Supporting Information

of

Tumor microenvironment (TME)-responsive nanoplatform for systemic saporin delivery and effective breast cancer therapy

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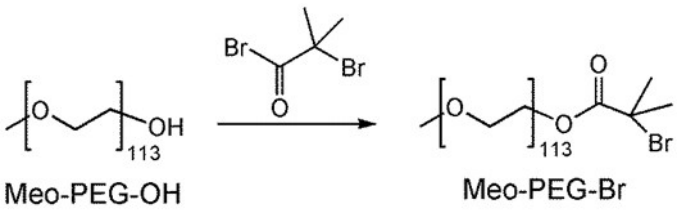
1. Materials

2-(Pentamethyleneimino) ethanol, methacryloyl chloride, and hydroquinone were purchased from Alfa Aesar Company and used directly. α-Bromoisobutyryl bromide, *N,N′*-dimethylformamide (DMF), methoxyl-polyethylene glycol (Meo-PEG_{113}-OH), saporin from *Saponaria officinalis* seeds, 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. Fluorescent dye Cy5.5 NHS ester was acquired from Lumiprobe. Methoxyl-poly(ethylene glycol)-*b*-poly(lactic acid-*co*-glycolic acid) (MeO-PEG_{5k}-*b*-$PLGA_{15k}$) was purchased from Xi'an ruxi Biological Technologi Co. Ltd and used as received. Amphiphilic cationic lipid-like compound alkyl-modified polyamidoamine (PAMAM) dendrimer (G0-C14) was synthesized via ring opening of 1,2-epoxytetradecane by generation 0 of (PAMAM) dendrimer according to our previous study [1]. Dulbecco's Modified Eagle's Medium (DMEM), penicillin, streptomycin, trypsin, and fetal bovine serum (FBS) were purchased from Invitrogen Corp. and used received. All other reagents and solvents are of analytical grade and used without further purification.

2. Synthesis of Meo-PEG-Br

The Meo-PEG-Br was synthesized according to our previous study [2]. In brief, 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), respectively. After drying over anhydrous $MgSO_4$, the solution was concentrated, and cold ether was added to precipitate the product. After re-precipitation thrice, the product was collected as white powder after drying under vacuum. The synthesis route of Meo-PEG-Br is shown in Scheme S1. The ^{1}HNMR spectrum of Meo-PEG-Br is

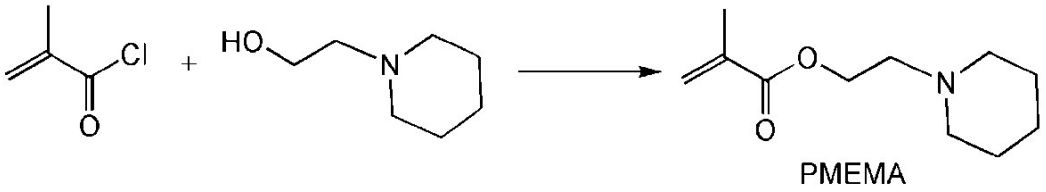
shown in Figure S1.



Scheme S1. Synthesis route of Meo-PEG-Br.

3. Synthesis of 2-(pentamethyleneimino) ethyl methacrylate (PMEMA)

PMEMA was synthesized according to previous report [3]. In brief, 2-(pentamethyleneimino) ethanol (0.1 mol, 12.9 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 route of PMEMA is shown in Scheme S2. ^{1}HNMR (400 MHz, $CDCl_3$, δ ppm): 6.08, 5.54 (s, 2H, CH_2=C(CH_3)-), 4.22-4.26 (m, 2H, -OC$H_2$$CH_2$N-), 2.79-2.87 (m, 2H, -$OCH_2CH_2$N-), 2.71-2.74 (m, 4H, -$OCH_2CH_2N$(C$H_2$$CH_2$$CH_2$)$_2$-), 1.92 (s, 3H, CH_2=C(CH_3)-), 1.55-1.64 (m, 8H, -OCH_2CH_2N(CH_2CH_2CH_2)$_2$-).

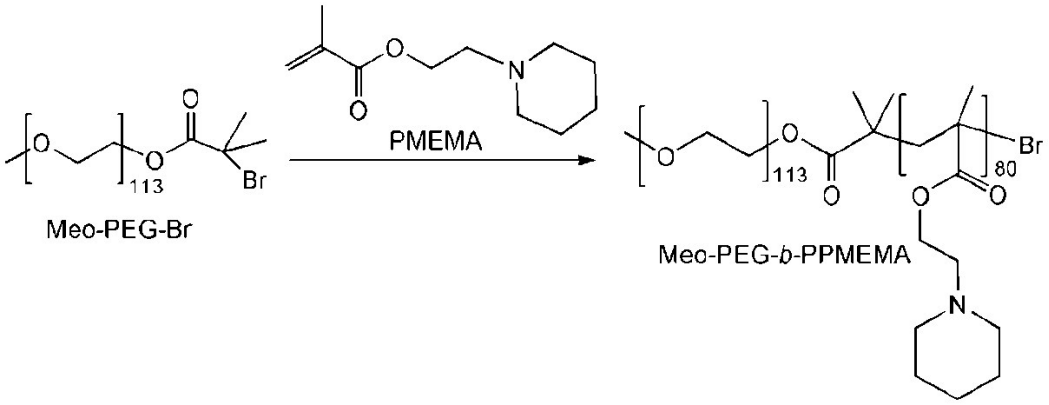


Scheme S2. Synthesis route of PMEMA.

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

Meo-PEG-*b*-PPMEMA block copolymer was synthesized by atom transfer radical polymerization (ATRP). PMEMA (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 route of Meo-PEG-*b*-PPMEMA 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. $M_{n,\ GPC}$ = 1.97 × 10^4 (PDI = 1.21); $M_{n,NMR}$ = 2.03 × 10^4.



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

5. ^{1}H Nuclear magnetic resonance (^{1}HNMR)

The ^{1}HNMR spectra of Meo-PEG-Br, PMEMA, and Meo-PEG-*b*-PPMEMA 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.

6. Gel permeation chromatography (GPC)

Number- and weight-average molecular weights (M_n and M_w, respectively) of the polymers were determined by 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.

7. Acid-base titration

Meo-PEG-*b*-PPMEMA polymer 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.

8. Synthesis of Cy5.5-labeled saporin

The Cy5.5-labeled saporin was synthesized via the reaction between NHS group of Cy5.5 NHS ester and the amino group of saporin. In brief, saporin (5 mg, 0.18 μmol) was dissolved in 2 mL of PBS solution (pH 7.4) and Cy5.5 NHS ester (1 mg, 1.5 μmol) dissolved in 0.5 mL of DMSO was added. After stirring in dark for 24 h, the mixture was transferred to a dialysis tube (MWCO 10K) and dialyzed against deionized water for 24 h. The final dye-labeled saporin (denoted Cy5.5-saporin) was collected after freeze-drying under vacuum.

9. Preparation of the saporin-loaded nanoparticles (NPs)

Meo-PEG-*b*-PPMEMA polymer was dissolved in DMF to form a homogenous solution with a concentration of 10 mg/mL. Subsequently, a mixture of 20 μg saporin (2 mg/mL aqueous solution)

and G0-C14 (5 mg/mL in DMF) was prepared and mixed with 200 μL of Meo-PEG-*b*-PPMEMA polymer solution. Under vigorous 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 saporin-loaded NPs were dispersed in 1 mL of PBS buffer (pH 7.4). Four different saporin-loaded NPs (denoted NP30, NP50, NP75, and NP100) were prepared and their feed composition is shown in Figure S5.

10. Characterizations of the saporin-loaded NPs

Size and zeta potential were determined by dynamic light scattering (DLS, Malvern Zetasizer). The morphology of the saporin-loaded 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 saporin encapsulation efficiency (EE%), Cy5.5-saporin-loaded NPs were prepared according to the method described above and dispersed in 1 mL of PBS buffer (pH 7.4). 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 free Cy5.5-saporin solution (20 μg/mL aqueous solution) with 20-fold DMSO. The fluorescence intensity of Cy5.5-saporin was measured using a Synergy HT multimode microplate reader and the saporin EE% is calculated as: EE% = $(FI_{NPs} / FI_{Standard}) \times 100$.

12. *In vitro* saporin release

Cy5.5-saporin-loaded NPs were prepared according to the method described above. Subsequently, the NPs were dispersed in 1 mL of PBS buffer (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 Cy5.5-saporin (E_x = 684 nm, E_m = 710 nm) was determined using a microplate reader. The cumulative saporin release was calculated as: Cumulative release (%) = (M_t / M_∞) × 100, where M_t is the amount of Cy5.5-saporin released from the NPs at time t and M_∞ is the amount of Cy5.5-saporin encapsulated into the NPs.

13. Cell culture

Human breast cancer cells (MDA-MB-231) were incubated in DMEM medium (pH 7.4) with 10% FBS at 37 ^oC in a humidified atmosphere containing 5% CO_2.

14. Confocal laser scanning microscope (CLSM)

Luc-MDA-MB-231 (50,000 cells) were seeded in round discs and incubated in 2 mL of DMEM 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, Cy5.5-saporin-loaded NPs were added, and the cells were allowed to incubate for 4 h. After removing the medium and subsequently washing with PBS buffer (pH 7.4) thrice, the endosomes and nuclei were respectively stained by Lysotracker green and Hoechst 33342 and the saporin uptake was viewed under a ZEISS 800 CLSM.

15. Flow cytometry

Luc-MDA-MB-231 (50,000 cells) were seeded in 6-well plate and incubated in 2 mL of DMEM 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, Cy5.5-saporin-loaded NPs were added, and the cells were allowed to incubate for 4 h. After removing the medium and subsequently washing with PBS buffer (pH 7.4) thrice, the cells were digested by trypsin and collected for flow cytometry quantitative analysis (BD FACSAria™ III, USA).

16. Apoptosis analysis

MDA-MB-231 cells were seeded in 6-well plates (50,000 cells per well) and incubated in 2 mL of DMEM 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 Cy5.5-saporin-loaded NPs were added. After incubation for 24 h, the cells were digested by trypsin and the cells were collected for PE Texas Red and FITC Annexin V staining using PE Annexin V Apoptosis Detection Kit I. The apoptosis analysis was performed using a BD FACSAria™ III Flow Cytometry Analyzer.

17. *In vitro* cytotoxicity

MDA-MB-231 cells were seeded in a 96-well plate with a density of 5000 cells/well. After the incubation in 100 μL of DMEM containing 10% FBS for 24 h, a fixed amount of saporin-loaded NPs dispersed 100 μL of fresh medium was added and the cells were allowed to incubate for another 24 h. After replacing the medium with 100 μL of fresh medium without FBS, 10 μL of AlamarBlue solution was added to each well. After incubating for another 1 h, the fluorescence intensity (E_x = 530 nm, E_m = 590 nm) was measured using a microplate reader. The average value of three independent experiments was collected and the cell viability was calculated as follows: Cell viability (%) = ($FI_{treated}$ / $FI_{control}$) × 100, where $FI_{control}$ is the fluorescence intensity obtained in the absence of the saporin-loaded NPs and $FI_{treated}$ is fluorescence intensity obtained in the presence of the saporin-loaded NPs.

18. Animals

Healthy female BALB/c normal mice and nude mice (4-5 weeks old) were purchased from the Sun Yat-Sen University Experimental Animal Center. All *in vivo* studies were performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee at Sun Yat-Sen University.

19. Pharmacokinetics study

Healthy female BALB/c mice were randomly divided into two groups (n = 3) and given an intravenous injection of either (i) free Cy5.5-saporin (0.5 mg/kg), or (ii) Cy5.5-saporin-loaded NPs (0.5 mg/kg saporin-equivalent dose). 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 Cy5.5-saporin in the blood was determined by a microplate reader.

20. MDA-MB-231 xenograft tumor model

MDA-MB-231 xenograft tumor model was constructed by subcutaneous injection with 200 μL of MDA-MB-231 cell suspension (a mixture of DMEM medium and Matrigel in 1:1 volume ratio) with a density 1×10^7 cells/mL into the back region of healthy female nude mice. When the volume of the MDA-MB-231 tumor xenograft reached ~70 mm^3, the mice were used for the following *in vivo* experiments.

21. Biodistribution

MDA-MB-231 tumor-bearing female nude mice were randomly divided into two groups (n = 3) and given an intravenous injection of either (i) free Cy5.5-saporin (0.5 mg/kg), or (ii) Cy5.5-saporin-loaded NPs (0.5 mg/kg saporin-equivalent dose). 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 (FI) of each tissue was quantified by Image-J. The final biodistribution is shown using the format of signal per organ.

22. Inhibition of tumor growth

MDA-MB-231 tumor-bearing female nude mice were randomly divided into five groups (n = 5) and intravenously injected with either (i) PBS, (ii) free saporin (0.5 mg/kg), (iii) NP50 platform (0.5 mg/kg saporin-equivalent dose), (iv) saporin-loaded PLGA NPs (0.5 mg/kg saporin-equivalent dose) or (v) the TME-responsive NPs without loading of saporin once every two days. All the mice were administrated by three 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.

23. Histology

After the aforementioned treatment by (i) PBS, (ii) free saporin, (iii) NP50 platform, (iv) saporin-loaded PLGA NPs or (v) the TME-responsive NPs without loading of saporin, the mice were sacrificed at end of the evaluation period, and the tumors and main organs were collected. After fixing with 4% PFA and then embedding in paraffin, the tissue was sectioned and stained with hematoxylin-eosin (H&E) and then viewed under an optical microscope.

24. Immune response

Healthy female BALB/c mice were randomly divided into four groups (n = 3) and given an intravenous injection of either (i) PBS, (ii) free saporin (0.5 mg/kg), (iii) NP50 platform (0.5 mg/kg saporin-equivalent dose), or (iv) the TME-responsive NPs without loading of saporin. 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.

25. 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] X. Xu, K. Xie, X. Q. Zhang, E. M. Pridgen, G. Y. Park, D. S. Cui, J. Shi, J. Wu, P. W. Kantoff, S. J. Lippard, R. Langer, G. C. Walker, O. C. Farokhzad. Enhancing tumor cell response to chemotherapy through nanoparticle-mediated codelivery of siRNA and cisplatin prodrug. *Proc. Natl. Acad. Sci. U S A* 2013, 110, 18638-18643.

[2] X. Xu, J. Wu, Y. Liu, M. Yu, L. Zhao, X. Zhu, S. Bhasin, Q. Li, E. Ha, J. Shi, O. C. Farokhzad. Ultra-pH-responsive and tumor-penetrating nanoplatform for targeted siRNA delivery with robust anti-cancer efficacy. *Angew. Chem. Int. Ed.* 2016, 55, 7091-7094.

[3] Y. Wang, K. Zhou, G. Huang, C. Hensley, X. Huang, X. Ma, T. Zhao, B. D. Sumer, R. J. DeBerardinis, J. Gao. A nanoparticle-based strategy for the imaging of a broad range of tumours by nonlinear amplification of microenvironment signals. *Nat. Mater.* 2014, 13, 204-212.

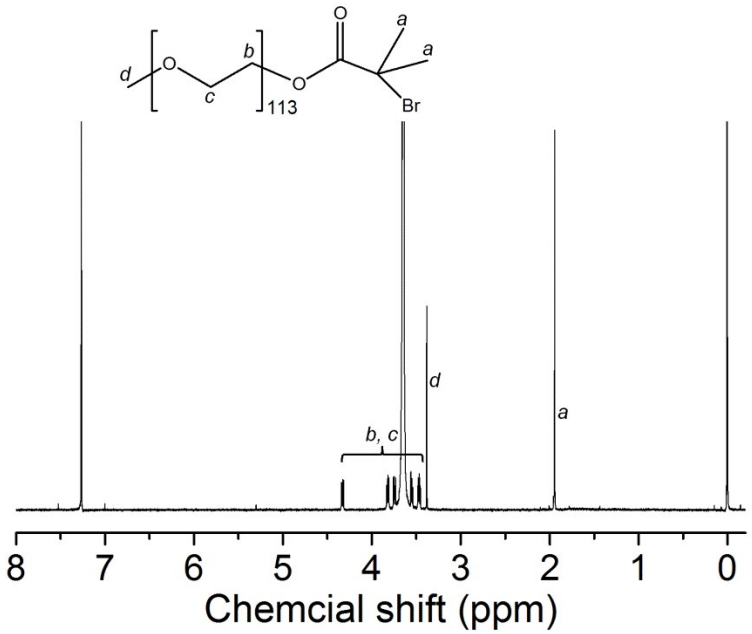


Figure S1. [1]HNMR spectrum of Meo-PEG-Br in $CDCl_3$.

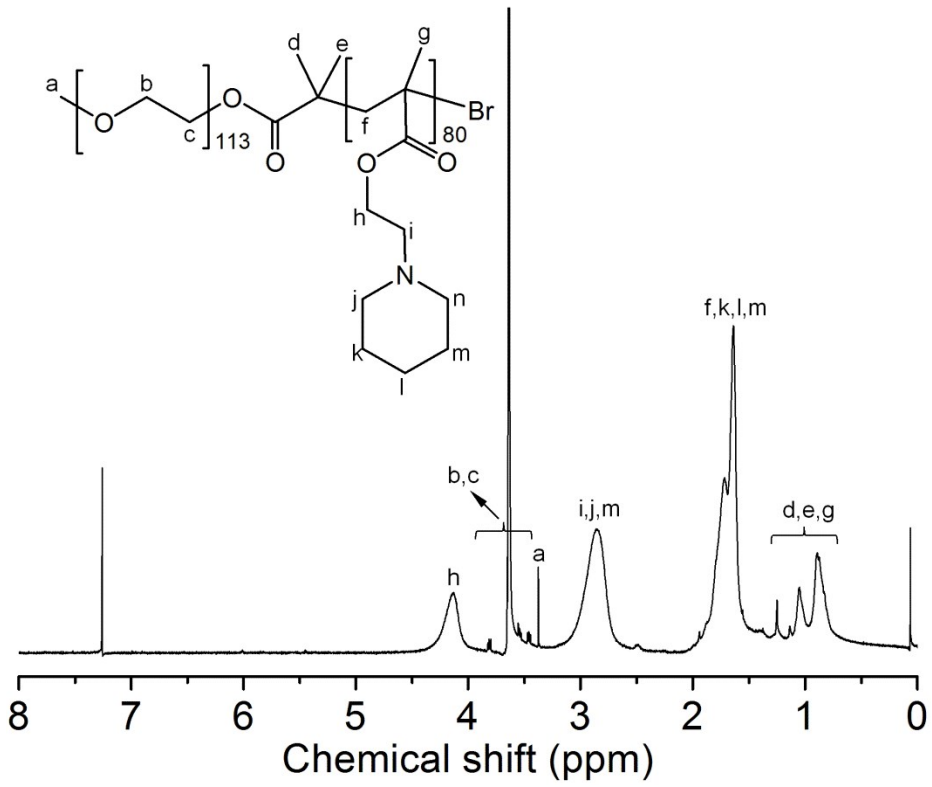


Figure S2. [1]HNMR spectrum of the Meo-PEG-*b*-PPMEMA polymer in $CDCl_3$.

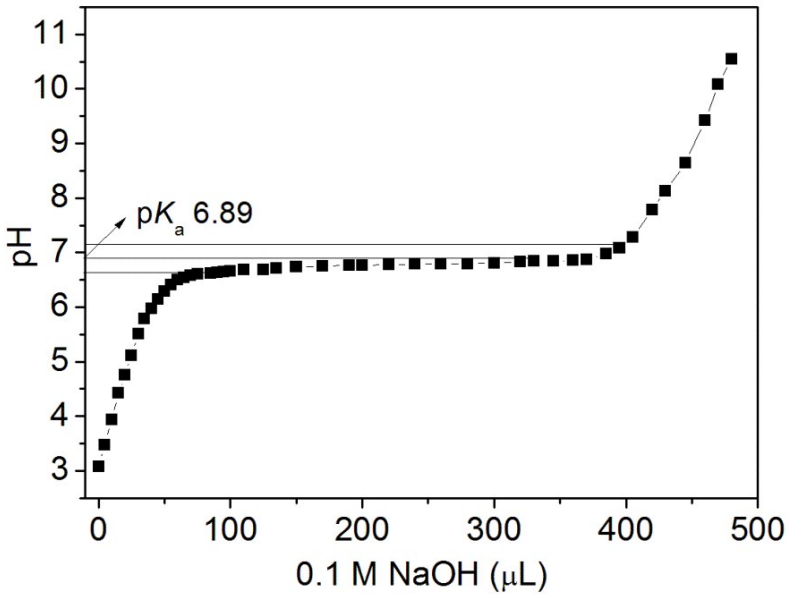


Figure S3. Acid-base titration profile of the Meo-PEG-*b*-PPMEMA polymer.

No.	NP30	NP50	NP75	NP100
Meo-PEG-*b*-PPMEMA (10 mg/mL)	200 μL	200 μL	200 μL	200 μL
Saporin (2 mg/mL)	10 μL	10 μL	10 μL	10 μL
G0-C14 (5 mg/mL)	30 μL	50 μL	75 μL	100 μL
Size (nm)	79.2	96.5	123.8	149.6
EE%	30.7	64.7	77.5	87.6
Zeta potential (mv)	-1.9	-5.4	-10.9	-15.7

Figure S4. Feed compositions and physicochemical properties of the saporin-loaded NPs.

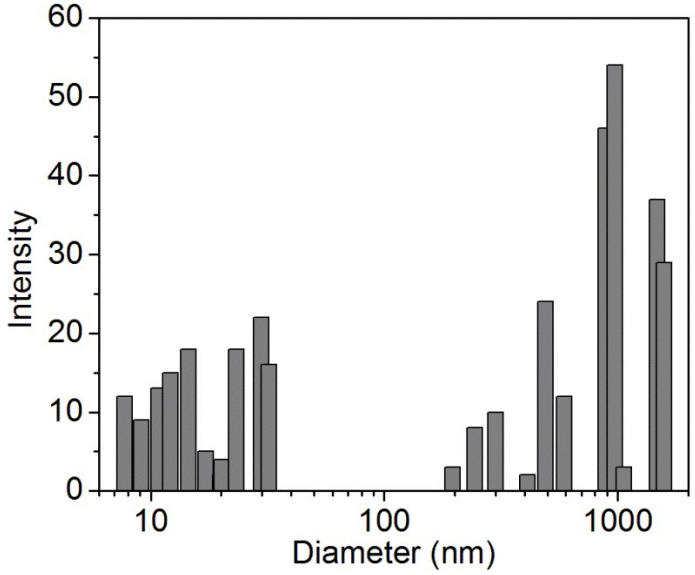


Figure S5. Size distribution of the NP50 platform incubated in PBS buffer at pH 6.8 for 5 min.

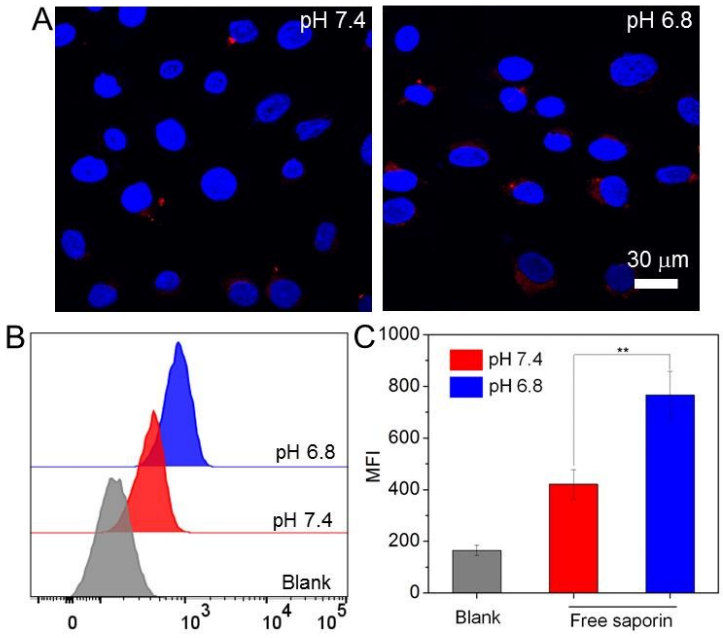


Figure S6. (A) CLSM images of MDA-MB-231 cells incubated with free saporin at pH 7.4 or 6.8 for 4 h. (B, C) Flow cytometry profile (B) and mean fluorescence intensity (MFI, C) of MDA-MB-231 cells incubated with free saporin at pH 7.4 or 6.8 for 4 h. $^{**}P < 0.01$.

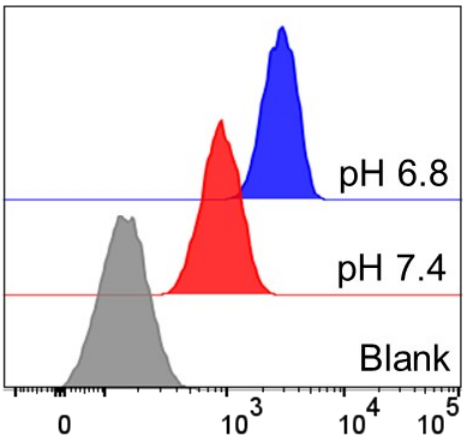


Figure S7. Flow cytometry profile of MDA-MB-231 cells treated with the NP50 platform at pH 7.4 or 6.8 for 4 h.

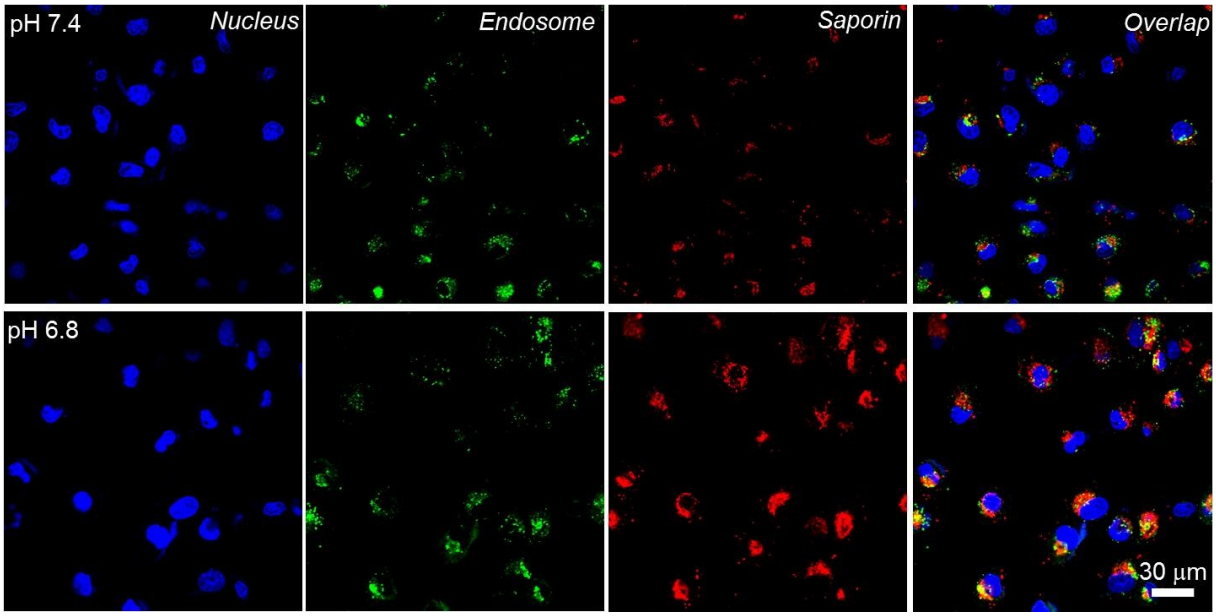
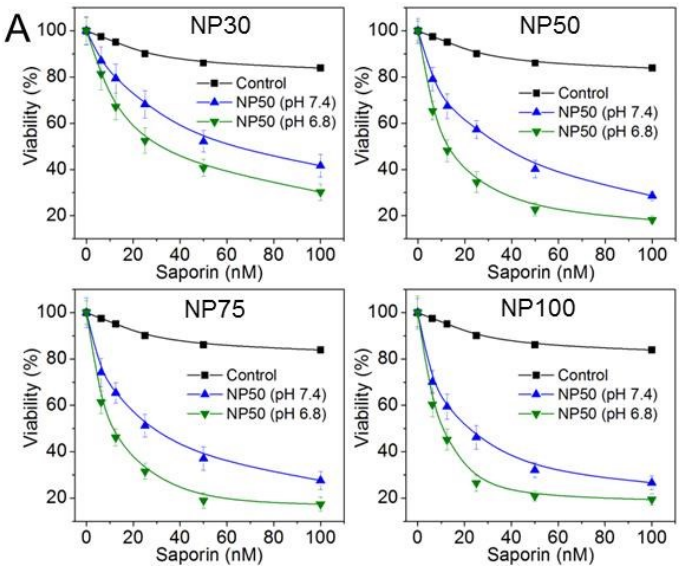


Figure S8. CLSM images of MDA-MB-231 cells incubated with the NP50 platform at pH 7.4 or 6.8 for 4 h. The nuclei and endosomes were stained with Hoechst 33342 (blue fluorescence) and Lysotracker green (green fluorescence), respectively.



B

	IC_{50} (pH 6.8)	IC_{50} (pH 7.4)
NP30	31.9 nM	63.5 nM
NP50	12.8 nM	36.3 nM
NP75	11.4 nM	28.5 nM
NP100	10.3 nM	21.9 nM

Figure S9. (A) Viability of MDA-MB-231 cells treated with the saporin-loaded NPs at pH 7.4 or 6.8 for 24 h. The TME pH-responsive NPs without loading of saporin were used as a control. (B) IC_{50} value of each NP formulation calculated from (A).

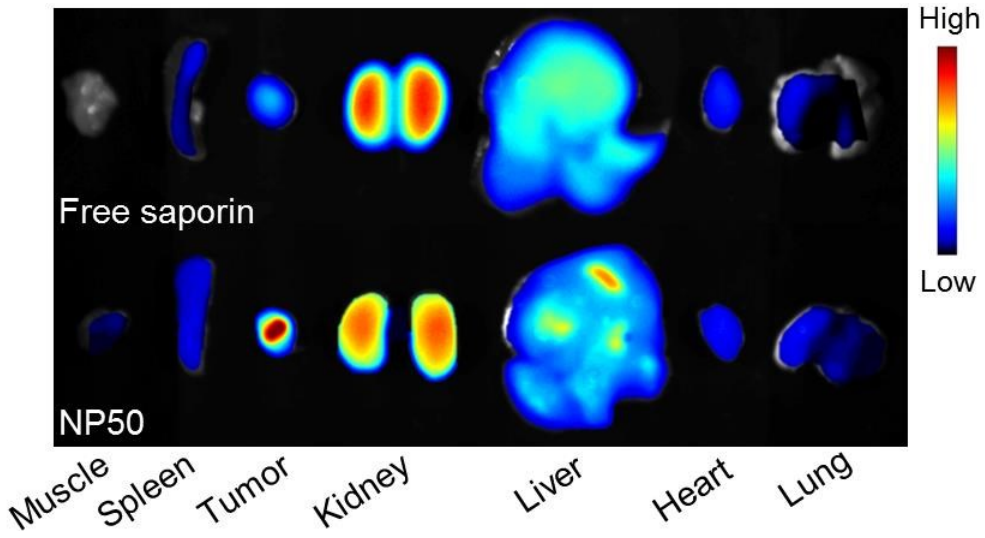


Figure S10. Overlaid fluorescence image of the tumors and main organs of MDA-MB-231 xenograft tumor-bearing mice at 24 h post injection of free saporin or the NP50 platform.

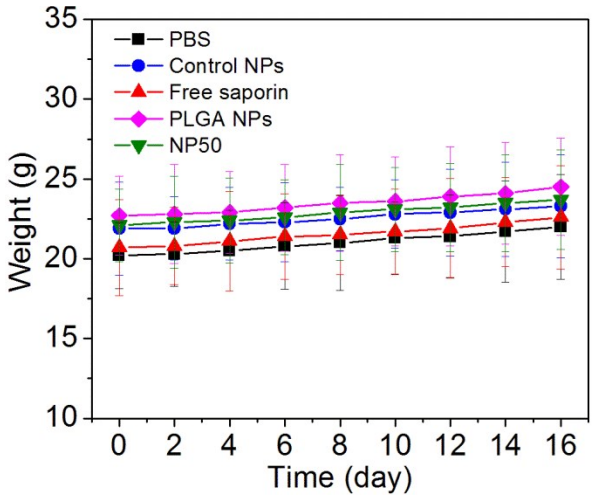


Figure S11. Body weight of MDA-MB-231 xenograft tumor-bearing mice treated with PBS, free saporin, NP50 platform, saporin-loaded PLGA NPs, and the TME pH-responsive NPs without loading of saporin (Control NPs).

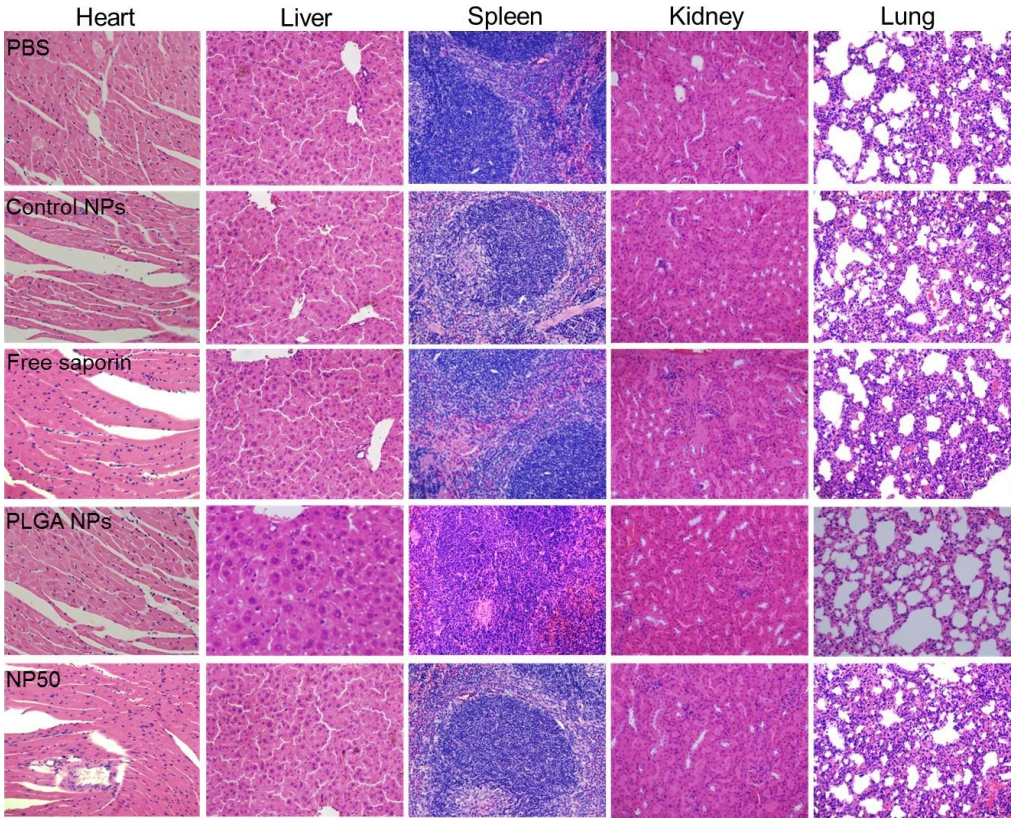


Figure S12. Histological section of the major organs of MDA-MB-231 xenograft tumor-bearing mice treated with PBS, free saporin, NP50 platform, saporin-loaded PLGA NPs, and the TME pH-responsive NPs without loading of saporin (Control NPs). Hematoxylin-eosin; 200 ×

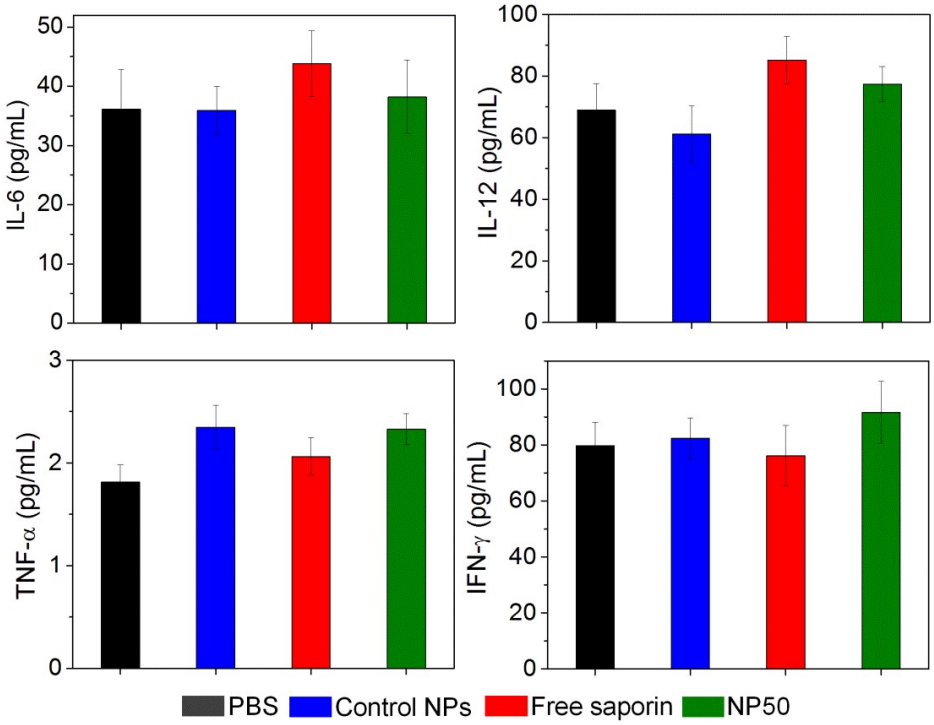


Figure S13. Serum levels of IL-6, IL-12, TNF-α, and IFN-γ at 24 h post injection of PBS, free saporin, NP50 platform, and the TME pH-responsive NPs without loading of saporin (Control NPs).