) and analyzed using 0.5% agarose gel at 4  $^{\circ}\text{C}$  (100 V) for about 45 min in  $1 \times \text{TAE}/\text{Mg}^{2+}$  buffer. The bands were stained with GelRed, visualized by UV illumination (254 nm), and photographed by BioRad ChemiDoc MP Imaging System.

### **Denaturing PAGE analysis.**

Denaturing PAGE analysis was performed in a gel that contains 20% or 10% acrylamide (19:1, acrylamide/bisacrylamide). Both the gel and running buffer containing 8.3 M urea, 89 mM Tris, 89 mM boric acid, and 2 mM EDTA (TBE buffer). Each sample solution was mixed with an equivalent amount of loading buffer containing  $1 \times \text{TBE}$  buffer, 8 M urea, 0.03% bromophenol blue, and 0.03% xylene cyanol FF and then loaded onto the gel. The gel was run on a FB-VE10-1 electrophoresis unit (Fisher Biotech) at room temperature and then stained with ethidium bromide, visualized by UV illumination (254 nm), and photographed by a digital camera.

### **Dynamic light scattering (DLS) analysis and Zeta potential**

100  $\mu\text{L}$  tailed-TET (5  $\mu\text{M}$ ), TET-nanogels (3  $\mu\text{M}$ ) with ratios of 1:1.4, 1:1.6, 1:1.8 and 1:2.0 were characterized by DLS measurements on a Zetasizer Nano ZS (Malvern Instruments Ltd.) to determine the size distribution. 500  $\mu\text{L}$  tailed-TET (5  $\mu\text{M}$ ) and TET-nanogel (3  $\mu\text{M}$ ) were characterized by Zeta potential measurements on a Zetasizer Nano ZS (Malvern Instruments Ltd.) to verify the potential.

### **Atomic force microscopy**

Tailed-TET and nanogels were diluted to 10 nM. 2  $\mu\text{L}$  tailed-TET or nanogel solution was spotted onto freshly cleaved mica surface (Electron Microscopy Sciences) and incubated for 10 s to ensure nanogels can be absorbed onto the substrate. Then the sample drop was washed off by 20  $\mu\text{L}$  magnesium acetate solution (2 mM) and dried by nitrogen quickly. The size of tailed-TET and nanogels was imaged by Atomic Force Microscope (\*Multimode Nanoscope IIIa AFM).

### **Stability of the nanogels in different pH**

3  $\mu\text{M}$  TET-nanogels were incubated in TAE/ $\text{Mg}^{2+}$  buffers with pH of 5.0, 6.5, 7.0, and 8.2 for 1 h, respectively, then nanogel samples were evaluated by 0.5% agarose gel electrophoresis at 4  $^{\circ}\text{C}$  (100 V). To further confirm the stability of TET-nanogels in different pH environment, the hydrodynamic sizes of the nanogels were also determined by DLS after incubating the sample in different pH environment for 1h.

### **Nonspecific enzymatic degradation.**

The stability performance of TET-nanogels (3  $\mu\text{M}$ ) in serum was demonstrated by incubation with DMEM containing 10 % fetal bovine serum (FBS) for different time (1 h, 2 h, 4 h, 8 h) at 37  $^{\circ}\text{C}$ , then the nanogels samples were analyzed by 0.5 % agarose gel electrophoresis at 4  $^{\circ}\text{C}$  (100 V). Meanwhile, nanogels (3  $\mu\text{M}$ ) with varied ratios of 1:1.4, 1:1.6 and 1:2.0 were also incubated with DMEM containing 10% FBS for 8 h and analyzed by 0.5% agarose gel electrophoresis. To further verify the stability performance of TET-nanogel, the size of TET-nanogel incubated with DMEM containing 10% FBS for different time was determined by DLS.

### **RNase A resistance performance of TET-nanogel**

3  $\mu\text{M}$  TET-nanogel was incubated with different concentrations of RNase A (0.05 U/mL, 0.1 U/mL, 0.2 U/mL, 0.4 U/mL, 0.8 U/mL) at 37  $^{\circ}\text{C}$  for 1 h. Then 20  $\mu\text{L}$  TET-nanogel was analyzed by 10 % denaturing PAGE gel. Similarly, to confirm the stability of nanogels with varied ratios in RNase A, nanogels with assembly ratios of 1:1.4, 1:1.6, 1:1.8 and 1:2.0 were incubated with 0.8 U/mL RNase A for 1 h at 37  $^{\circ}\text{C}$ , after which samples were analyzed by 10% denaturing PAGE gel.

### **Efficient release behavior of siRNA**

RNase H was used here to examine whether siRNA can release from nanogels or not from two factors: time and concentration. Firstly, 3  $\mu\text{M}$  TET-nanogel was incubated with different concentration RNase H (50 U/mL, 20 U/mL, 10 U/mL, 5 U/mL, 1 U/mL) at 37  $^{\circ}\text{C}$  for 1 h, then they

mixed with 20 U/mL RNase H at 37 °C for different time (0.5 h, 1 h, 2 h, 4 h). Both of the two were characterized by 10% denaturing PAGE gel.

### **Cellular culture**

MCF-7 cells (a human breast cancer cell line) and EGFP-HeLa cells were cultured in DMEM containing 10 % FBS and antibiotics (50 units/mL penicillin and 50 units/mL streptomycin) at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub>.

### **Flow cytometric analysis of cellular uptake**

For cellular uptake study, TET-nanogel was labeled with fluorescent dye by using Cy5-labelled siRNA linker. MCF-7 cells ( $2 \times 10^4$  cells per well) were plated into 24-well culture plates and cultured at 37 °C for 24 h. Then removed the culture medium DMEM and the cells were incubated with the nanogels, tailed-TET, and siRNAs labeled with Cy5 (with a final Cy5 concentration of 2 μM) in Opti-MEM medium at 37 °C for 2 h. Then the solution was removed and the cells were washed with PBS three times and harvested by trypsin treatment, the harvested cells were washed with PBS thrice by centrifuging for 5 min at 1000 rpm, then resuspending the cells with 500 μL PBS for flow cytometry analysis using LSRFortessa (Becton Dickinson). Fluorescence was determined by counting 10000 events, and the behavior of cellular uptake was analyzed by flow cytometry. The uptake behavior of nanogels with assembly ratios of 1:1.4, 1:1.6 and 1:2.0 were also evaluated similarly.

### **Confocal laser scanning microscopy**

MCF-7 cells were plated in 24-well culture plates with a clean coverslip put in each well at  $5 \times 10^4$  cells per well and cultured in DMEM for over 24 h. Subsequently, removed DMEM medium by washing with PBS and replacing with Opti-MEM medium. Cy5 labeled TET-nanogel, naked siRNA and tailed-TET (with final Cy5 concentration was 2 μM) were incubated with MCF-7 cells at 37 °C for 2 h. Followed by removing the Opti-MEM medium and washing with ice-cold PBS for three times, then cells were fixed with 4% formaldehyde for 30 min at room temperature in each well and washed thrice with ice-cold PBS again. Cell nucleus was stained with Hoechst 33342 for 15 min. The slides were rinsed twice with PBS, mounted and scanned by a laser scanning confocal microscope (Leica TCS SP8 STED 3X). The fluorescence intensities of the confocal images were quantified by ImageJ software (NIH).

### **Cytotoxicity of TET-nanogel and Lipo2000 determined by MTT assay.**

The cytotoxicity of TET-nanogel and Lipo2000 was estimated by MTT assay. Breast cancer cells MCF-7 and fibroblast cells L929 were plated in 96-well culture plates at a density of  $1 \times 10^4$  cells per well and cultured for 24 h. Then, cells were incubated with TET-nanogels containing various siRNA concentrations: 0.5 μM, 1 μM, 2 μM, and 3 μM at 37 °C for 48 h. To evaluate the cytotoxicity of Lipo2000, according to the Lipofectamine® 2000 DNA Transfection Reagent Protocol (Thermo

Fisher), different doses of Lipo2000 were used to transfect equivalent concentration (0.5  $\mu$ M, 1  $\mu$ M, 2  $\mu$ M, and 3  $\mu$ M) of siRNA, and the final Lipo2000 reagent used per well was 0.2  $\mu$ L, 0.3  $\mu$ L, 0.4  $\mu$ L, and 0.5  $\mu$ L respectively, followed by incubating with MCF-7 cells and L929 cells for 48 h. 20  $\mu$ L of MTT solution (5 mg/mL) was added to each well and the plates were incubated at 37  $^{\circ}$ C for 4 h. Next, the MTT formazan crystals were dissolved with 250  $\mu$ L DMSO at room temperature. Finally, the absorbance was measured in a microplate reader at a wavelength of 490 nm.

### **Endocytosis pathway of TET-nanogel**

To explore the cellular uptake mechanism of TET-nanogel, MCF-7 cells were preincubated with uptake inhibitors of 1 mM Me- $\beta$ -CD, 450 mM sucrose and 50 nM wortmannin for 30 min to inhibit caveolae, clathrin, and micropinocytosis endocytosis, respectively. Then, the cells were incubated with Cy5-labeled TET-nanogel for 2 h and the inhibitors were maintained throughout the experiments. After that, the cellular uptake amount of Cy5-labeled TET-nanogel was evaluated by flow cytometry.

### **Colocalization study of TET-nanogel and lysosomes.**

MCF-7 cells were plated in 24-well culture plates with a clean coverslip ( $2 \times 10^4$  cells per well) and cultured for 24 h. Then remove culture medium and washed with PBS, the cells were incubated with Opti-MEM medium containing Cy5-labeled TET-nanogel (equivalent Cy5 concentration: 2  $\mu$ M) at 37  $^{\circ}$ C for 1 h. Then the medium was replaced with fresh medium and further incubated for another 0 h, 7 h, 15 h, 23 h respectively. Thereafter, the medium was replaced with fresh medium containing 60 nM LysoTracker DND-26 (green) and incubated 30 min. After that, the solutions were removed and the cells were carefully washed with ice-cold PBS for three times, fixed with 4% formaldehyde for 30 min at room temperature in each well, washed thrice with ice-cold PBS again. The slides were rinsed with PBS three times, finally mounted and scanned by a laser scanning confocal microscope (Leica TCS SP8 STED 3X). The Lecia imaging software was employed to quantify the extent of colocalization between the fluorescence signals of Cy5-labelled TET-nanogel and lysosomes.

### **Gene silencing**

Flow cytometry analysis and laser scanning confocal microscope were used for quantificationally analysis and visualized images of the gene silencing efficacy. EGFP-HeLa cells were plated in 24-well culture plates with a clean coverslip put in each well at  $2 \times 10^4$  cells per well and cultured for 24 h in fresh DMEM medium, then removed culture medium and replaced with Opti-MEM medium. TET-nanogel, naked siEGFP, siEGFP transfected by Lipo2000, and TET@siEGFP were added to



each cell at the equivalent siRNA concentration (100 nM). After 8 h incubation, the Opti-MEM medium was replaced with fresh DMEM medium and further incubated for 48 h. For flow cytometric analysis, the solutions were removed and the cells were washed with PBS three times and harvested by trypsin treatment, the harvested cells were washed with PBS thrice by centrifuging for 5 min at 1000 rpm, then resuspend the cells with 500  $\mu$ L PBS for flow cytometry analysis using LSRFortessa (Becton Dickinson). Fluorescence was determined by counting 10000 events, and the behavior of cellular uptake was analyzed by Flow cytometric. For CLSM imaging, after 8 h incubation, the cells were carefully washed with ice-cold PBS for three times, followed by removing of culture solution, fixing with 4% formaldehyde for 30 min at room temperature in each well, and then washing thrice with ice-cold PBS again. The slides were rinsed thrice with PBS, mounted and imaged by a laser scanning confocal microscope (Leica TCS SP8 STED 3X).

### **Western blot analysis**

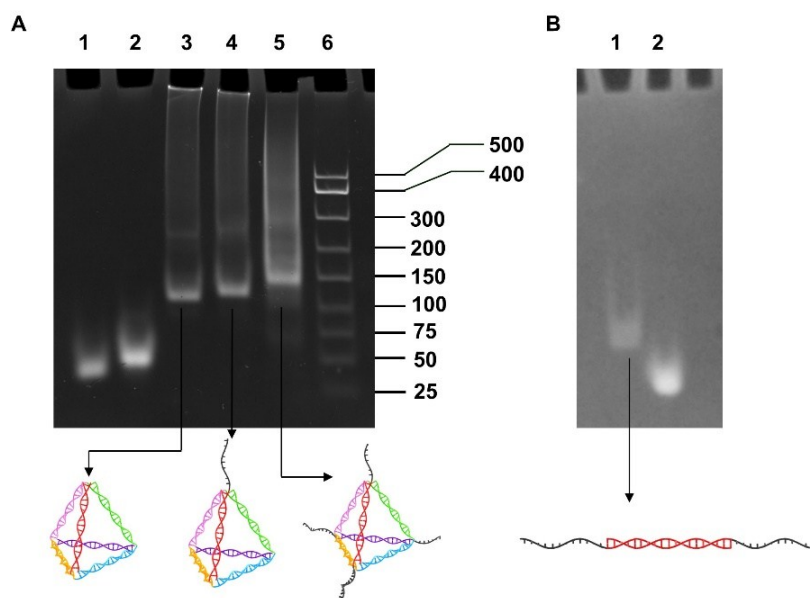
Western blot was used to examine the EGFP protein expression. EGFP-HeLa cells were plated in 5  $\times$  100 mm culture dishes and cultured for 24 h, then removed culture medium and added TET-nanogel, siEGFP transfected by Lipo2000, naked siEGFP and TET@siEGFP at the equivalent siRNA concentration (100 nM). After 8 h, the medium was replaced with fresh Opti-MEM medium and further incubated for 72 h. Followed by removing the solutions and washing the cells thrice with ice-cold PBS and treating with 500  $\mu$ L lysis buffer containing 1 $\times$  protease and phosphatase inhibitor. The cells lysates were incubated on ice for 30 min and shocked repeatedly. Then the cell lysates were centrifugated for 10 min at 12000 rpm. The BCA protein assay kit was used to measure the protein concentrations. The lysate with the same amount of total protein was incubated with an equal volume of dithiothreitol (DTT)-containing loading buffer (54 mg/mL). After boiling for 5 min, samples with an equal amount of total protein were separated by 4-20% Precast gradient gel. The intact gel was then transferred to Immobilon-P membrane. The membranes were incubated in 5 % BSA in PBST for 2 h, followed by incubating with 1% BSA in PBS with primary antibodies against  $\beta$ -actin, EGFP overnight, followed by incubating in 1% BSA in PBS with secondary antibodies (1 : 3000) diluted in TBST for 30 min.

### **Quantitative real-time PCR (qRT-PCR) analysis**

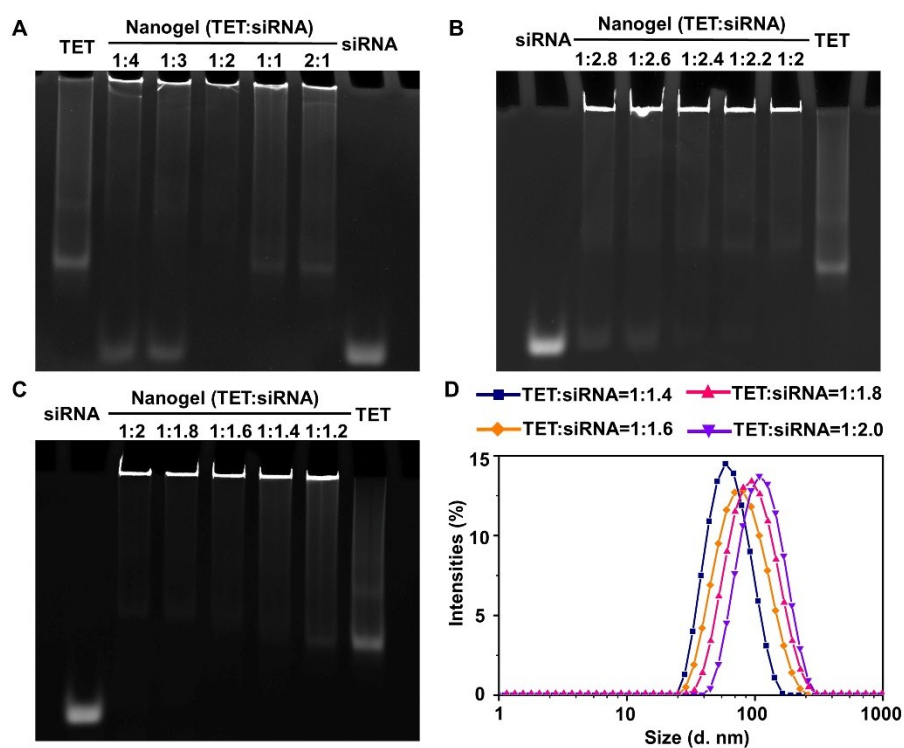
qRT-PCR was applied to examine the mRNA expression. EGFP-HeLa cells were plated in 5 culture dishes with 100 mm diameters and cultured for 24 h, followed by removing culture medium and adding TET-nanogel, siEGFP transfected by Lipo2000, naked siEGFP, and TET@siEGFP at the equivalent siRNA concentration (100 nM). After 8 h, the medium was replaced with fresh medium and further incubated for 48 h. Then the solutions were removed and the cells were washed with ice-cold PBS for three times and treated with 1 mL trizol buffer. After collecting the cells, total RNA from EGFP-HeLa cells were extracted according to the manufacturer's protocol. 2.5  $\mu$ L of cDNA, which was obtained by inverse transcription from extracted RNA (2  $\mu$ L), was subjected to qRT-PCR analysis targeting EGFP and GAPDH. Then the Stepone Plus Real-Time PCR system

was used for mRNA quantification.

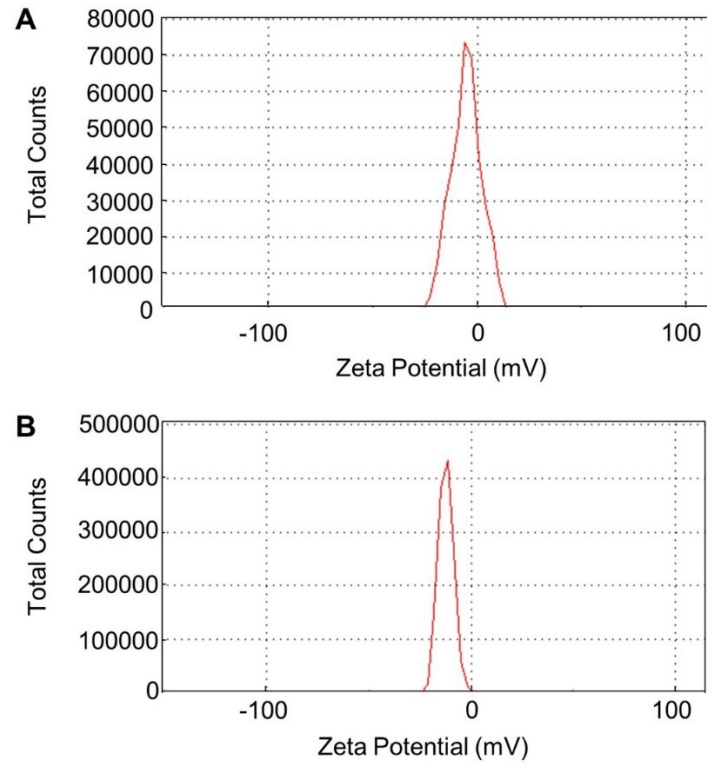
## 2. Supplementary Figures



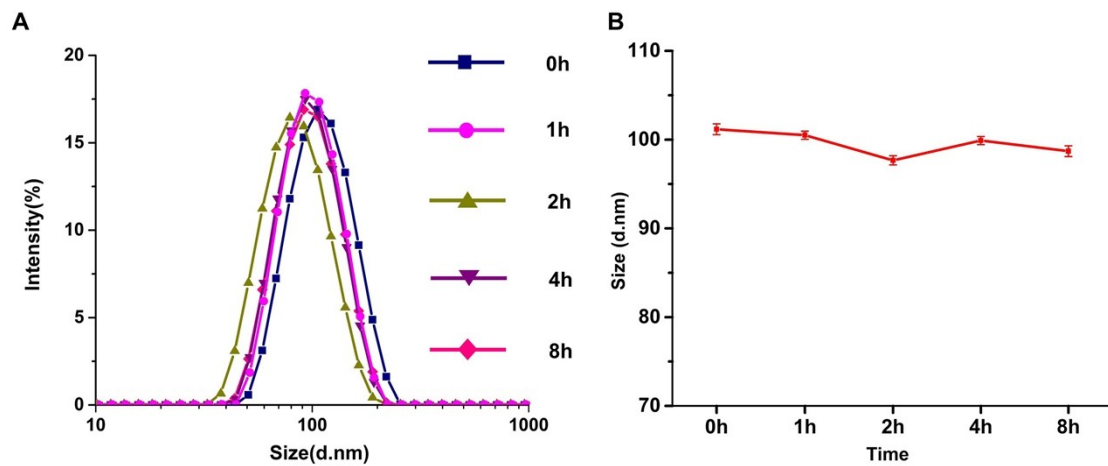
**Fig. S1. (A)** 8% native PAGE demonstration of tailed-TET with sticky ends on each vertex. **Lane 1.** Single strand DNA. **Lane 2.** Single strand DNA with a sticky end. **Lane 3.** TET structure without any sticky ends. **Lane 4.** TET structure with only one sticky end. **Lane 5.** tailed-TET nanostructure with four sticky ends. **Lane 6.** DNA marker A (25 bp-500 bp). The position of the sample in lane 5 shows that we obtained the desired DNA tetrahedron nanostructure. **(B)** 4% native PAGE demonstration of siRNA linkers. **Lane 1.** Self-assembled siRNA linkers. **Lane 2.** Single strand siRNA.



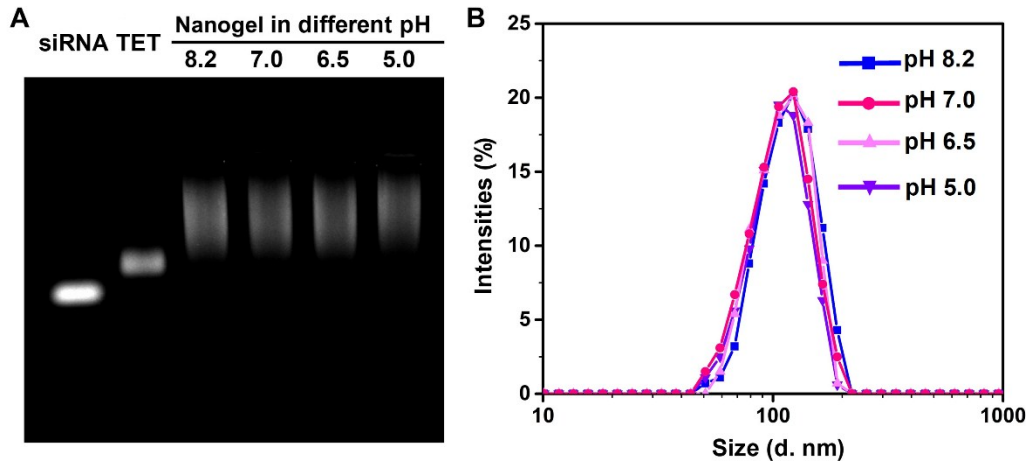
**Fig. S2.** (A), (B), and (C) The self-assembly of TET-nanogels using tailed-TET and siRNA linker with different ratios. The products were checked by 4% polyacrylamide gel electrophoresis under native condition. Above results show that when the ratio of tailed-TET and siRNA linker was far from 1:2, leftover tailed-TET or siRNA linker appeared on the gel images. While tuning the ratio of tailed-TET to siRNA linker slightly above or under 1:2, extra structural units will remain on the particle surface, terminating the growth of the nanogel. (D) Hydrodynamic sizes of TET-nanogels with different assembly ratios as determined by DLS analysis. The results of DLS revealed that when the ratios were 1:1.4, 1:1.6, 1:1.8, and 1:2, the sizes of the nanogels were 65 nm, 82 nm, 98 nm, and 108 nm, respectively.



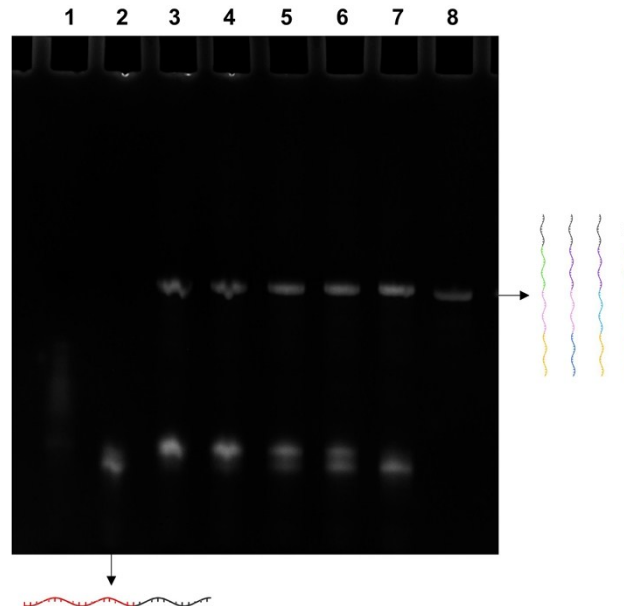
**Fig. S3.** (A) The zeta potential of tailed-TET. (B) The zeta potential of TET-nanogel.



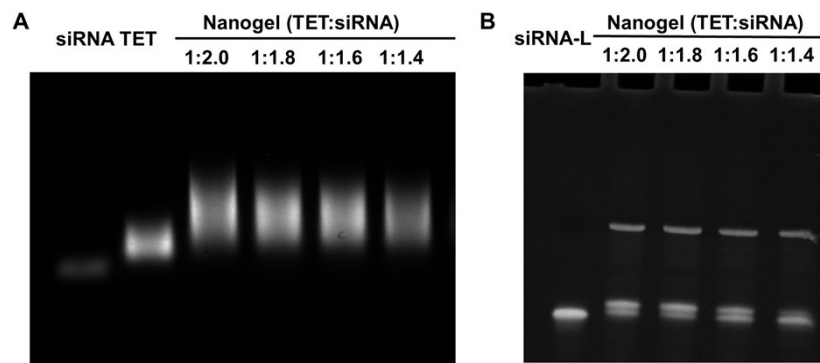
**Fig. S4.** (A) DLS analysis and (B) Size variation of TET-nanogel in serum incubated for different time.



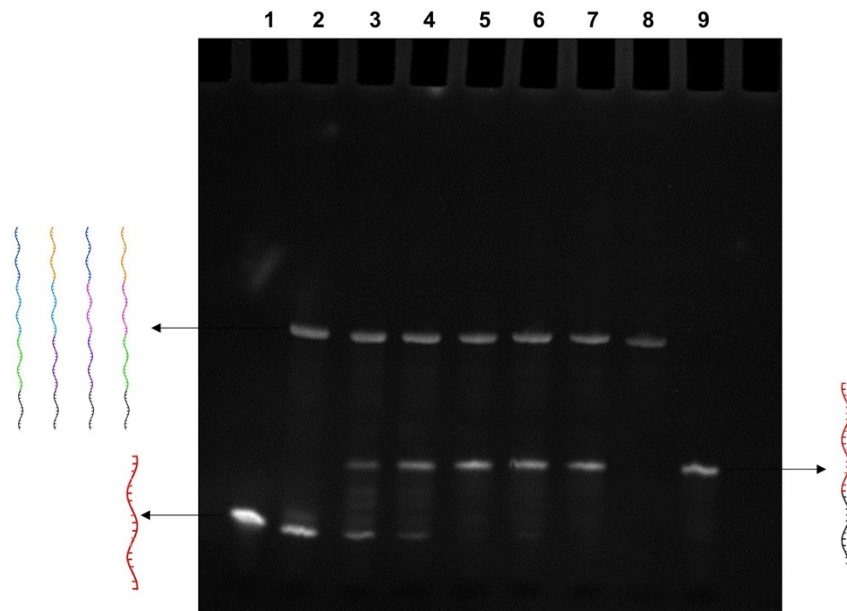
**Fig. S5.** (A) 0.5% agarose gel electrophoresis of the TET-nanogels after incubating the samples in buffered solutions with different pH for 1 h. (B) DLS analysis of the TET-nanogels after incubating the samples in different pH environment for 1 h.



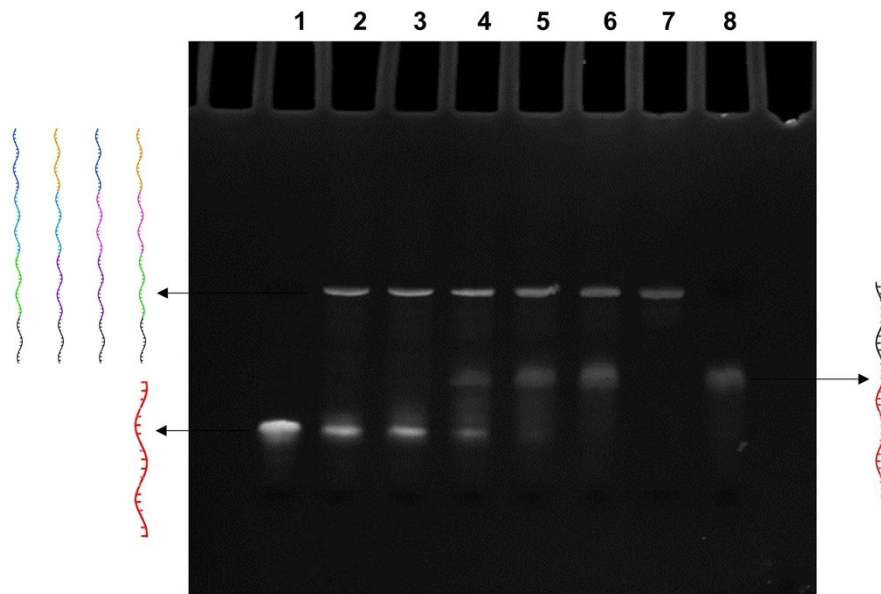
**Fig. S6.** 10% denaturing PAGE gel analysis of nanogels with different concentrations of RNase A at 37 °C for 1 h. **Lane 1.** Naked siEGFP incubated with 0.1 U/mL RNase A for 5 min. **Lane 2.** naked siEGFP with sticky ends without RNase A. **Lane 3.** TET-nanogel incubated with 0.05 U/mL RNase A for 1 h. **Lane 4.** TET-nanogel incubated with 0.1 U/mL RNase A for 1 h. **Lane 5.** TET-nanogel incubated with 0.2 U/mL RNase A for 1 h. **Lane 6.** TET-nanogel incubated with 0.4 U/mL RNase A for 1 h. **Lane 7.** TET-nanogel incubated with 0.8 U/mL RNase A for 1 h. **Lane 8.** DNA single strand of tailed-TET (69 bases). Naked siEGFP degraded after 5 min incubation with 0.1 U/mL RNase A. By contrast, almost no degradation happened even when TET-nanogel incubating with 0.8 U/mL RNase A for 1h, suggesting that TET-nanogel could protect siRNA against enzymatic degradation.



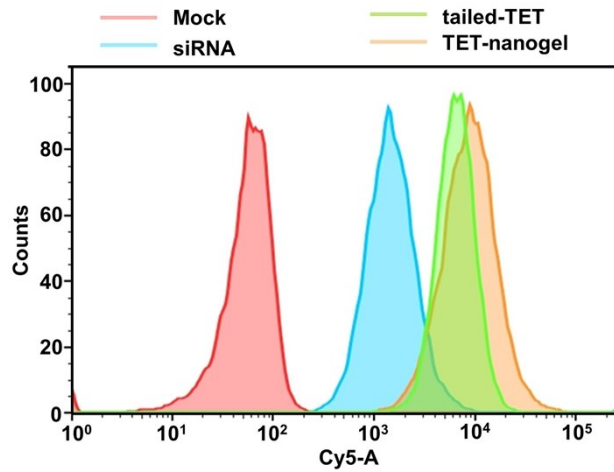
**Fig. S7.** (A) 0.5% agarose gel electrophoresis of the self-assembled nanogels after being incubated with 10% FBS-containing DMEM for 8 h at 37 °C. (B) 10% denaturing PAGE analysis of the self-assembled nanogels after being incubated with 0.1 U/mL RNase A at 37 °C for 1 h.



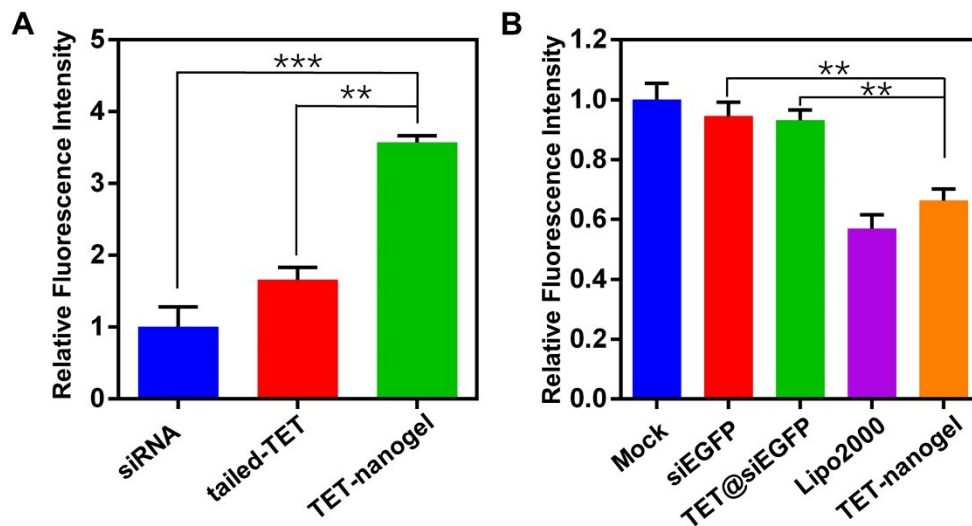
**Fig. S8.** 10% denaturing PAGE gel analysis of nanogels with different concentrations of RNase H at 37 °C for 1 h. **Lane 1.** Naked siEGFP without sticky ends. **Lane 2.** TET-nanogel incubated with 50 U/mL RNase H for 1 h. **Lane 3.** TET-nanogel incubated with 20 U/mL RNase H for 1 h. **Lane 4.** TET-nanogel incubated with 10 U/mL RNase H for 1 h. **Lane 5.** TET-nanogel incubated with 5 U/mL RNase H for 1 h. **Lane 6.** TET-nanogel incubated with 1 U/mL RNase H for 1 h. **Lane 7.** TET-nanogel without treating. **Lane 8.** DNA single strand of tailed-TET (69 bases). **Lane 9.** siRNA with sticky ends. When incubating with 10 U/mL RNase H, a smear band appeared in the position of siRNA, suggesting that siRNA started to be released. When incubating with 50 U/mL RNase H, no band appeared in the position of siRNA-linkers, indicating that siRNA could be completely released.



**Fig. S9.** 10% denaturing PAGE gel analysis of nanogels with 20 U/mL RNase H at 37 °C for different time. **Lane 1.** Naked siRNA without sticky ends. **Lane 2.** TET-nanogel incubated with 20 U/mL RNase H for 4 h. **Lane 3.** TET nanogel incubated with 20 U/mL RNase H for 2 h. **Lane 4.** TET-nanogel incubated with 20 U/mL RNase H for 1 h. **Lane 5.** TET-nanogel incubated with 20 U/mL RNase H for 0.5 h. **Lane 6.** TET-nanogel without treating. **Lane 7.** DNA single strand of TET (69 bases). **Lane 8.** siRNA with sticky ends. Note that a smear band appeared in the position of the released siRNA after incubating for only 0.5 h, demonstrating siRNA release in a relatively short time. When incubating the TET-nanogel with RNase H for 2 h, there was no band in the position of siRNA-linkers, suggesting that siRNA could be fully released.

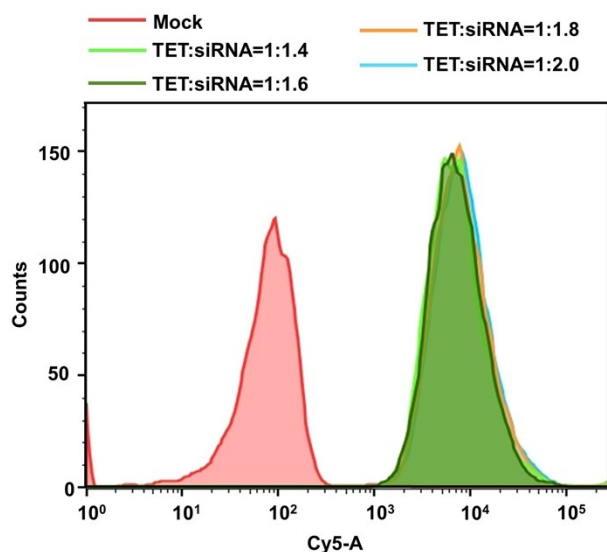


**Fig. S10.** Cellular uptake of the TET-nanogel as determined by flow cytometry analysis.

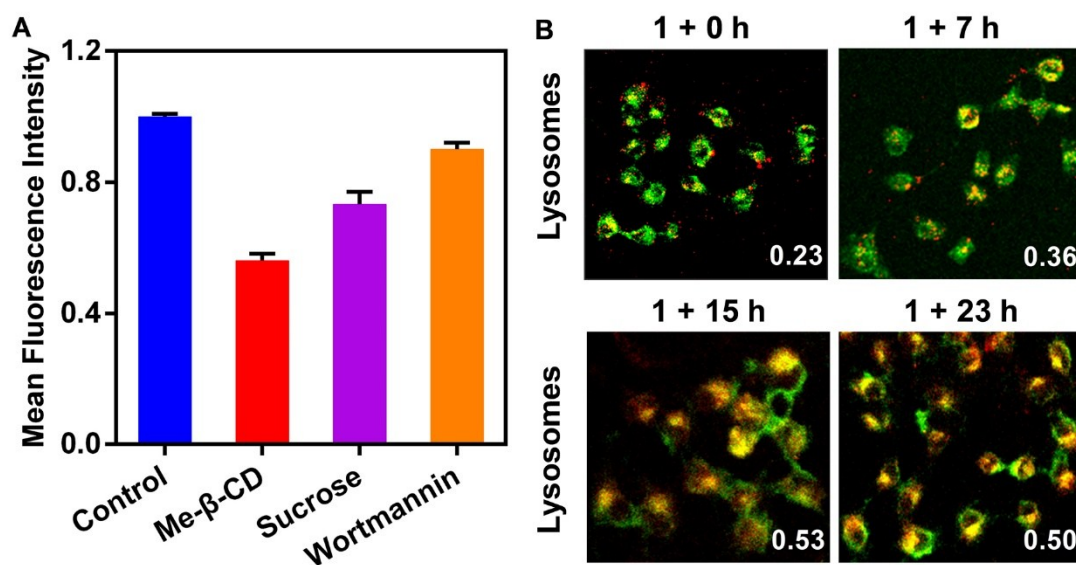


**Fig. S11.** (A) Relative fluorescence intensity of MCF-7 cells incubated with siRNA, tailed-TET, and TET-nanogel in Figure 2C analyzed by ImageJ. (B) Relative fluorescence intensity of EGFP-HeLa cells incubated with siEGFP, TET@siEGFP, Lipo2000 and TET-nanogel (siRNA concentration: 100 nM) in Figure 3D as analyzed by ImageJ. significance: \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

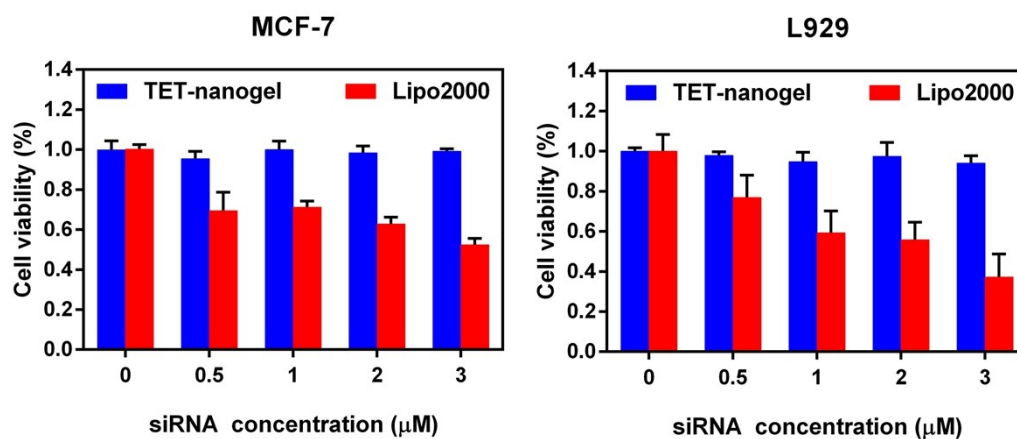




**Fig. S12.** Flow cytometry analysis of cellular uptake of Cy5-labeled TET-nanogels assembled with different ratios. Nanogels assembled with different ratios exhibited almost the same mean fluorescence, indicating that within the range of 60-110 nm, size variation did not significantly influence the cellular uptake efficiency of the nanogels.



**Fig. S13.** (A) Uptake inhibition of TET-nanogel in MCF-7 cells preincubated with different uptake inhibitors of Me- $\beta$ -CD (1 mM, caveolae-mediated endocytosis inhibitor), sucrose (450 nM, clathrin-mediated endocytosis inhibitor) and wortmannin (50 nM, macropinocytosis inhibitor). Fluorescence from Cy5 was measured by flow cytometric assay. (B) Colocalization images of Cy5-labelled TET-nanogel (red) and lysosomes (green). The cells were treated with Cy5-labelled TET-nanogel (Cy5 concentration: 2  $\mu$ M) for 1 h. After removing the TET-nanogel, the cells were further incubated with fresh culture medium at 37  $^{\circ}$ C for 0 h, 7 h, 15 h, 23 h, respectively. Colocalization coefficients are displayed on images.



**Fig. S14.** MTT assays of fibroblast cells L929 and breast cancer cells MCF-7 treated with nanogels and Lipo2000 at different siRNA concentrations.

### Reference

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2. F. Ding, Q. Mou, Y. Ma, G. Pan, Y. Guo, G. Tong, C. H. Choi, X. Zhu and C. Zhang, *Angew. Chem., Int. Ed.*, 2018, **57**, 3064-3068 (*Angew. Chem.*, 2018, **130**, 3118-3122).