Supporting Information

Flexible bowl-shaped magnetic assembly for multifunctional gene delivery systems

Ranran Wang,[a,b]† Xiaoguang Dai,[a,b]† Shun Duan,[a,b] Nana Zhao,[a,b,*] and Fu-Jian Xu[a,b,*]

[a]State Key Laboratory of Chemical Resource Engineering, Beijing University of Chemical Technology, Beijing, 100029, China
[b]Key Lab of Biomedical Materials of Natural Macromolecules (Beijing University of Chemical Technology), Ministry of Education, Beijing Laboratory of Biomedical Materials, Beijing Advanced Innovation Center for Soft Matter Science and Engineering, Beijing University of Chemical Technology, Beijing 100029, China

*To whom correspondence should be addressed
Email: xufj@mail.buct.edu.cn (F. J. Xu), zhaonn@mail.buct.edu.cn (N. Zhao)
†Both authors contributed equally to this work.

Experimental Section

Materials

Iron(III) acetylacetonate (97%) was obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). Oleic acid (90%) was purchased from Alfa Aesar Co. Oleylamine was obtained from Tokyo Chemical Industry (TCI). Sodium hydroxide (NaOH, 96%), ethanol (99%) and chloroform ($CHCl_3$) were obtained from Beijing Chemical Co., China. Tetraethylorthosilicate (TEOS, 98%) were obtained from Energy Chemical Co., Ltd (Shanghai, China). β-cyclodextrin (CD, 99%), cetyltrimethylammonium bromide (CTAB, 99%), 3-aminopropyl triethoxysilane (APTES), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC, 98%), N-hydroxysuccinimide (NHS, 98%), copper (I) bromide (CuBr, 98%), glycidyl methacrylate (GMA, 98%), branched polyethylenimine (PEI; Mw~25000 Da), 2-bromoisobutyryl bromide (BIBB, 98%), triethylamine (TEA), N,N,N',N,'N''-pentamethyl diethylenetriamine (PMDETA) and ethanolamine (EA, 98%) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). GMA were used after removal of the inhibitors in a ready-to-use disposable inhibitor-removal column (Sigma Aldrich). 3-(4,5-Dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT), fluorescein diacetate (FDA), propidiumiodinate (PI) penicillin and streptomycin were purchased from Sigma Chemical Co. (St. Louis, MO). 4T1 and HEK293 cell lines were purchased from the American Type Culture Collection (ATCC, Rockville, MD). The plasmid (encoding Renilla luciferase, pRL-CMV) was from Promega Co. (Cergy Pontoise, France), which was cloned originally from the marine organism Renilla reniformis. The plasmid DNA (pDNA) was amplified in Escherichia coli and purified according to the protocol of Qiagen GmbH (Hilden, Germany). All other chemicals were used as received and were of analytical grade.

Radioimmunoprecipitation assay (RIPA) buffer and Annexin V-FITC/PI Apoptosis Kit were purchased from Beijing Solarbio Science & Technology Co., Ltd. China.

Synthesis of MNP-PGEA Nanohybrids

10 mg of MNP was dissolved in the mixture of ethanol and water (9:1, v/v). 0.2 mL of APTES was then added into the solution and the mixture was stirred for 6 h at room temperature. Subsequently, 0.2 mL of TEA was added for another 18 h. The MNP-NH_2 product was purified by centrifugation at 10000 rpm and washed with water and ethanol for three times. Then, MNP-NH_2 was reacted with Ad-COOH with the activation of EDAC/NHS to produce MNP-Ad. In detail, 0.31 g of NHS, 0.6 g of EDAC, 0.52 g of Ad-COOH and 0.8 mL of TEA were dissolved in 5 mL of ethanol. The reaction was maintained for 4 h under stirring at 37 ^{o}C. 10 mg of MNP-NH_2 dissolved in 2 mL of ethanol was added and the solution was maintained for another 48 h. The resultant MNP-Ad was purified by centrifugation at 10000 rpm and washed three times with the mixture of ethanol and water (1:1, v/v). Cationic polymer CD-PGEA was synthesized following the procedure described in our previous work.[S1] In a typical host–guest self-assembly, 5 mg of MNP-Ad dissolved in 0.5 mL of ethanol was slowly added to the 5 mL of aqueous solution of CD-PEGA (8 and 10 mg/mL for *b*-MNP-PGEA and *s*-MNP-PGEA, respectively), and the mixture was stirred at room temperature for 24 h. The final product MNP-PGEA was collected by centrifugation.

Characterization

Transmission electron microscopy (TEM), thermal gravimetric analysis (TGA), particle size and zeta potential measurements, as well as atomic force microscopy (AFM) were used to characterize the obtained MNP, MNP-PGEA and MNP-PGEA/pDNA products. For TEM measurements, the samples was droped onto a formvar-covered copper grid, followed by drying naturally. TEM was performed

using a Tecnai G2 analytical electron microscope (FEI Company, Hillsboro, OR) operating at 200 kV. A Tarsus TG 209 F3 thermogravimetric analyzer (Netzsch, Germany) was used to determine the amount of grafted polymers on MNP-PGEA. The particle size and zeta potential of the MNP-PGEA and MNP-PGEA/pDNA complexes were measured using a Zetasizer Nano ZS (Malvern Instruments, Southborough, MA). AFM images were visualized with a Bruker Dimension Icon AFM (Bruker, Santa Barbara, CA) in the smart mode under ambient conditions. Agarose gel electrophoresis was used to test the pDNA condensation ability of MNP-PGEA nanohybrids.[S2] The electrophoresis test was carried out in TAE running buffer in a Sub-Cell system (Bio-Rad Lab, Hercules, CA). DNA bands were visualized and photographed by a UV transilluminator and BioDco-It imaging system (UVP Inc., Upland, CA).

***In vitro* cytotoxicity**

The cytotoxicity of MNP-PGEA/pDNA was evaluated in 4T1 and HEK293 cell lines. MNP-PGEA/pDNA complexes with various N/P ratios were prepared by mixing different amounts of pristine MNP-PGEA with 0.33 μg of pDNA. For MTT assay, 4T1 and HEK293 cells were cultured Dulbecco's modified eagle medium (DMEM), which was supplemented with 10% fetal bovine serum (FBS), 100 units/mL of penicillin and 100 mg/mL of streptomycin under a 5% CO_2 atmosphere with 95% relative humidity at 37 ^oC. The cells were seeded in a 96-well microtiter plate (Nunc Co., Wiesbaden, Germany) at a density of 10^4 cells per well and incubated in 100 μL of DMEM per well for 24 h. The culture media were then replaced with fresh media containing serial dilutions of MNP-PGEA/pDNA complexes at different N/P ratios and the cells were incubated for 4 h. The concentration of MNP-PGEA changed from 17.4 to 104.4 μg/mL as the N/P ratios were from 5 to 30 was. Then, the complexes

were removed completely and the cells were cultured in fresh normal medium for additional 20 h. Thereafter, 10 μL of sterile-filtered MTT stock solution in PBS (5 mg/mL) was added to each well to produce a final MTT concentration of 0.5 mg/mL. After that, the unreacted dye was removed by aspiration and 10 μL of MTT (5 mg/mL in PBS) was added to each well and cultured for 4 h. Finally, 100 μL of DMSO was added to dissolve the produced formazan crystals. Cells cultured in a medium without complexes were used as control. Bio-Rad Model 680 Microplate Reader (UK) was adopted to measure the absorbance of final product formazan at a wavelength of 570 nm.

***In vitro* gene transfection assay**

Transfection assays were performed in both 4T1 and HEK293 cell lines while plasmid pRL-CMV encoding Renilla luciferase and pEGFP-N1 encoding enhanced green fluorescent protein were used as the reporter gene. The cells were cultured for 24 h, and then the medium was replaced with 500 μL of fresh DMEM medium containing MNP-PGEA/pDNA at different N/P ratios ([MNP-PGEA] of 10.4 to 62.6 μg/mL). After 4 h, each well was replaced with 500 μL of the fresh media containing 10% FBS and cultured for another 20 h, resulting in a total of transfection time of 24 h. The cultured cells were washed twice with PBS, and 70 μL of cell lysate (Promega Co., Cergy Pontoise, France) was added. A commercial kit (Promega Co., Cergy Pontoise, France), and a luminometer (Berthold Lumat LB 9507, Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany) were used to evaluate luciferase gene expression. The gene transfection results were expressed using Relative light units (RLUs) per milligram of cell protein lysate (RLU/mg protein). The plasmid pEGFP-N1 was employed to as the reporter gene to observe the intuitive gene transfection mediated by *b*-MNP-PGEA/pDNA in 4T1 cell line. The transfected

cells were imaged using a Leica DMIL fluorescence microscope and the percentage of the EGFP-positive cells was determined from flow cytometry.

Cellular internalization

Cellular internalization was evaluated in 4T1 cell line by flow cytometry and fluorescence microscopy. The MNP-PGEA/pDNA complexes containing 125 μg of nanohybrids and 6 μg of pDNA (N/P=20) were added in the presence and absence of magnetic field. Fluorescent dye YOYO-1 was employed to label pDNA and 4',6-diamidino-2-phenylindole (DAPI) was used to stain cell nuclei.[S3] The images were obtained by a Leica DMI3000 B fluorescence microscope. To analyze the fluorescence intensity, the cells were trypsinized and analyzed by flow cytometry (MoFlo XDP, Beckman).

Photothermal effect of b-MNP-PGEA nanohybrids

To record the temperature variation of *b*-MNP-PGEA solutions with different concentrations, the solutions in a quartz cuvette were irradiated by a NIR laser at 808 nm (Daheng New Epoch Technology, Inc., Beijing, China) with a power density of 2.5 W/cm^2 for 10 min. AIR thermal camera (FLIR Systems Inc., Ohio, USA) was employed to record the temperatures at each time point. For *in vitro* photothermal experiment, 4T1 cells were first seeded into 24-well plates with 500 μL of fresh DMEM (5×10^4 cells per well) and incubated for 24 h. Then, the medium was replaced with 500 μL of fresh medium containing *b*-MNP-PGEA (41.76 μg/mL) and the cells were incubated for 4 h. The medium was replaced with fresh medium and irradiated by an 808 nm laser with an output power density of 2.5 W/cm^2 for 10 min. Thereafter, the cells were incubated with FDA and PI in a dark room for 10 min, prior to being imaged using a Leica fluorescence microscope. Meanwhile, 4T1 cells without *b*-MNP-PGEA under irradiation were stained and imaged as a control.

For *in vivo* photothermal experiments, female BALB/c nude mice (6 weeks old, weight 18-20 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., LTD (Beijing, China). Animal studies were approved by Ethical Committee of Chinese Academy of Medical Sciences (CAMS) and Peking Union Medical College and performed under legal protocols. 100 μL of 1×10^6 C6 single-cells suspension in PBS was subcutaneously injected into the back of the nude mice to prepare tumor-bearing mice. The tumor-bearing mice were taken for *in vivo* experiments when the volume of the tumors reached ~100 mm^3. 100 μL of *b*-MNP-PGEA solution (3.13 mg/mL) was intratumorally injected. After 10 min, the tumor site was irradiated by 808 nm NIR irradiation for 10 min. The whole-body infrared thermal images at different time points were obtained by an infrared camera.

Complementary PTT/gene therapy *in vitro* and *in vivo*

To evaluate the cell apoptosis induced by p53 gene transfection in 4T1 cells line, 4T1 cells were seeded in 96-well plates at a density of 10^4 cells per well in 100 μL of medium and incubated for 24 h. 100 μL of *b*-MNP-PGEA/p53 complex (6.96 μg of *b*-MNP-PGEA, 0.33 μg of p53) at the N/P ratio of 20 was added to the well and incubated for 4 h. Then 100 μL of fresh medium was added to replace the medium and incubated for another 44 h. In parallel, 4T1 cells incubated with 0.33 μg of p53 was explored as control group. In order to evaluate the effect of combined therapy of PTT/gene therapy *in vitro*, 4T1 cells were incubated with 100 μL of *b*-MNP-PGEA/p53 (6.96 μg of *b*-MNP-PGEA, 0.33 μg of p53, N/P=20) complexes for 4 h, respectively. After that, the media was replaced with fresh medium and irradiated by 808 nm laser with a power density of 2.5 W/cm^2 for 10 min and incubated for another 44 h.

For tumor therapy, the tumor bearing BALB/C nude mice were randomly divided into five groups with four mice in each group, which were treated by 100 μL of PBS (control group, G1), *b*-MNP-PGEA/p53 (313.2 μg of *b*-MNP-PGEA, 15 μg of p53, N/P=20, G2), *b*-MNP-PGEA+NIR (313.2 μg, G3), *b*-MNP-PGEA/p53+NIR (313.2 μg, G4) and *b*-MNP-PGEA/p53+NIR (M) (313.2 μg of *b*-MNP-PGEA, 15 μg of p53, N/P=20, G5), respectively. The treatments were administrated once every other day for twelve days. The tumors in G3, G4 and G5 groups were irradiated with 808 nm laser (2.5 W/cm^2, 10 min) only once after the first injection. A caliper was used to measure the sizes of tumors after treatment every two days. The volume was calculated by the formula: tumor length × (tumor width)^2)/2. Relative volume V/V_0 (V_0 as the initial tumor volume before treatment) was used to evaluate the relative tumor growth ratio. After 12 days all the mice were euthanized and the tumors were weighed, imaged, and dissected, prior to H&E and immunohistochemical analysis, which were performed according to the procedures reported previously.[S4]

MRI of b-MNP-PGEA *in vitro* and *in vivo*

Aqueous solutions of *b*-MNP-PGEA nanohybrids at different concentrations ([Fe]: 0, 0.0025, 0.05, 0.1, 0.2 mM) were first investigated to evaluate their contrast enhancement effects. For *in vitro* MRI, approximately 8×10^5 4T1 cells were seeded in 6-well plate and incubated for 24 h. Then, the cells were incubated with *b*-MNP-PGEA at different concentrations ([Fe]: 0, 5, 10, 20, and 30 μM, respectively) for 4 h with or without magnetic field. Then, the cells were washed, trypsinized, centrifuged, resuspended in 0.2 mL of PBS, and placed in Eppendorf tubes, which was placed in a self-designed scanning holder. The MRI experiments were performed on a 7.0-T MRI instrument (BioSpec 70/20 USR 7.0 T, Bruker, Germany). The following parameters were adopted: repetition time (TR) of 3000 ms; echo time (TE) of 50 ms; field of

view (FOV) read of 3.5 cm^2; matrix: 256 × 256; number of excitations (NEX): 2.0; slice thickness = 1.0 mm.

For *in vivo* MRI measurements, 4T1 tumor-bearing BALB/C nude mice were first anesthetized with pentobarbital sodium by intraperitoneal injection. 100 μL of *b*-MNP-PGEA solution (3.13 mg/mL) was intratumorally injected. MR images of tumor were obtained before and 10 min after injection. Parameters adopted: TR/TE = 3000/50 ms; FOV: 4 cm^2; matrix: 256×256; slice thickness = 1.0 mm.

Statistical analysis

All experiments were repeated at least three times. Data were presented as means ± standard deviation. Statistical significance was evaluated by using Student t-test when only two groups were compared. If more than two groups were compared, evaluation of significance was performed using one-way analysis of variance (ANOVA) followed by Bonferroni's *post hoc test*. $*p < 0.05$ was considered to be statistical significance. $**p < 0.01$ and $***p < 0.001$ were considered to be extreme significance.

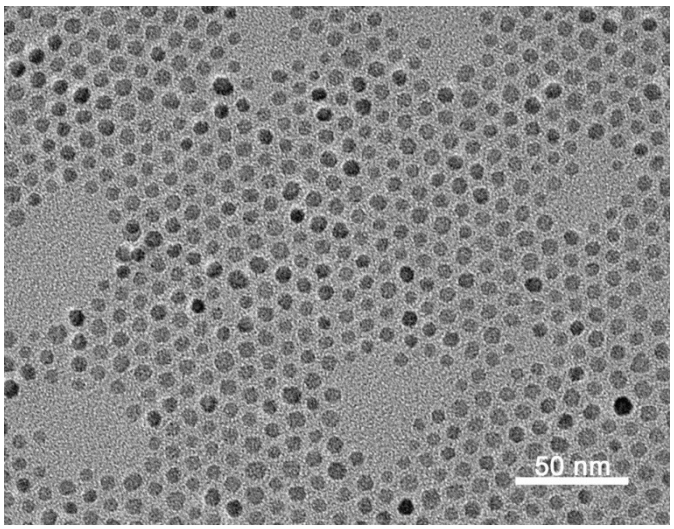

Figure S1. TEM images of the Fe_3O_4 nanoparticles.

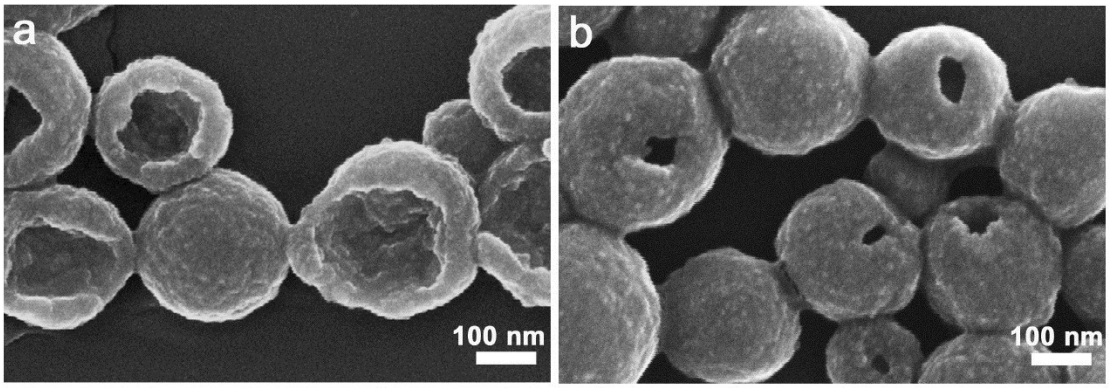

Figure S2. SEM images of (a) *b*-MNP and (b) *s*-MNP.

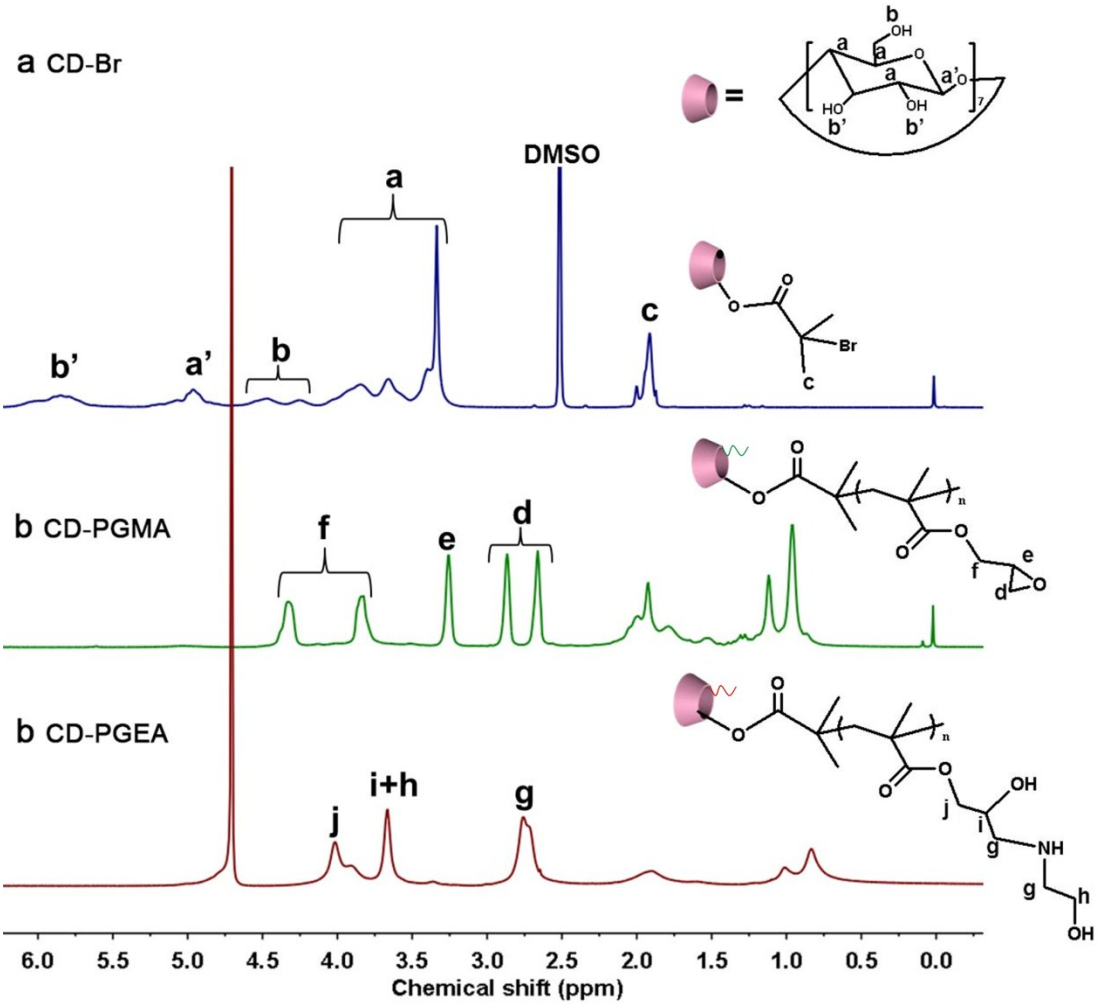

Figure S3. ^{1}H NMR spectra of (a) CD-Br in DMSO-d6, (b) CD-PGMA in $CDCl_3$, and (c) CD-PGEA in D_2O.

Molecular structures of CD-Br, CD-PGMA and CD-PGEA. ^{1}H-NMR spectra was adopted to characterize the chemical structure of CD-Br, CD-PGMA and CD-PGEA. For CD-Br (Figure S3a), the structure was confirmed by the peak at the chemical shift

of δ = 1.90 ppm (c, C(Br)-CH_3) and the peak at the chemical shift of δ = 4.90 ppm (a', O-CH-O). On the basis of the area ratio of the peak c and peak a', the substitutional ratio of hydroxyl groups of CD was determined as 2, which verified that a CD-Br core with two ATRP initiation sites was successfully synthesized. For CD-PGMA (Figure S3b), the peak at the chemical shift of δ = 3.70 ppm and 4.33 ppm (f) corresponded to the methylene protons (CH_2−O−C=O) of PGMA. The protons of epoxy groups in PGMA was associated with the peak at the chemical shift of δ = 3.23 ppm (e) and δ = 2.67, 2.82 ppm (d). The area ratio of peak e and peak d was around 1:2, which demonstrate the epoxide ring was retained during the ATRP process. The peaks associated with CD core exhibit weak peaks because of the less contribution of CD to the CD-PGMA. From gel-permeation chromatography (GPC) results, CD-PGMA with *M*n of 1.6×10^4 g/mol and polymer dispersity index (PDI) of 1.34 was obtained. For CD-PGEA (Figure S3c), the peaks of epoxide rings (e and d) disappeared and the peaks associated with methylene protons in CH_2−O−C=O shifted and combined as a single peak at δ = 4.0 ppm (j). The peaks at δ = 3.70 ppm (h+i) appear which was associated with the protons of methylidyne (CH-OH) and methylene (O-CH_2). The methylene protons (NH-CH_2) was demonstrated by the peak at δ = 2.84 ppm (g). The above ^{1}H-NMR spectra confirm the successful ring-opening reaction to produce CD-PGEA.

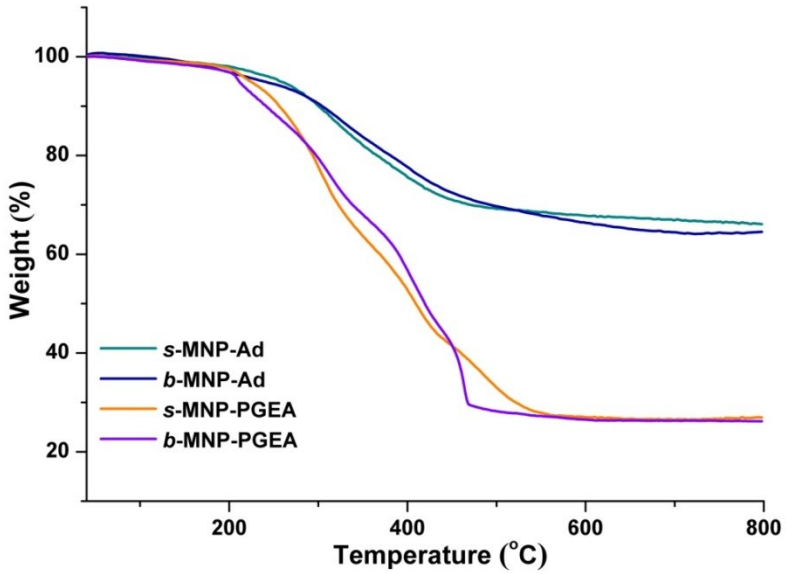

Figure S4. Thermogravimetric analysis of MNP-Ad and MNP-PGEA.

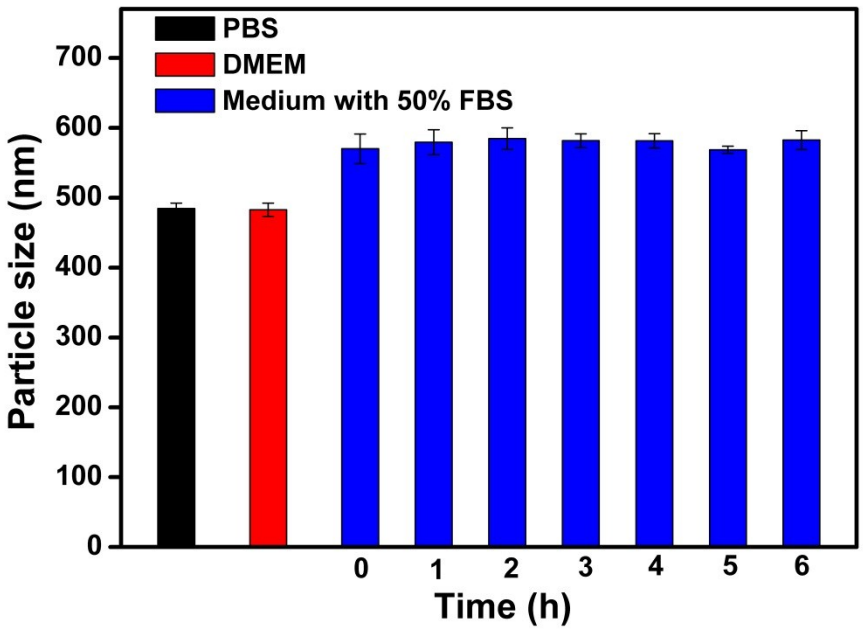

Figure S5. Hydrodynamic particle size of *b*-MNP-PGEA in PBS, DMEM and medium with 50% fetal bovine serum (FBS) (mean ± SD, n=3).

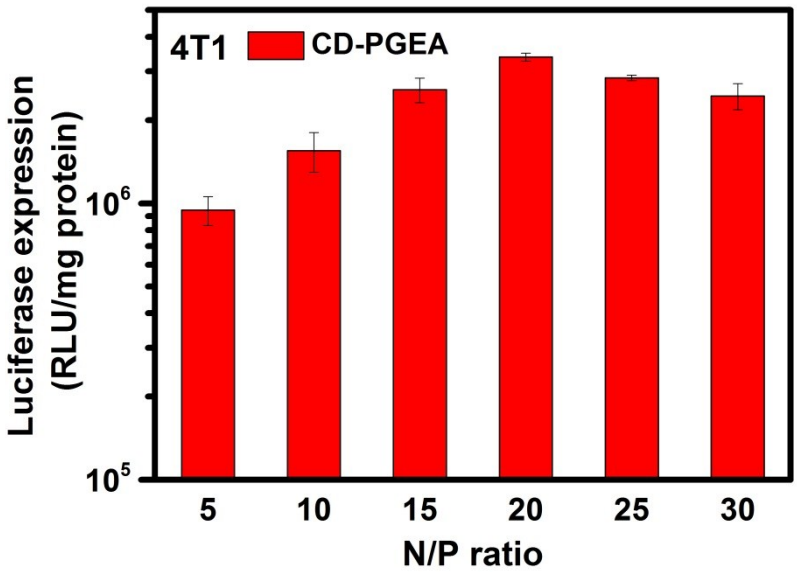

Figure S6. Luciferase gene transfection efficiency assay mediated by the CD-PGEA/pDNA complexes at various N/P ratios.

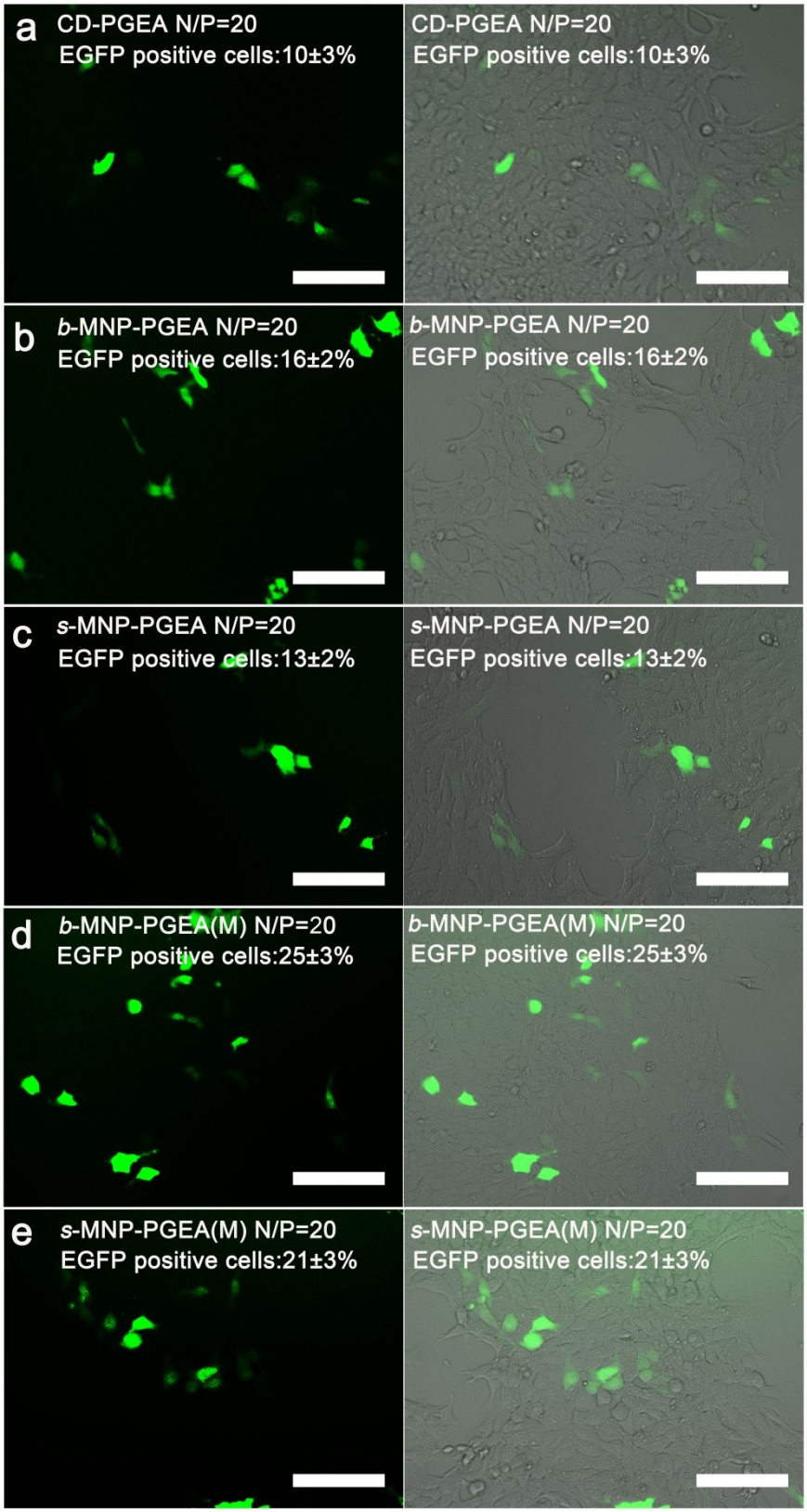

Figure S7. Representative microscopic images of EGFP gene expression mediated by (a) CD-PGEA, (b) *b*-MNP-PGEA, (c) *s*-MNP-PGEA, (d) *b*-MNP-PGEA(M), and (e) *s*-MNP-PGEA(M) at the N/P ratio of 20 in 4T1 cell line. Scale bar: 100 μm.

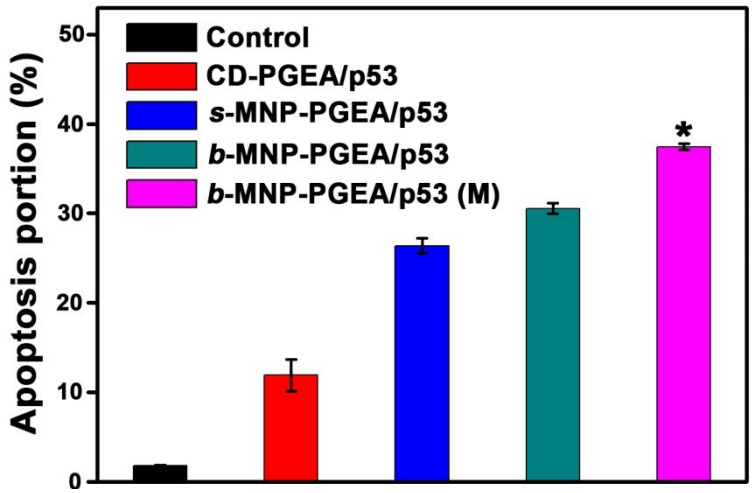

Figure S8. Percentage of apoptotic cells after 96 h transfection (mean ± SD, $n = 3$, $^*p < 0.01$).

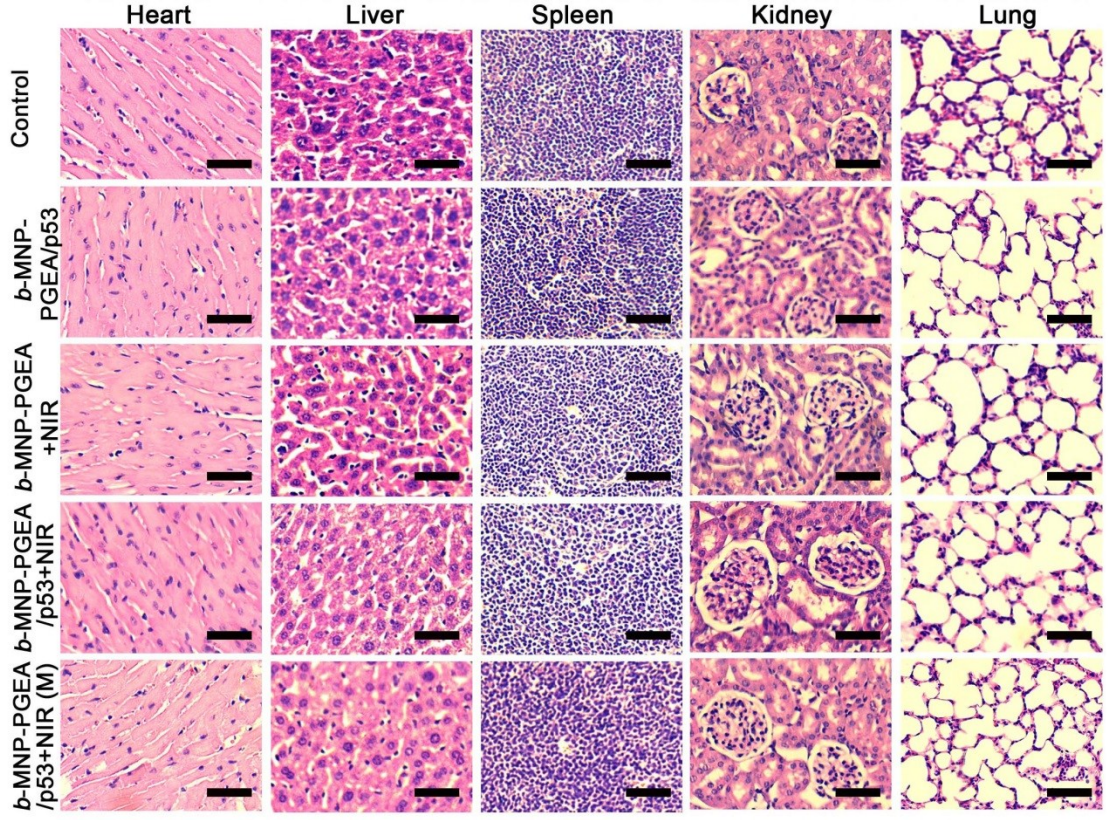

Figure S9. Histology analysis of major organs (hear, liver, spleen, kidney, and lung) of different groups of mice. Scale bars: 50 μm.

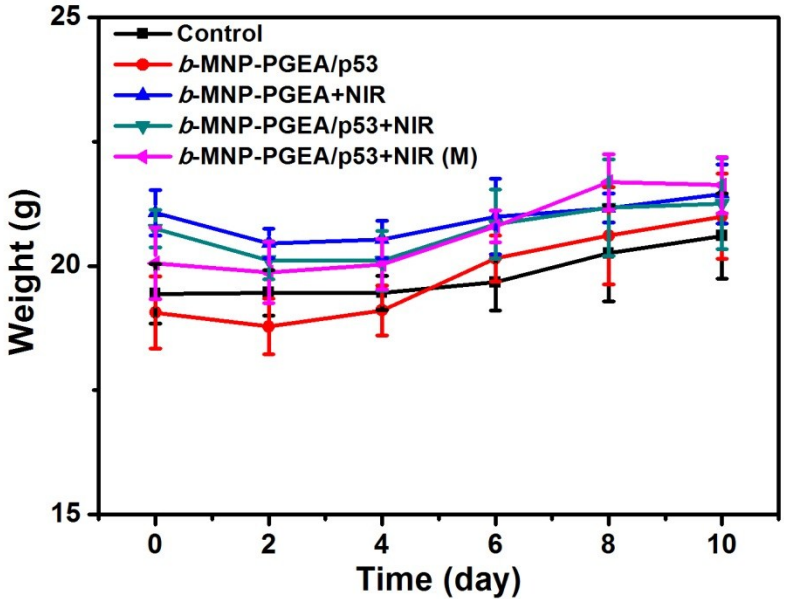

Figure S10. Body weight evolution of tumor-bearing mice following different administration. (n = 4)

References

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S3 Y. Hu, W. Yuan, N. Zhao, J. Ma, W. T. Yang and F. J. Xu, *Biomaterials*, 2013, **34**, 5411.

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