Supporting Information

for

Tumor Microenvironment-Responsive Multistaged Nanoplatform for Systemic RNAi and Cancer Therapy

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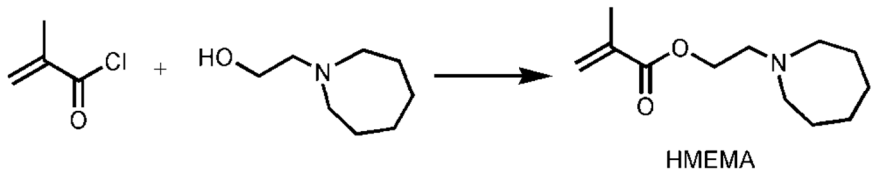
1. Materials

2-(Hexamethyleneimino) ethanol, methacryloyl chloride, and hydroquinone were purchased from Alfa Aesar Company and used directly. α-Bromoisobutyryl bromide, *N,N′*-dimethylformamide (DMF), triethylamine (TEA), *N,N,N′,N′,N′*-pentamethyldiethylenetriamine (PMDETA), copper (I) bromide (CuBr), isopropyl alcohol, dichloromethane (DCM), tetrahydrofuran (THF), and diethyl ether were provided by Sigma-Aldrich and used as received. Methoxyl-polyethylene glycol (Meo-PEG_{113}-OH) was purchased from JenKem Technology. Tumor-targeting and cell-penetrating peptide-amphiphiles (TCPA1: $C_{17}H_{35}CONH$-GR_8GRGDS-OH; TCPA2: $C_{17}H_{35}CONH$-($C_{17}H_{35}CONH$)-KR_8GRGDS-OH) were obtained from GL Biochem Ltd. 2-Aminoethyl methacrylate (AMA) were purchased from Polyscience Company. Cyanine5.5 NHS ester was purchased from Lumiprobe. Lipofectamine 2000 (Lipo2K) was purchased from Invitrogen. Steady-Glo luciferase assay system was provided by Promega. Fluorescent dye DY677-labelled siLuc and siBRD4 were acquired from GE Dharmacon. The siRNA sequences are as follows: siLuc, 5'-CUU ACG CUG AGU ACU UCG AdTdT-3' (sense) and 5'-UCG AAG UAC UCA GCG UAA GdTdT-3' (antisense); siBRD4, 5'-AAA CAC AAC UCA AGC AUC GUU -3' (sense) and 5'-CGA UGC UUG AGU UGU GUU UUU-3' (antisense). DY677 was labelled at the 5'-end of both the sense and antisense strands of siLuc. Fluorescein and its quencher (Dabcyl)-labelled siLuc was also provided by GE Dharmacon. Fluorescein was labelled at the 5'-end of the sense strand and Dabcyl was labelled at 3'-end of the antisense strand. HeLa cells stably expressing firefly luciferase (Luc-HeLa) were obtained from Alnylam Pharmaceuticals, Inc. The cells were incubated in RPMI 1640 medium (Invitrogen) with 10% fetal bovine serum (FBS, Sigma-Aldrich). All other reagents and solvents are of analytical grade and used without further purification.

2. Synthesis of 2-(hexamethyleneimino) ethyl methacrylate (HMEMA)

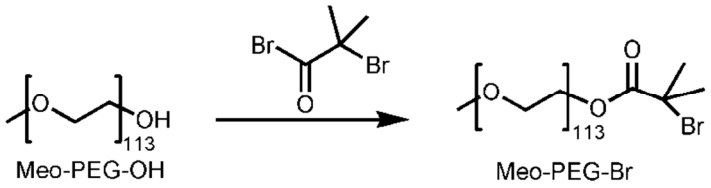
HMEMA was synthesized according previous report [1]. In brief, 2-(hexamethyleneimino) ethanol (0.1 mol, 14.3 g), TEA (0.12 mol, 12.1 g), and inhibitor hydroquinone (0.001 mol, 0.11 g) were dissolved in 100 mL of THF and then methacryloyl chloride (0.1 mol, 10.5 g) was added dropwise. After refluxing for 2 h, the precipitation was removed and the THF solvent was removed by rotary evaporator. The resulting residue was distilled under vacuum as a colorless liquid. The synthesis of HMEMA is shown in Scheme S1. The ^{1}HNMR spectrum of HMEMA is shown in Figure S1.



Scheme S1. Synthesis route of HMEMA.

3. Synthesis of Meo-PEG-Br

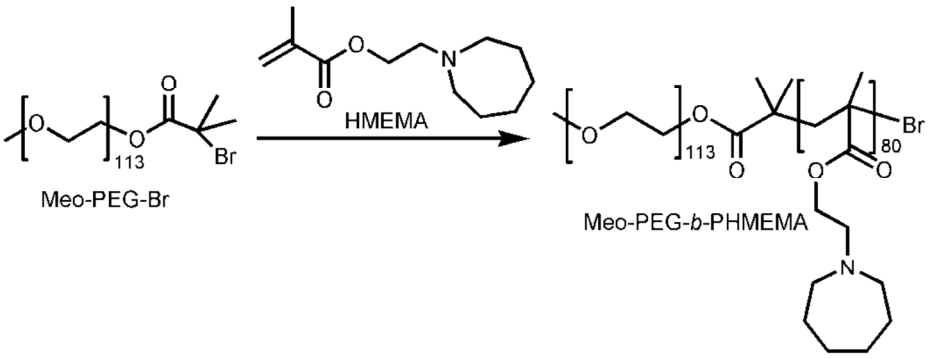
Meo-PEG_{113}-OH (8 g, 1.6 mmol) and TEA (1.3 mL, 9.6 mmol) were dissolved in 250 mL of DCM. In an ice-salt bath, α-bromoisobutyryl bromide (l mL, 8 mmol) dissolved in 10 mL of DCM was added dropwise. After stirring for 24 h, the mixture was washed with 1 M NaOH (3 × 50 mL), 1 M HCl (3 × 50 mL), and deionized water (3 × 50 mL). After drying over anhydrous $MgSO_4$, the solution was concentrated, and cold ether was added to precipitate the product. After re-precipitating thrice and drying under vacuum, the product was collected as white powder. The synthesis of Meo-PEG-Br is shown in Scheme S2. The ^{1}HNMR spectrum of Meo-PEG-Br is shown in Figure S2.



Scheme S2. Synthesis route of Meo-PEG-Br.

4. Synthesis of methoxyl-polyethylene glycol-*b*-poly (2-(hexamethyleneimino) ethyl methacrylate) (Meo-PEG-*b*-PHMEMA)

Meo-PEG-*b*-PHMEMA block copolymer was synthesized by atom transfer radical polymerization (ATRP). HMEMA (12 mmol), Meo-PEG-Br (0.15 mmol), and PMDETA (0.15 mmol) were added to a polymerization tube. DMF (3 mL) and 2-propanol (3 mL) were then added to dissolve the monomer and initiator. After three cycles of freeze-pump-thaw to remove oxygen, CuBr (0.15 mmol) was added under nitrogen atmosphere and the polymerization tube was sealed under vacuum. After polymerization at 40 ^{o}C for 24 h, tetrahydrofuran (THF) was added to dilute the product, which was then passed through a neutral Al_2O_3 column to remove the catalyst. The resulting THF solution was concentrated and the residue was dialyzed against THF, followed by deionized water. The expected polymer was collected as a white powder after freeze-drying under vacuum. The synthesis of Meo-PEG-*b*-PHMEMA is shown in Scheme S3. The ^{1}HNMR spectrum is shown in Figure S3. The molecular weight was determined by gel permeation chromatography (GPC) using THF as eluent. $M_{n,\ GPC} = 2.34 \times 10^4$ (PDI = 1.25); $M_{n,NMR} = 2.15 \times 10^4$.



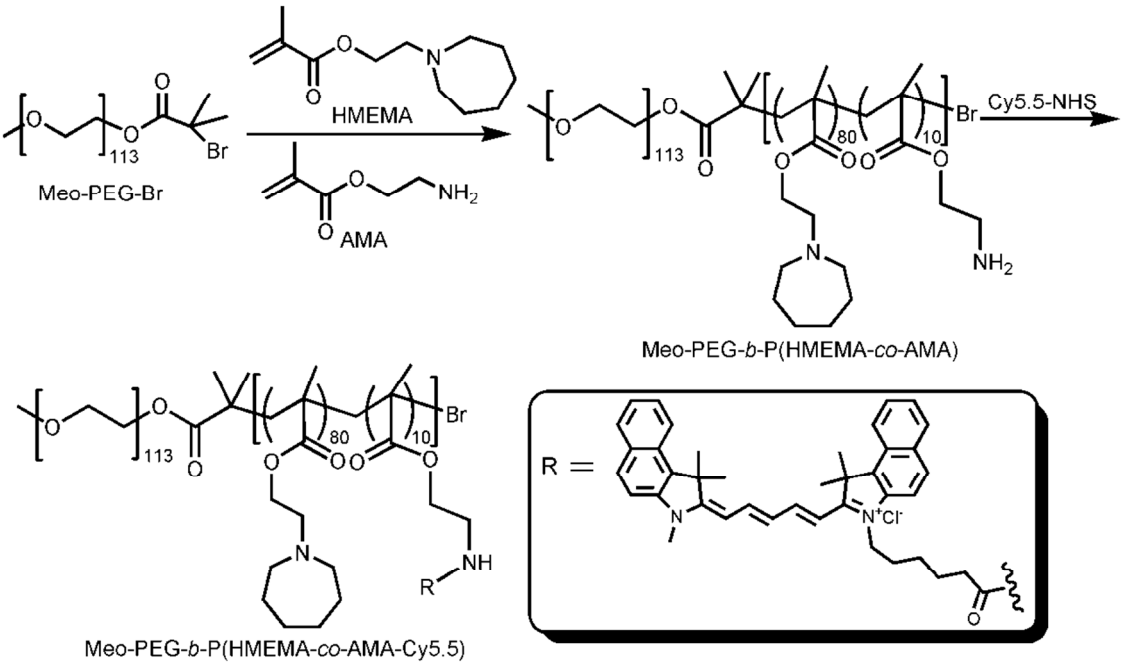
Scheme S3. Synthesis route of Meo-PEG-*b*-PHMEMA.

5. Synthesis of methoxyl-polyethylene glycol-*b*-poly (2-(hexamethyleneimino) ethyl methacrylate-*co*-2-aminoethyl methacrylate) (Meo-PEG-*b*-P(HMEMA-*co*-AMA))

Meo-PEG-*b*-P(HMEMA-*co*-AMA) copolymer was synthesized by ATRP. HMEMA (6 mmol), Meo-PEG-Br (0.075 mmol), and PMDETA (0.075 mmol) were added to a polymerization tube. DMF (1.5 mL) and 2-propanol (1.5 mL) were then added to dissolve the monomer and initiator. After three cycles of freeze-pump-thaw to remove oxygen, CuBr (0.075mmol) was added under nitrogen atmosphere and the polymerization tube was sealed under vacuum. After polymerization at 40 ^{o}C for 24 h, tetrahydrofuran (THF) was added to dilute the product, which was then passed through a neutral Al_2O_3 column to remove the catalyst. The resulting THF solution was concentrated and the residue was dialyzed against THF, followed by deionized water. The expected polymer was collected as a white powder after freeze-drying under vacuum. The synthesis of Meo-PEG-*b*-P(HMEMA-*co*-AMA) is shown in Scheme S4. The ^{1}HNMR spectrum is shown in Figure S4. The molecular weight was determined by gel permeation chromatography (GPC) using THF as eluent. $M_{n,\,GPC} = 2.42 \times 10^4$ (PDI = 1.33); $M_{n,NMR} = 2.23 \times 10^4$.

6. Synthesis of Meo-PEG-*b*-P(HMEMA-*co*-AMA-Cy5.5)

Meo-PEG-*b*-P(HMEMA-*co*-AMA) (0.5 g) and Cy5.5 NHS ester (1.5-fold molar excess relative to the AMA repeating unit) were well dissolved in 15 mL of DMF. After constantly stirring in dark for 48 h, the solution was dialyzed against DMF for 48 h followed deionized water for 72 h. The product was collected after freeze-drying. The synthesis of Meo-PEG-*b*-P(HMEMA-*co*-AMA-Cy5.5) is shown in Scheme S4.



Scheme S4. Synthesis route of Meo-PEG-*b*-P(HMEMA-*co*-AMA) and Meo-PEG-*b*-P(HMEMA-*co*-AMA-Cy5.5).

7. Gel permeation chromatography (GPC)

Number- and weight-average molecular weights (M_n and M_w, respectively) of the polymers were determined by a gel permeation chromatographic system equipped with a Waters 2690D separations module and a Waters 2410 refractive index detector. THF was used as the eluent at a

flow rate of 0.3 mL/min. Waters millennium module software was used to calculate molecular weight based on a universal calibration curve generated by polystyrene standard of narrow molecular weight distribution.

8. ^{1}H Nuclear magnetic resonance (^{1}HNMR)

The ^{1}HNMR spectra of the polymers were recorded on a Mercury VX-300 spectrometer at 400 MHz (Varian, USA), using $CDCl_3$ as a solvent and TMS as an internal standard.

9. Acid-base titration

Meo-PEG-*b*-PHMEMA was dispersed in deionized water, and a concentrated HCl aqueous solution was added until the copolymer was completely dissolved (1 mg/mL). Subsequently, 1 M NaOH aqueous solution was added in 1-5 μL increments. After each addition, the solution was constantly stirred for 3 min, and the solution pH was measured using a pH meter. The pK_a of the copolymer was determined as the pH at which 50% of the copolymer turns ionized.

10. Evaluation of pH sensitivity

A DMF solution of Meo-PEG-*b*-PHMEMA (5 mg/mL) and Meo-PEG-*b*-P(HMEMA-*co*-AMA-Cy5.5) (5 mg/mL) was mixed in a volume ratio of 1:1. Under vigorously stirring (1000 rpm200 μL of the mixture was added dropwise to 5 mL of deionized water. After collection and purification using ultrafiltration device (EMD Millipore, MWCO 100 kDa), the NPs formed were dispersed in 1 mL of phosphate buffered saline (PBS) at different pHs. Three minutes later, the fluorescence intensity with an excitation of 675 nm was measured on a Synergy HT multi-mode microplate reader (BioTek Instruments). The normalized fluorescence intensity (NFI) vs.

pH profile was used to quantitatively assess the pH responsiveness. NFI is calculated as follows:

$$NFI = (F - F_{min}) / (F_{max} - F_{min})$$

where F is the fluorescence intensity of the NPs at any given pH value and F_{max} and F_{min} are the maximal and minimal fluorescence intensity of the NPs, respectively.

11. Preparation of the siRNA loaded nanoparticles (NPs) and siRNA-TCPA complexes

Meo-PEG-*b*-PHMEMA was dissolved in DMF to form a homogenous solution with a concentration of 10 mg/mL. Subsequently, a mixture of 1 nmol siRNA (0.1 nmol/μL aqueous solution) and TCPA (5 mg/mL in DMF) in an N/P molar ratio of 1:20 was prepared and mixed with 200 μL of Meo-PEG-*b*-PHMEMA solution. Under vigorously stirring (1000 rpm), the mixture was added dropwise to 5 mL of deionized water. The NP dispersion formed was transferred to an ultrafiltration device (EMD Millipore, MWCO 100 K) and centrifuged to remove the organic solvent and free compounds. After washing with PBS buffer (pH 7.4) (3 × 5 mL), the siRNA loaded NPs were dispersed in 1 mL of PBS buffer (pH 7.4).

To prepare the siRNA-TCPA complexes, a mixture of 1 nmol siRNA (0.1 nmol/μL aqueous solution) and TCPA (5 mg/mL in DMF) in an N/P molar ratio of 1:20 was prepared and then added to rapidly stirring (1000 rpm) deionized water.

12. Characterizations of NPs

Size and zeta potential were determined by dynamic light scattering (DLS, Brookhaven Instruments Corporation). The morphology of NPs was visualized on a Tecnai G^2 Spirit BioTWIN transmission electron microscope (TEM). Before observation, the sample was stained with 1% uranyl acetate and dried under air. To determine siRNA encapsulation efficiency (EE%),

DY677-labelled siLuc (DY677-siRNA) loaded NPs were prepared according to the method aforementioned. A small volume (5 μL) of the NP solution was withdrawn and mixed with 20-fold DMSO. The standard was prepared by mixing 5 μL of naked DY677-siRNA solution (1 nmol/mL in pH 7.4 PBS buffer) with 20-fold DMSO. The fluorescence intensity of DY677-siRNA was measured using a microplate reader and the siRNA EE% is calculated as: EE% = $(FI_{NPs} / FI_{Standard}) \times 100$.

13. Digestion assay

NPs loaded with fluorescein- and Dabcyl-labelled siLuc were prepared according to the method aforementioned, and then dispersed in 1 mL of PBS buffer at pH 7.4 or 6.8. Subsequently, 20 U RNase was added and the sample was incubated in 37 ^{o}C. At predetermined time intervals, the fluorescent emission spectra were examined using a microplate reader with excitation at 480 nm and emission data range between 490 and 650 nm.

14. Gel retardation assay

The DY677-siRNA-loaded NPs and DY677-siRNA-TCPA complexes were prepared as described above. Then the siRNA-loaded NPs and siRNA-TCPA complexes were incubated with PBS at different pHs for 30 min, and then separated on 2% E-Gel precast agarose gels (Life Technologies). To observe the siRNA migration, the gels were visualized under DY677 channel using the Maestro 2 In-Vivo Imaging System (Cri Inc) and compared to naked DY677-siRNA.

15. *In vitro* siRNA release

DY677-siRNA-loaded NPs were prepared as described above. Subsequently, the NPs were

dispersed in 1 mL of PBS (pH 7.4) and then transferred to a Float-a-lyzer G2 dialysis device (MWCO 100 kDa, Spectrum) that was immersed in PBS buffer (pH 7.4 or 6.8) at 37 ^{o}C. At a predetermined interval, 5 μL of the NP solution was withdrawn and mixed with 20-fold DMSO. The fluorescence intensity of DY677- siRNA was determined using a microplate reader.

16. Cell culture

Luc-HeLa and prostate cancer cells (LNCaP, PC3, DU145 and 22RV1) were incubated in RPMI1640 medium (pH 7.4) with 10% FBS at 37 ^{o}C in a humidified atmosphere containing 5% CO_2.

17. Confocal laser scanning microscope (CLSM)

Luc-HeLa (50,000 cells) were seeded in round discs and incubated in 2 mL of RPMI1640 medium (pH7.4) containing 10% FBS for 24 h. After replacing the medium with 2 mL of fresh medium at pH 7.4 or 6.8, DY677-siRNA-loaded NPs were added, and the cells were allowed to incubate for 2 h. After removing the medium and subsequently washing with PBS buffer (pH 7.4) thrice, lysotracker green was added to stain the endosomes and the nuclei were stained by Hoechst 33342. The uptake of siRNA loaded NPs were viewed under a FV1000 CLSM (Olympus).

18. Flow cytometry

Luc-HeLa (50,000 cells) were seeded in 6-well plate and incubated in 2 mL of RPMI1640 medium (pH 7.4) containing 10% FBS for 24 h. After replacing the medium with 2 mL of fresh medium at pH 7.4 or 6.8, DY677-siRNA-loaded NPs were added to three independent wells, and

the cells were allowed to incubate for 2 h. After removing the medium and subsequently washing with PBS buffer (pH 7.4) thrice, all the cells were trypsinized and combined together for flow cytometry (FACS) quantitative analysis (DXP11 Analyzer). For the FACS analysis, 20,000 cells were analyzed and the MFI of these cells was automatically calculated by the software.

19. Luc silencing

Luc-HeLa cells were seeded in 96-well plates (5,000 cells per well) and incubated in 0.1 mL of RPMI1640 medium (pH 7.4) with 10% FBS for 24 h. Thereafter, the medium was replaced by fresh medium at siLuc-loaded NPs were added. After 24 h incubation, the cells were washed with PBS buffer (pH 7.4) and allowed to incubate in fresh medium (pH 7.4) for another 48 h. The Luc expression in HeLa cells was determined using Steady-Glo luciferase assay kits. Cytotoxicity was measured using AlamarBlue assay according to the manufacturer's protocol. The luminescence or fluorescence intensity was measured using a microplate reader, and the average value of five independent experiments was collected.

20. *In vitro* BRD4 silencing

LNCaP cells were seeded in 6-well plates (50,000 cells per well) and incubated in 2 mL of RPMI1640 medium (pH 7.4) containing 10% FBS for 24 h. Subsequently, the medium was replaced by fresh medium at pH 7.4 or 6.8, and then siBRD4 loaded NPs were added to three independent wells. After incubation for 24 h, the cells were washed with PBS buffer (pH 7.4) and further incubated in fresh medium (pH 7.4) for another 48 h. Thereafter, all the cells of three independent wells were combined and the proteins were extracted using modified radioimmunoprecipitation assay lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40

substitute, 0.25% sodium deoxycholate, 1mM sodium fluoride, 1mM Na_3VO_4, 1mM EDTA), supplemented with protease inhibitor cocktail and 1 mM phenylmethanesulfonyl fluoride (PMSF). The BRD4 expression was examined using the western blot analysis described below.

21. Western blot analysis

Equal amounts of protein, as determined with a bicinchoninic acid (BCA) protein assay kit (Pierce/Thermo Scientific) according to the manufacturer's instructions, were added to SDS-PAGE gels and separated by gel electrophoresis. After transferring the proteins from gel to polyvinylidene difluoride membrane, the blots were blocked with 3% BSA in TBST (50 mM Tris-HCl pH 7.4, 150 mM NaCl, and 0.1% Tween 20) and then incubated with a mixture of BRD4 rabbit antibody (Abcam) and beta-actin rabbit antibody (Cell Signaling). The BRD4 expression was detected with horseradish peroxidase (HRP)-conjugated secondary antibody (anti-rabbit IgG HRP-linked antibody, Cell Signaling) and an enhanced chemiluminescence (ECL) detection system (Pierce).

22. Immunofluorescence staining

LNCaP cells (50,000 cells) were seeded in round disc and incubated in 2 mL of RPMI1640 medium (pH 7.4) containing 10% FBS for 24 h. After replacing the medium with fresh medium (pH 7.4 or 6.8), siBRD4 loaded NPs were added and the cells were allowed to incubated for 24 h. Subsequently, the cells were washed with PBS buffer (pH 7.4) and fresh medium (pH 7.4) was added. After 48 h incubation, the cells were fixed with 4% paraformaldehyde. The cells were then permeabilized by incubation in 0.2% Triton X-100 in PBS buffer (pH 7.4) for 5 minutes, followed by washing with pH 7.4 PBS buffer (3 × 5 min). Thereafter, the cells were blocked with

blocking buffer (2% normal goat serum, 2% BSA, and 0.2% gelatin in pH 7.4 PBS buffer) at room temperature for 1 h. After washing the cells with pH 7.4 PBS buffer (3 × 5 min), BRD4 rabbit antibody (Abcam) diluted in 1% BSA solution was added and the cells were incubated for 1 h. Subsequently, the cells were with pH 7.4 PBS buffer (3 × 5 min), and then further incubated with Alex Fluro 647-linked secondary antibody and Alex Fluro 488-conjugated phalloidin for another 1 h. After washing with pH 7.4 PBS buffer (3 × 5 min), the cells were viewed under a FV1000 CLSM.

23. Apoptosis analysis

LNCaP cells were seeded in 6-well plates (50,000 cells per well) and incubated in 2 mL of RPMI1640 medium (pH 7.4) containing 10% FBS for 24 h. Subsequently, the medium was replaced by fresh medium at pH 7.4 or 6.8, and then siBRD4 loaded NPs were added to three independent wells. After incubation for 24 h, the cells were washed with PBS buffer (pH 7.4) and further incubated in fresh medium (pH 7.4) for another 48 h. Thereafter, all the cells were trypsinized and combined together for 7-amino-actinomycin (7-AAD) and PE Annexin V staining using PE Annexin V Apoptosis Detection Kit I (BD Pharmingen™). The apoptosis analysis was performed using a DXP11 Flow Cytometry Analyzer. During the FACS analysis, 20,000 cells were analyzed for apoptosis analysis.

24. *In vitro* cell proliferation

LNCaP cells were seeded in 6-well plates (20,000 cells per well) and incubated in 2 mL of RPMI1640 medium (pH 7.4) containing 10% FBS for 24 h. Thereafter, cell number was measured by AlamarBlue assay at day 0 according to the manufacturer's protocol. After

removing the Alamarblue assay agent and adding 2 mL of fresh medium, the cells were treated with the siBRD4-loaded NPs at pH 7.4 or 6.8 for 24 h and then washed with PBS buffer (pH 7.4) for further incubation with fresh medium. At predetermined intervals (day 2, 4, and 6), the cell number was measured by AlamarBlue assay. After each measurement, the AlamarBlue assay agent was removed and 2 mL of fresh medium (pH 7.4) was replaced for further incubation. Because the AlamarBlue assay agent is non-toxic, cell proliferation can be continuously monitored in real time before cells reaching high confluency (> 90%). The average value of three independent experiments was collected and cell proliferation rate at each time point was calculated as follows:

$$\text{Proliferation rate at day x} = (\text{Cell number at day x})/(\text{Cell number at day 0})$$

25. Animals

Healthy male BALB/c mice and Athymic nude mice (4-5 weeks old) were purchased from Charles River Laboratories. All *in vivo* studies were performed in accordance with National Institutes of Health animal care guidelines and in strict pathogen-free conditions in the animal facility of Brigham and Women's Hospital. Animal protocol was approved by the Institutional Animal Care and Use Committees on animal care (Harvard Medical School).

26. Pharmacokinetics study

Healthy male BALB/c mice were randomly divided into three groups (n = 3) and given an intravenous injection of either (i) naked DY677-siRNA, (ii) DY677-siRNA-TCPA2 complexes or (iii) DY677-siRNA-loaded NPs at a 1 nmol siRNA dose per mouse. At predetermined time intervals, orbital vein blood (20 μL) was withdrawn using a tube containing heparin, and the

wound was pressed for several seconds to stop the bleeding. The fluorescence intensity of DY677-labelled siRNA in the blood was determined by microplate reader. The blood circulation half-life ($t_{1/2}$) was calculated according to previous report [2].

27. LNCaP xenograft tumor model

LNCaP xenograft tumor model was constructed by subcutaneous injection with 200 μL of LNCaP cell suspension (a mixture of RPMI 1640 medium and Matrigel in 1:1 volume ratio) with a density 1×10^7 cells/mL into the back region of healthy male Athymic nude mice. When the volume of the LNCaP tumor xenograft reached ~70 mm^3, the mice were used for the following *in vivo* experiments.

28. Biodistribution

LNCaP tumor-bearing male Athymic nude mice were randomly divided into three groups (n = 3) and given an intravenous injection of either (i) naked DY677-siRNA, (ii) DY677-siRNA-TCPA2 complexes or (iii) DY677-siRNA-loaded NPs at a 1 nmol siRNA dose per mouse. Twenty-four hours after the injection, the mice were imaged using the Maestro 2 In-Vivo Imaging System (Cri Inc). Organs and tumors were then harvested and imaged. To quantify the accumulation of NPs in tumors and organs, the fluorescence intensity of each tissue was quantified by Image-J.

29. *In vivo* BRD4 silencing

LNCaP tumor-bearing male Athymic nude mice were randomly divided into two groups (n = 3) and intravenously injected with (i) siLuc-loaded NPs or (ii) siBRD4-loaded NPs for three consecutive days. Twenty-four hours after the final injection, mice were sacrificed and tumors

were harvested for western blot analysis, and immunohistochemistry and TUNEL staining. For the western blot analysis, the proteins in the tumor were extracted using modified radioimmunoprecipitation assay lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40 substitute, 0.25% sodium deoxycholate, 1mM sodium fluoride, 1mM Na_3VO_4, 1mM EDTA), supplemented with protease inhibitor cocktail and 1 mM phenylmethanesulfonyl fluoride (PMSF). Western blot was performed according to the method described above.

30. Immunohistochemistry (IHC) staining

IHC staining was performed on formalin-fixed paraffin-embedded tumor sections. Briefly, tumor slides were first heated to 60 ^{o}C for 1 h, desparaffinized with xylene (3 × 5 min), and washed with different concentrations of alcohol. After retrieval of antigen using DAKO target retrieval solution at 95-99 oC for 40 min, followed by washing, the slides were blocked with peroxidase blocking buffer (DAKO Company) for 5 min. After washing buffer (DAKO Company), the slides were incubated with BRD4 rabbit antibody (Abcam) diluted in DAKO antibody solution for 1 h. The slides were then washed and incubated with peroxidase-labeled polymer for 30 min. After washing and staining with DAB+ substrate-chromogen solution and hematoxylin, the slides we remounted and viewed under a MVX10 MacroView Dissecting scope equipped with OlympusDP80 camera.

31. Immune response

Healthy male BALB/c mice were randomly divided into three groups (n = 3) and given an intravenous injection of either (i) PBS, (ii) naked siBRD4 or (iii) siBRD4 loaded NPs at a 1 nmol siRNA dose per mouse. Twenty-four hours after injection, blood was collected and serum

isolated for measurements of representative cytokines (TNF-α, IL-6, IL-12, and IFN-γ) by enzyme-linked immunosorbent assay or ELISA (PBL Biomedical Laboratories and BD Biosciences) according to the manufacturer's instructions.

32. Histology

Healthy male BALB/c mice were randomly divided into three groups (n = 3) and administered daily intravenous injections of either (i) PBS, (ii) naked siBRD4 or (iii) siBRD4-loaded NPs at a 1 nmol siRNA dose per mouse. After three consecutive injections, the main organs were collected 24 h post the final injection, fixed with 4% paraformaldehyde, and embedded in paraffin. Tissue sections were stained with hematoxylin-eosin (H&E) and then viewed under an optical microscope.

33. Inhibition of tumor growth

LNCaP tumor-bearing male Athymic nude mice were randomly divided into four groups (n = 5) and intravenously injected with (i) PBS, (ii) naked siBRD4, (iii) siLuc-loaded NPs, or (iv) siBRD4-loaded NPs at a 1 nmol siRNA dose per mouse once every three days. All the mice were administrated by administered four consecutive injections and the tumor growth was monitored every two days by measuring perpendicular diameters using a caliper and tumor volume was calculated as follows:

$$V = W^2 \times L/2$$

where W and L are the shortest and longest diameters, respectively.

34. Statistical analysis

Statistical significance was determined by a two-tailed Student's t test assuming equal variance. A p value < 0.05 is considered statistically significant.

References

[1] Zhou K, Wang Y, Huang X, Luby-Phelps K, Sumer BD, Gao J. *Angew. Chem. Int. Ed.* **2011**, *50*, 6109-6114.

[2] Winter H, Ginsberg A, Egizi E, Erondu N, Whitney K, Pauli E, Everitt D. *Antimicrob. Agents Chemother.* **2013**, *57*, 5516-5520.

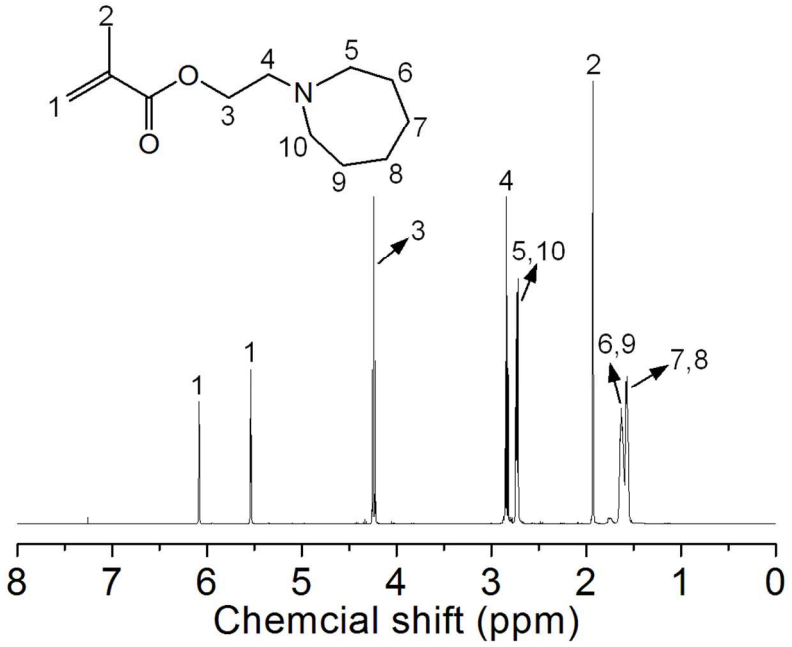


Figure S1. ^{1}HNMR spectrum of HMEMA in $CDCl_3$.

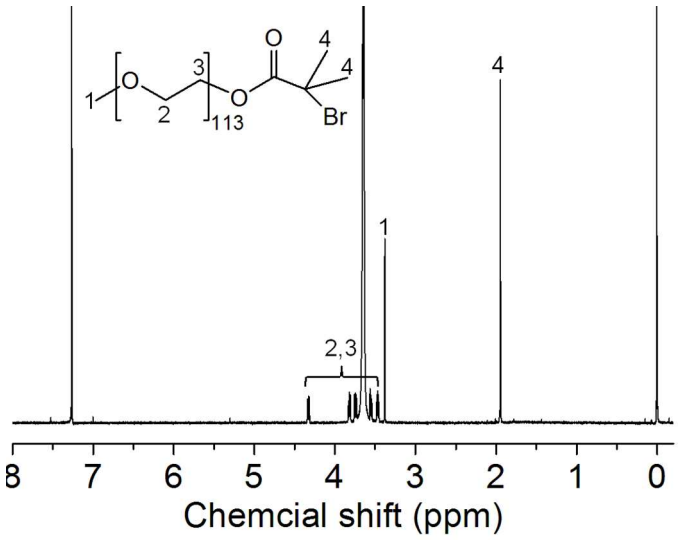


Figure S2. ^{1}HNMR spectrum of Meo-PEG-Br in $CDCl_3$.

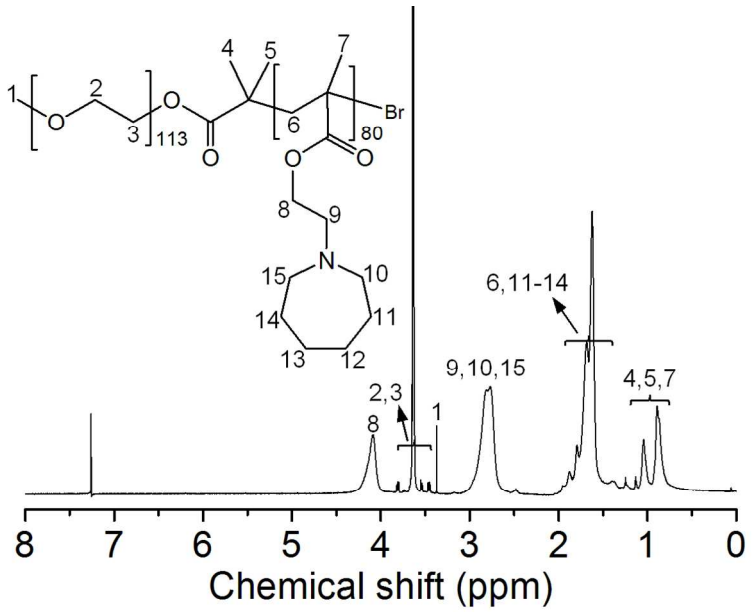


Figure S3. ^{1}HNMR spectrum of Meo-PEG-*b*-PHMEMA in $CDCl_3$.

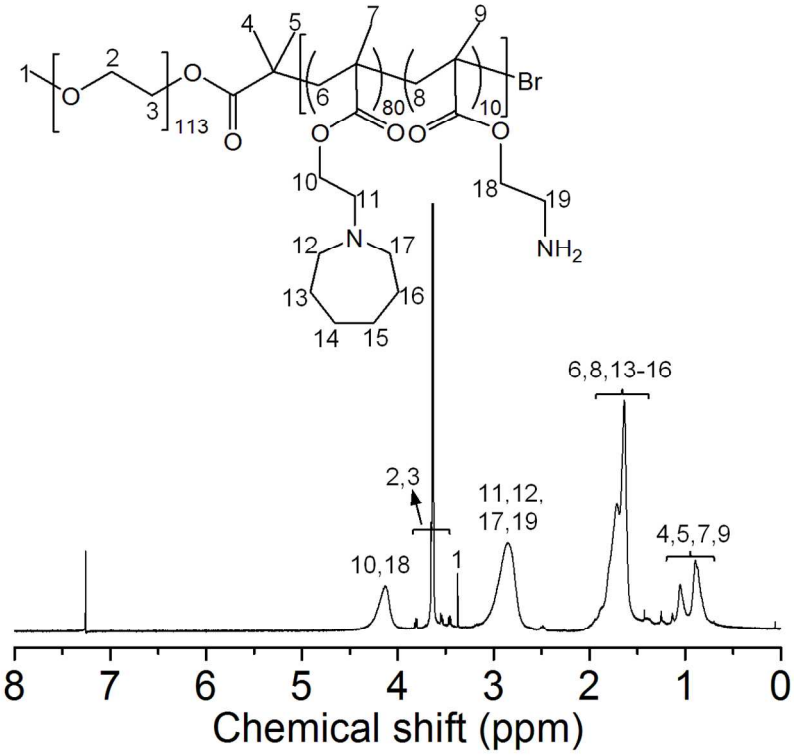


Figure S4. ^{1}HNMR spectrum of Meo-PEG-*b*-P(HMEMA-*co*-AMA) in $CDCl_3$.

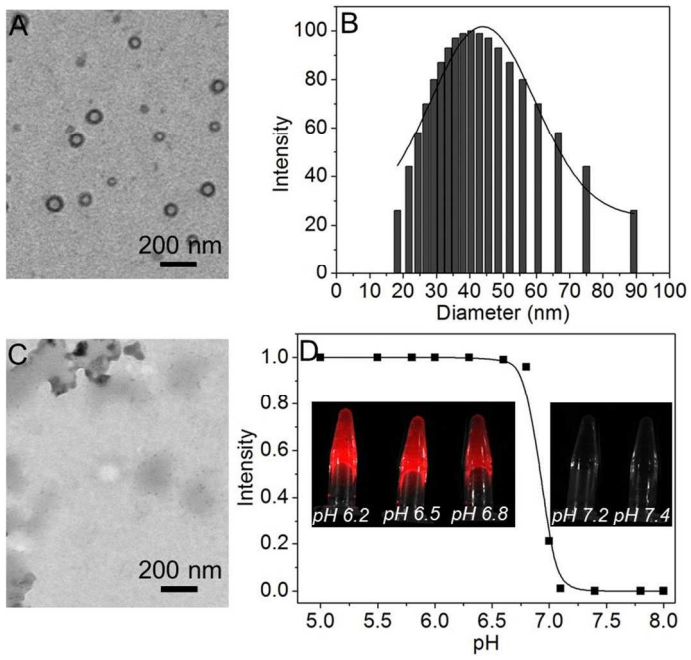


Figure S5. (A, B) TEM image (A) and size distribution (B) of the Cy5.5-labelled NPs in PBS buffer (pH 7.4). (C) TEM image of the Cy5.5-labelled NPs incubated in PBS buffer (pH 6.8) for 3 min. (D) Normalized fluorescence intensity as a function of pH for the Cy5.5-labelled NPs.

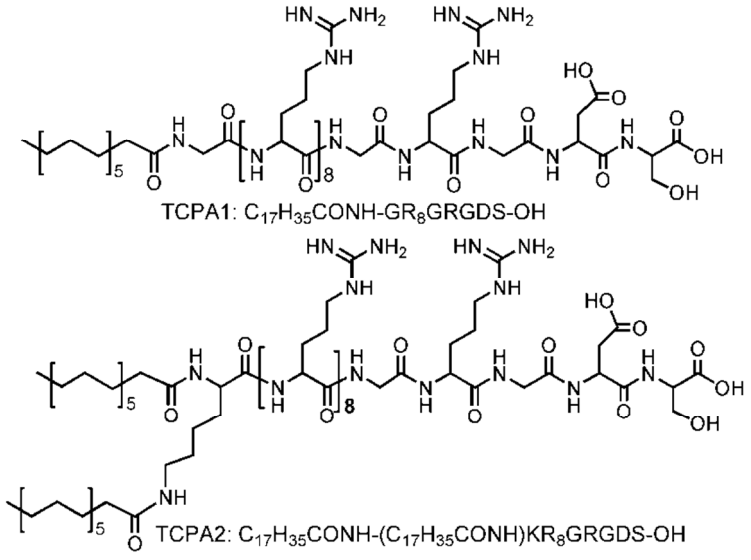


Figure S6. Molecular structures of TCPA1 and TCPA2.

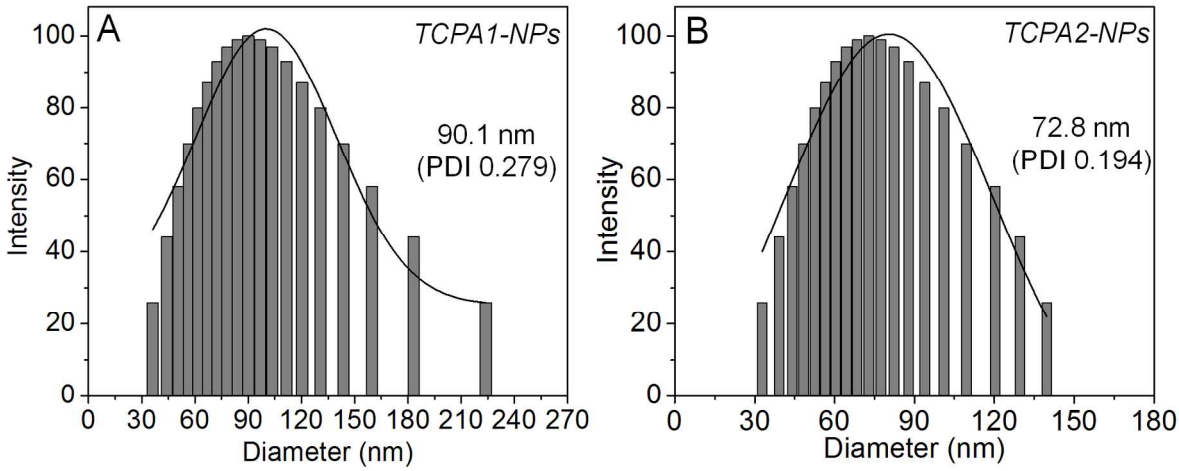


Figure S7. Size distribution of siLuc-loaded TCPA1-NPs (A) and TCPA2-NPs (B).

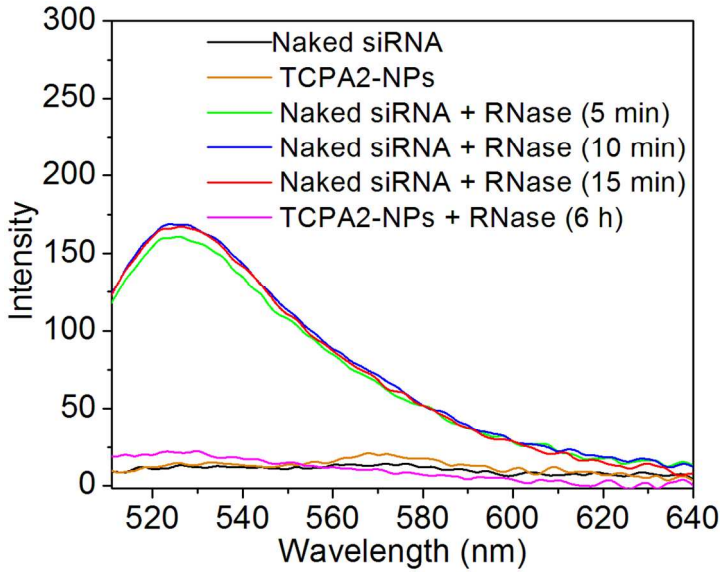


Figure S8. Fluorescent emission spectra of naked siLuc and siLuc-loaded TCPA2-NPs incubated in PBS buffer (pH 7.4) containing 20U RNase for different times (Fluorescein was labelled at 5'-end of the sense strand and its quencher Dabcyl was labeled at the 3'-end of the antisense strand).

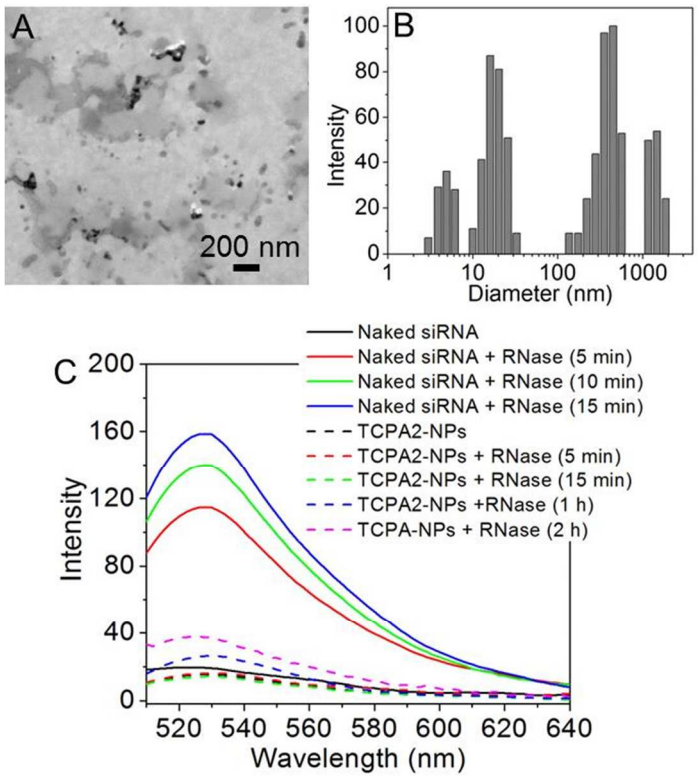


Figure S9. (A, B) TEM image (A) and size distribution (B) of siLuc-loaded TCPA2-NPs incubated in PBS buffer (pH 6.8) for 3 min. (C) Fluorescent emission spectra of naked siLuc (fluorescein and Dabcyl labelled) and siLuc-loaded TCPA2-NPs incubated in PBS buffer (pH 6.8) containing 20 U RNase for different times.

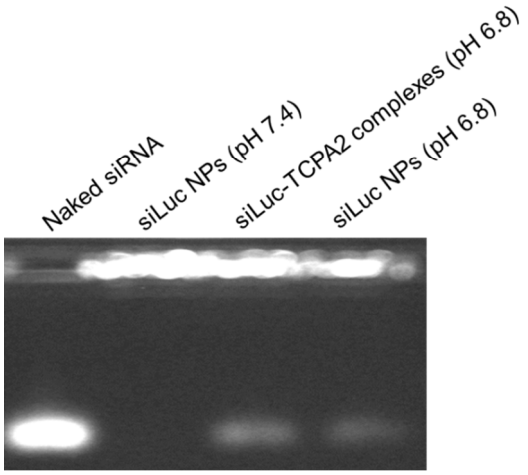


Figure S10. Agarose gel electrophoresis retardation assay of the siRNA-loaded TCPA2 NPs at pH 7.4 and 6.8.

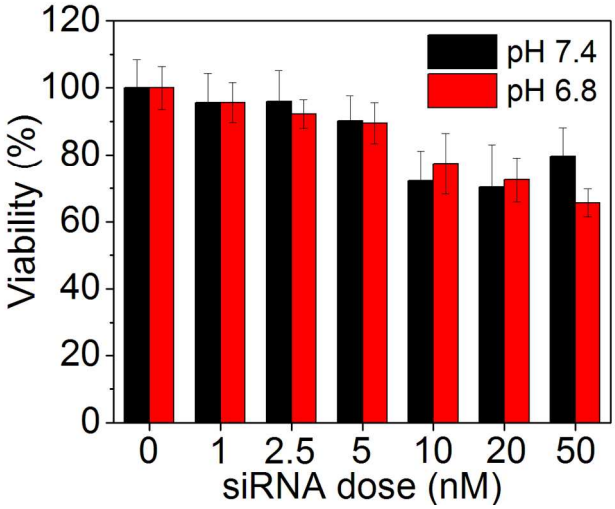


Figure S11. Viability of Luc-HeLa cells treated with siLuc-loaded TCPA2-NPs at different siRNA doses.

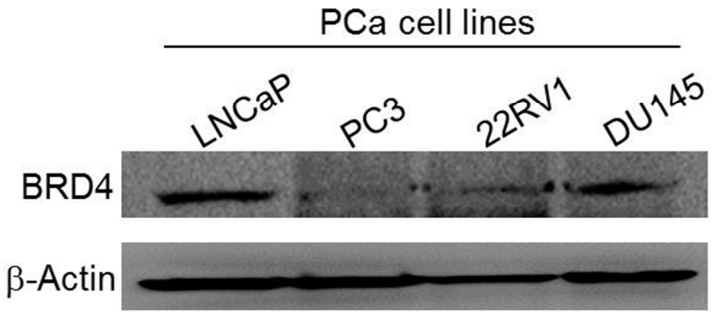


Figure S12. BRD4 expression in PCa cells (LNCaP, PC3, 22RV1 and DU145) determined by western blot.

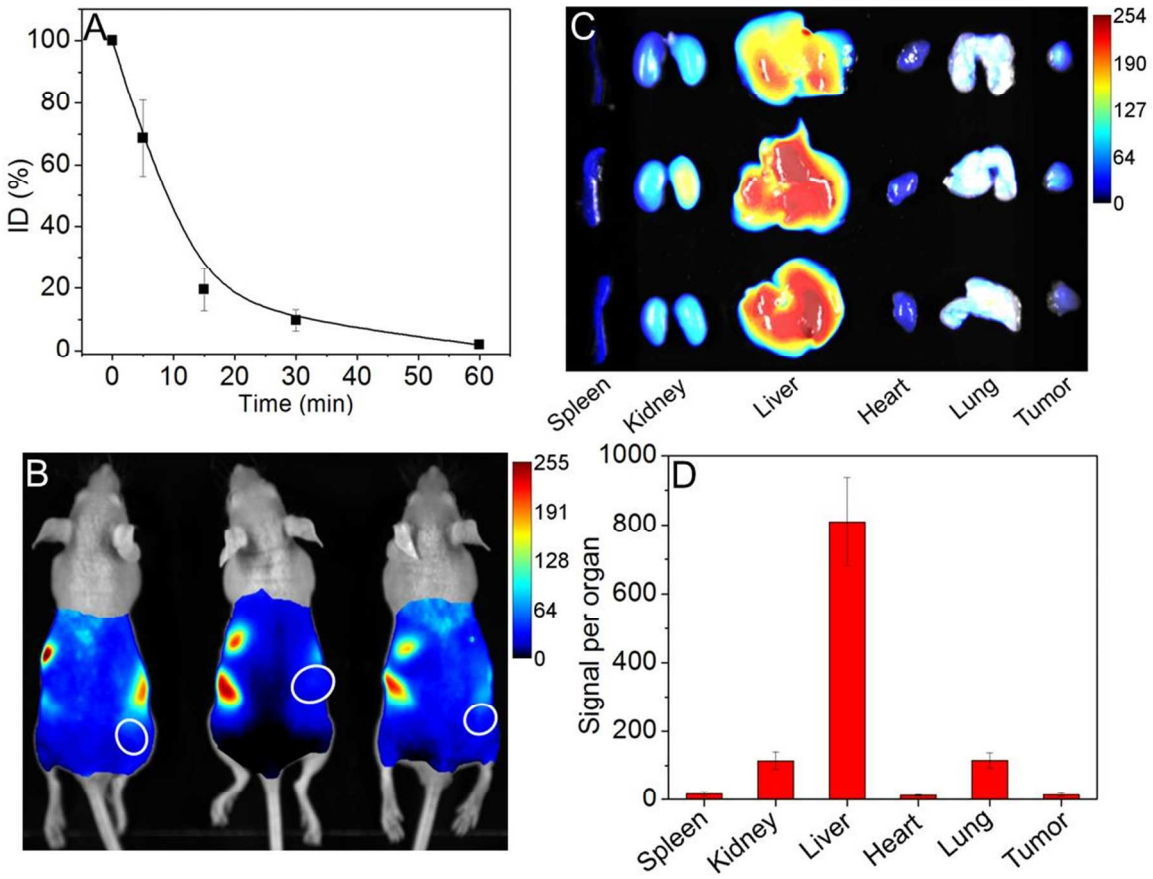


Figure S13. (A) Blood circulation profile of DY677-siRNA-TCPA2 complexes. (B) Overlaid fluorescent image of the LNCaP xenograft tumor-bearing nude mice 24 h post injection of the DY677-siRNA-TCPA2 complexes. Tumors are indicated by ellipses. (C) Overlaid fluorescent image of the tumors and main organs of the LNCaP xenograft tumor-bearing nude mice sacrificed 24 h post injection of the DY677-siRNA-TCPA2 complexes. (D) Biodistribution of the DY677-siRNA-TCPA2 complexes obtained from (C).

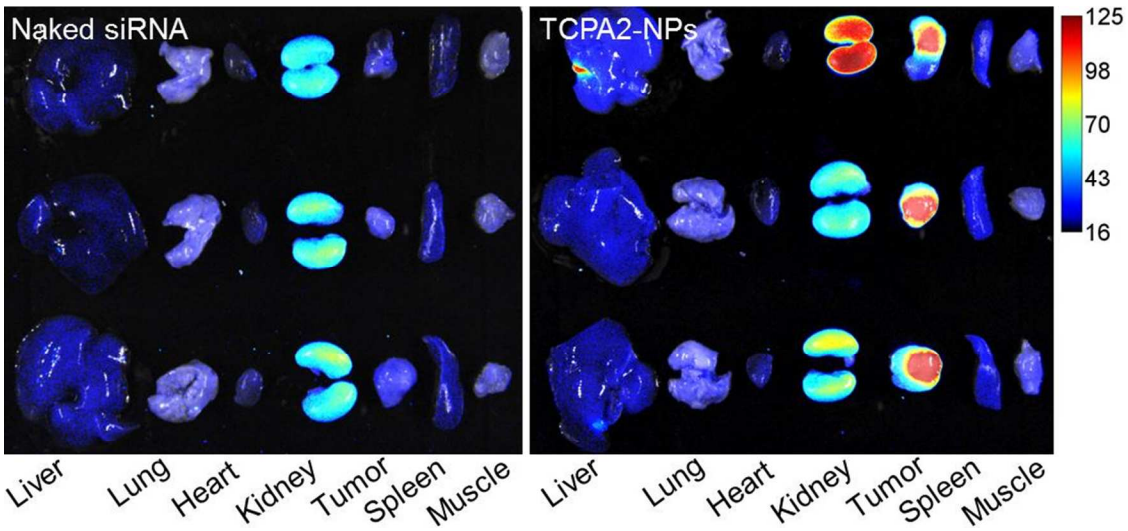


Figure S14. Overlaid fluorescent image of the tumors and main organs of the LNCaP xenograft tumor-bearing nude mice sacrificed 24 h post injection of naked DY677-siRNA and DY677-siRNA-loaded TCPA2-NPs.

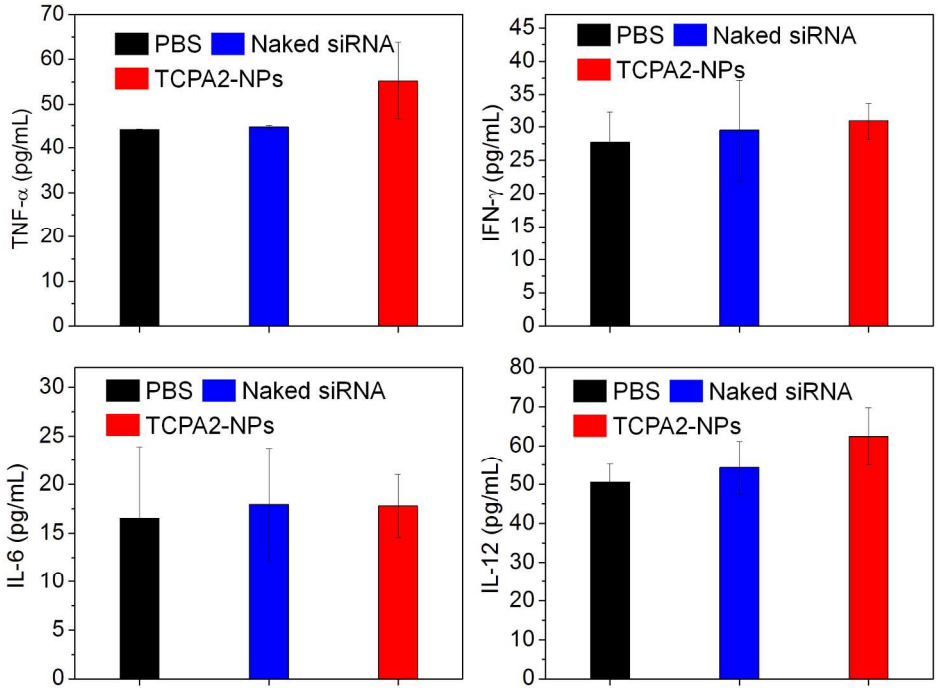


Figure S15. Serum levels of TNF-α, IFN-γ, IL-6 and IL-12 24 h post injection of PBS, naked siBRD4, and siBRD4-loaded TCPA2-NPs.

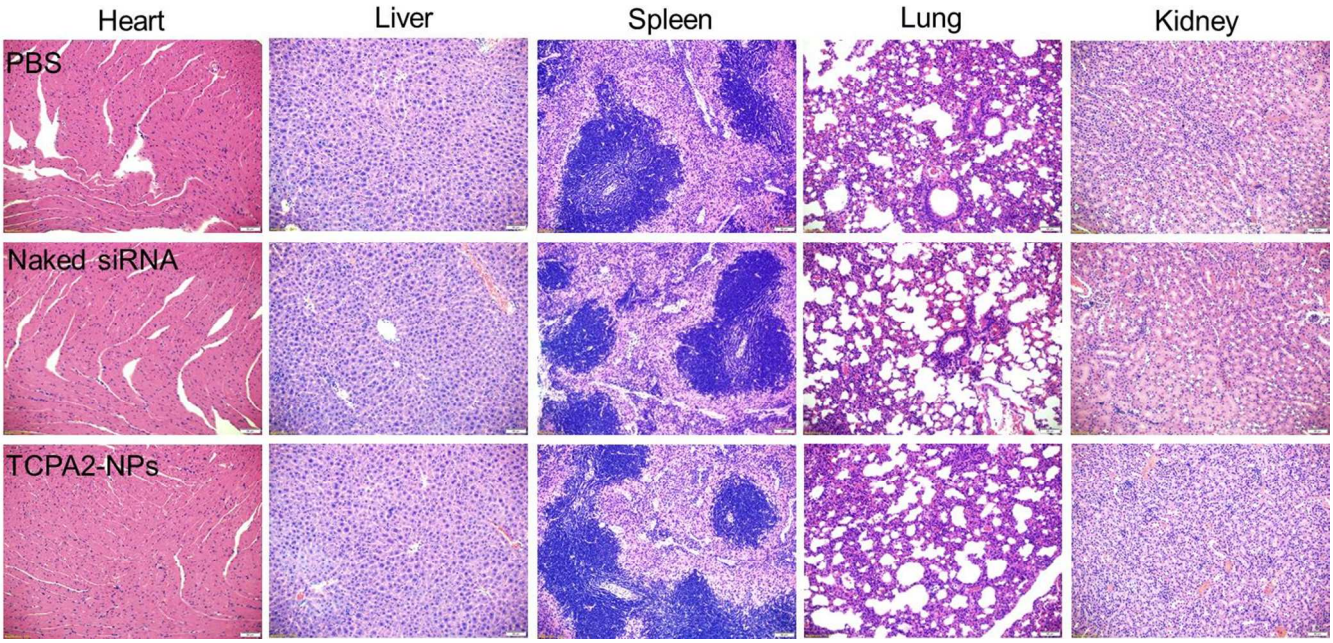


Figure S16. Histological sections of the major organs of mice intravenously injected with PBS, naked siBRD4, and siBRD4-loaded TCPA2-NPs. Hematoxylin-eosin; magnification 100 ×

Table S1. Size, zeta potential, and siRNA encapsulation efficiency (EE%) of the siRNA loaded NPs made with of Meo-PEG-*b*-PHMEMA and TCPA1 or TCPA2

NPs	siRNA EE (%)	Size (nm)	Zeta potential (mv)	
			pH 7.4	pH 6.8
TCPA1-NPs	39	90.1	9.27	29.2
TCPA2-NPs	52	72.8	5.69	27.6