Supplementary Information (SI) for Journal of Materials Chemistry B.

Guanidyl-rich α-helical polypeptide enables efficient cytosolic pro-protein delivery and CRISPR-Cas9 genome editing

Ziyin Zhao[1#], Haoyu Zhang[1#], Wei Li[1#], Yehan Wang[1], Yifei Wang[2], He Yang[2], Lichen Yin[1*], Xun Liu[2*]

[1] Institute of Functional Nano and Soft Materials (FUNSOM), Jiangsu Key Laboratory of Carbon-Based Functional Materials and Devices, Soochow University, Suzhou 215123, China

[2] Department of Thoracic Cancer, The Second Affiliated Hospital of Soochow University, Suzhou, 215004, China

[#]These authors contributed equally to this work

E-mail: lx8810@suda.edu.cn (X. Liu); lcyin@suda.edu.cn (L. Yin)

Materials, cells, and animals

2-Acetylphenylboronic acid (ABA) and adenosine triphosphate (ATP) were purchased from Energy Chemical (Shanghai, China). Bovine serum albumin (BSA) and horseradish peroxidase (HRP) were purchased from Beyotime (Shanghai, China). Fluorescein isothiocyanate (FITC), rhodamine B isothiocyanate (RBITC), saporin, Cas9, ovalbumin (OVA), and bovine pancreatic ribonuclease A (RNase A) were purchased from Sigma-Aldrich (Shanghai, China). α-Chymotrypsin (α-Chyt) was purchased from Perfemiker (Shanghai, China). Human IgG was purchased from Solarbio (Beijing, China). PULSin was purchased from Polyplus-transfection (Illkirch, France). Lipofectamine CMAX transfection reagent (CMAX) was purchased from Thermo Fisher Scientific (Germany). Chlorpromazine (CPZ), genistein (GNT), and wortmannin (WTM) were purchased from TCI (Shanghai, China). Methyl-β-cyclodextrin (mβCD) was purchased from J&K Scientific (Shanghai, China). Hoechst 33258, Lysotracker deep red (LDR), and 3-(4,5-dimethylthiahiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Invitrogen (Carlsbad, CA, USA). Phospho-Akt1 (Ser473) recombinant rabbit monoclonal antibody (anti-pAkt) was purchased from Abways Biotechnology (Shanghai, China). Caspase 3/7 live cell real-time detection kit was purchased from KeyGEN Biotech (Nanjing, China). Anti-poly(ADP-ribose) polymerase1 (PARP) rabbit polyclonal antibody was purchased from GeneTex (San Antonio, TX, USA). sgRNA was purchased from Tsingke Biotechnology Co., Ltd. (Beijing, China). T7 endonuclease I (T7E1) was purchased from New England Biolabs (Beijing, China). FITC-labeled proteins (protein-FITC) and RBITC-labeled IgG were prepared according to previous literature[1, 2].

HeLa (human cervix adenocarcinoma), A549 (Human lung carcinoma), CT-26 (murine colon carcinoma), B16F10 (murine skin melanoma), 293T (human embryonic fibroblast) cells were purchased from the American Type Culture Collection. PANC-1 cells (human pancreatic carcinoma) were purchased from FuHeng Cell Center. MPVEC cells (murine pulmonary microvascular endothelial) were purchased from YaJi Biological Technology. HeLa-GFP cells were purchased from Fenghui Biotechnology (Changsha, China) established by infecting HeLa cells with lentivirus harboring a GFP-expressing cassette. HL-1 cells (murine cardiomyocytes) were gifted from Prof. Yongbing Chen in Soochow university. HRMEC cells (human retinal microvascular endothelial) were gifted from Prof. Qian

Chen in Soochow university. HeLa, A549, 293T, PANC-1, MPVECs, HRMEC, and HeLa-GFP cells were cultured in DMEM (Gibco, Grand Island, NY) containing 10% heat-inactivated fetal bovine serum (FBS) at 37 °C with 5% CO_2. CT-26 and B16F10 cells were cultured in 1640 (Gibco, Grand Island, NY) containing 10% heat-inactivated FBS at 37 °C with 5% CO_2. HL-1 cells were cultured in MEM (Pricella, Wuhan, China) containing 10% heat-inactivated FBS at 37 °C with 5% CO_2.

Female BALB/c nude mice (6-8 weeks) were purchased from Shanghai Slaccas Experimental Animal Co. Ltd. (Shanghai, China) and housed in an SPF room. Animal experiments were conducted following protocols approved by Soochow University Laboratory Animal Center, China. The approval number for the laboratory is SYXK(Su)2017-0043.

β-gal activity assay.

X-gal staining assay was employed to test the cytosolic delivery efficiency and intracellular enzymatic activity of LPP/A-β-gal NCs. Briefly, HeLa cells were seeded on 96-well plates at 1.5×10^4 cells/well and cultured for 24 h. After replacement with serum-free DMEM, β-gal, LPP/β-gal NCs, PULSin/β-gal NCs, or LPP/A-β-gal NCs in PBS (final concentration of 2 μg β-gal/mL) were added to each well and incubated with cells for 4 h. Cells were then washed three times with PBS, fixed with paraformaldehyde (4%, 15 min) at room temperature, and further incubated overnight with 5-bromo-4-chloro-3-indolyl-β-$_D$-galactoside (X-gal, 100 μL, 0.05 mg/mL in working solution, PBS/DMF = 400/1, v/v) at 37 ℃ in the darkness without CO_2. Cells were further washed with PBS and observed by an optical microscope.

The intracellular activity of β-gal was further determined using a quantitative β-gal assay. HeLa cells on 96-well plates were treated with LPP/A-β-gal NCs as described above. Cells were then lysed with the passive lysis buffer (100 μL) for 1 h at room temperature. The lysate (25 μL) was incubated with *o*-nitrophenyl-β-$_D$-galactopyranoside (ONPG, 25 μL, 0.05 mg/mL in working solution, PBS/DMF = 400/1, v/v) at 37 °C for 30 min, followed by addition of Na_2CO_3 solution (1 mol/L, 150 μL) to stop the reaction. The absorbance of the reaction solution was immediately measured at 420 nm. Results were represented as the relative activity of native β-gal solution at the same β-gal amount.

HRP activity assay.

HeLa cells were seeded on 24-well plates at 1×10^5 cells/well and cultured for 24 h. After replacement with fresh serum-free DMEM, HRP, LPP/HRP NCs,

PULSin/HRP NCs, or LPP/A-HRP NCs were added at the final concentration of 2 μg HRP/mL. After incubation at 37 ℃ for 4 h, cells were washed three times with PBS. Then, colorless TMB solution (10 μg/mL, 200 μL) containing hydrogen peroxide (0.3%, 6 μL) was added to each well, and the cell culture medium was imaged by an optical camera after a 5-min incubation at room temperature. The absorbance of the reaction solution was immediately measured at 630 nm. Native HRP at the same concentration was tested using the TMB working solution, and was used to represent 100% enzymatic activity.

RNase A activity assay.

The activity of RNase A was measured using the RNase A ELISA kit. LPP/A-RNase A NCs and acid-treated LPP/A-RNase A NCs (pH 6.0) were treated using ELISA kit according to the manufacturer's instructions and the absorbance of the reaction solutions was measured immediately at 450 nm. Native RNase A at the same concentration was also treated using the ELISA kit and was used to represent 100% enzymatic activity.

***In vitro* anticancer efficacy of LPP/A-RNase A NCs and LPP/A-saporin NCs.**

HeLa cells were seeded on 96-well plates at 1×10^4 cells/well and cultured for 24 h. After replacement with serum-free DMEM, RNase A and LPP/A-RNase A NCs (final concentrations of 0, 0.3125, 0.625, 1.25, 2.5, 5, 10, and 20 μg RNase A/mL) were added to each well and incubated with cells for 4 h. The culture medium was replaced by fresh medium containing 10% FBS, and cells were further incubated for 44 h before viability assessment using the MTT assay. The *in vitro* anticancer efficacy of saporin and LPP/A-saporin NCs on HeLa cells were similarly evaluated at the final concentrations of 0, 0.25, 0.5, 0.75, and 1 μg saporin/mL.

Cytosolic delivery of LPP/A-anti-pAkt NCs.

LPP/A-anti-pAkt NCs-induced apoptosis of HeLa cells was firstly detected. Briefly, cells on 24-well plates were incubated with anti-pAkt, LPP/anti-pAkt NCs, or LPP/A-anti-pAkt NCs (2 μg anti-pAkt/mL) for 4 h as described above and additional 8 h in serum-containing medium. After replacement with fresh medium, cells were stained with the caspase3/7 live cell fluorescence real-time detection kit followed by observation with CLSM. Then, caspase-induced PARP cleavage was detected by Western blot. Briefly, HeLa cells on 6-well plates were incubated with anti-pAkt, PULSin/anti-pAkt NCs, or LPP/A-anti-pAkt NCs (2 μg anti-pAkt/mL) for 4 h in serum-free medium and additional 20 h in serum-containing medium. The amount of

PARP and cleaved PARP in cells was then determined by Western blot using the anti-PARP rabbit monoclonal primary antibody (1: 2000 dilution) and HRP-conjugated goat anti-rabbit IgG secondary antibody (1: 20000 dilution). Finally, the *in vitro* cytotoxicity of anti-pAkt and LPP/A-anti-pAkt NCs at the final concentrations of 0, 0.3125, 0.625, 1.25, 2.5, 5, 10, and 20 μg anti-pAkt/mL in HeLa cells were determined following the same procedures as described above for LPP/A-RNase A NCs.

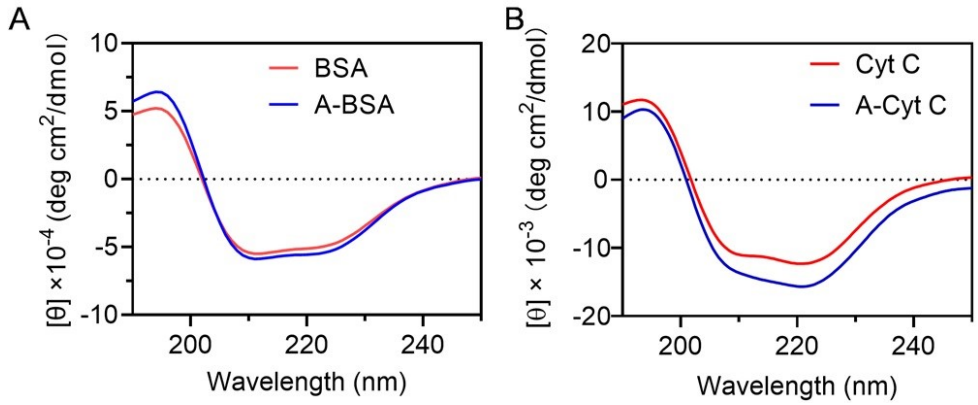

Figure S1. CD spectra of A-BSA (A) and A-Cyt C (B).

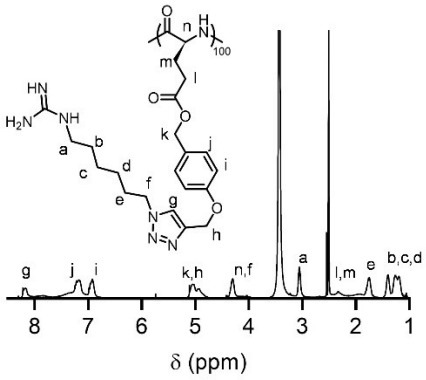

Figure S2. ^{1}H NMR spectrum of LPP (400 MHz, DMSO$_{d6}$).

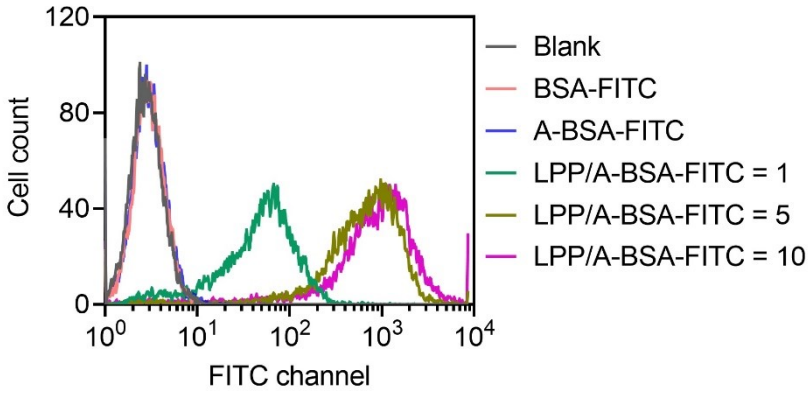

Figure S3. Flow cytometric diagrams of HeLa cells after 4-h incubation with LPP/A-BSA-FITC NCs (2 μg BSA-FITC/mL) at different LPP/A-BSA-FITC weight ratios.

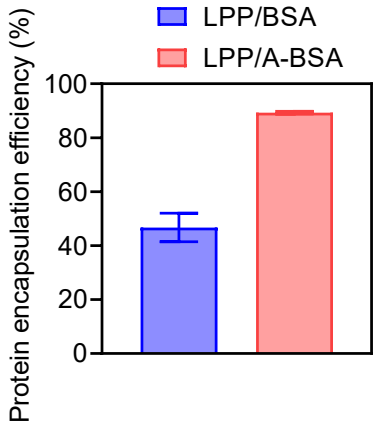

Figure S4. Protein encapsulation efficiency of LPP/BSA NCs and LPP/A-BSA NCs (w/w=5/1) in PBS (pH 7.4, n = 3).

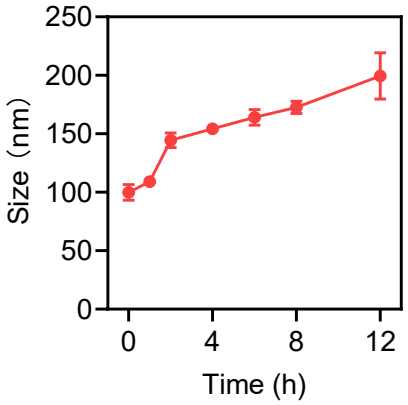

Figure S5. Size alteration of LPP/A-BSA NCs in PBS (pH 7.4) after incubation at 37 °C for different time (n = 3).

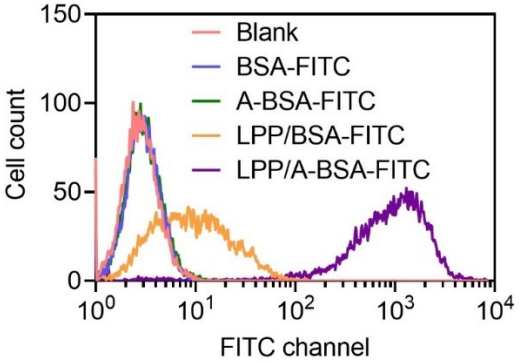

Figure S6. Flow cytometric diagrams of HeLa cells after 4-h incubation with BSA-FITC, A-BSA-FITC, LPP/BSA-FITC NCs, or LPP/A-BSA-FITC NCs (2 μg BSA-FITC/mL).

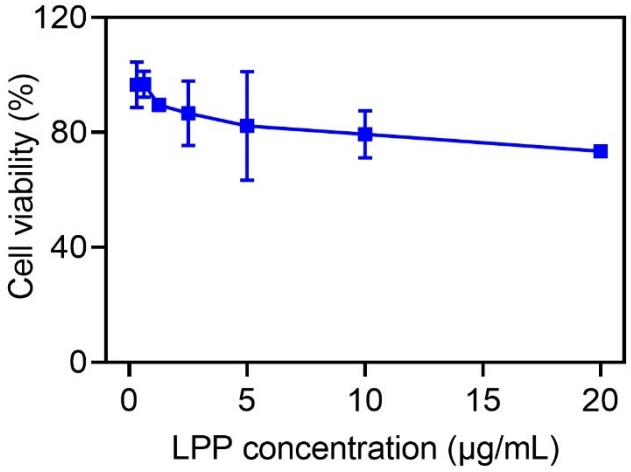

Figure S7. Viability of HeLa cells following 4-h incubation with LPP/A-BSA NCs in serum-free DMEM at different LPP concentrations and additional 20-h incubation in FBS-containing DMEM (n = 3).

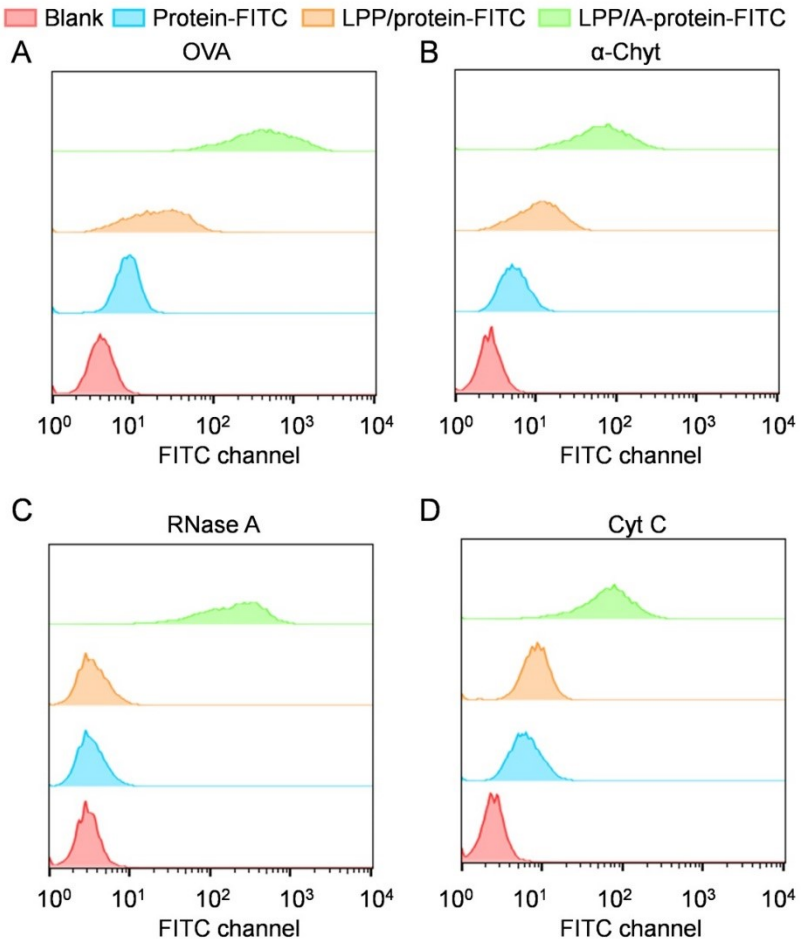

Figure S8. Flow cytometric diagrams of HeLa cells after 4-h incubation with various LPP/A-protein-FITC NCs (2 μg protein-FITC/mL). Proteins include OVA (A), α-Chy t (B), RNase A (C), and Cyt C (D).

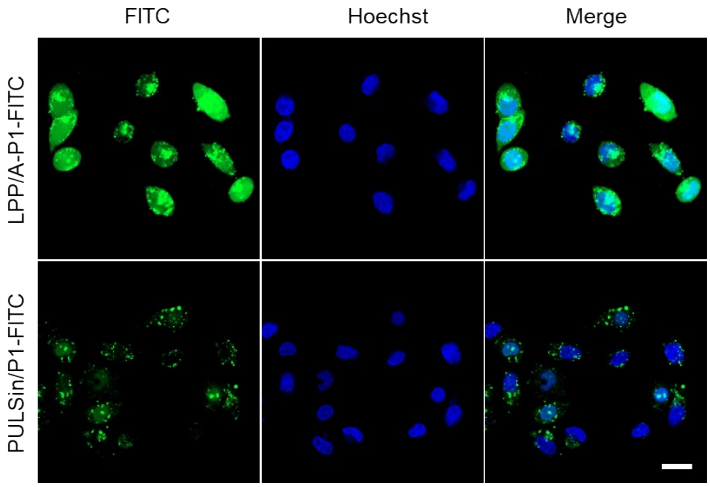

Figure S9. CLSM images of HeLa cells following 4-h incubation with LPP/A-P1-FITC NCs or PULSin/P1-FITC NCs (2 μg P1-FITC/mL, scale bar = 20 μm). Cell nuclei were stained with Hoechst 33258.

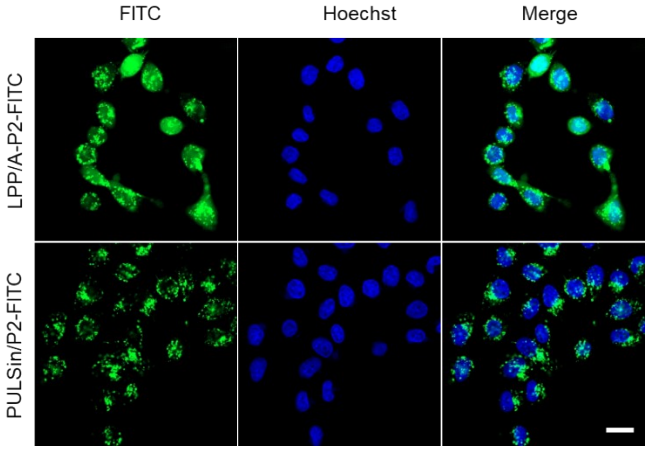

Figure S10. CLSM images of HeLa cells following 4-h incubation with LPP/A-P2-FITC NCs or PULSin/P2-FITC NCs (2 μg P2-FITC/mL, scale bar = 20 μm). Cell nuclei were stained with Hoechst 33258.

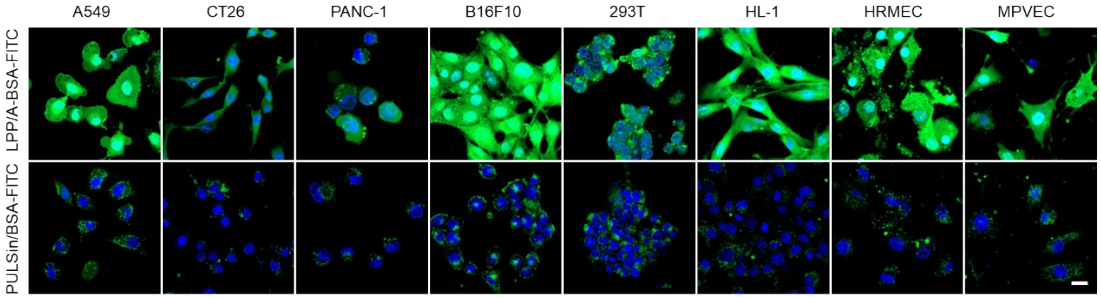

Figure S11. CLSM images of different cancer cells (A549, CT26, PANC-1, and B16F10) and non-cancerous cells (293T, HL-1, HRMEC, and MPVEC) treated with LPP/A-BSA-FITC NCs or PULSin/A-BSA-FITC NCs (2 μg BSA-FITC/mL, scale bar = 20 μm) for 4 h. Cell nuclei were stained with Hoechst 33258.

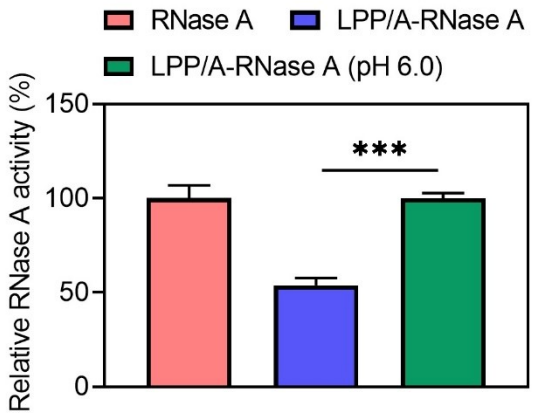

Figure S12. Relative enzyme activity of RNase A, LPP/A-RNase A NCs, and acid-treated LPP/A-RNase A NCs (pH 6.0, 2 h, n = 3).

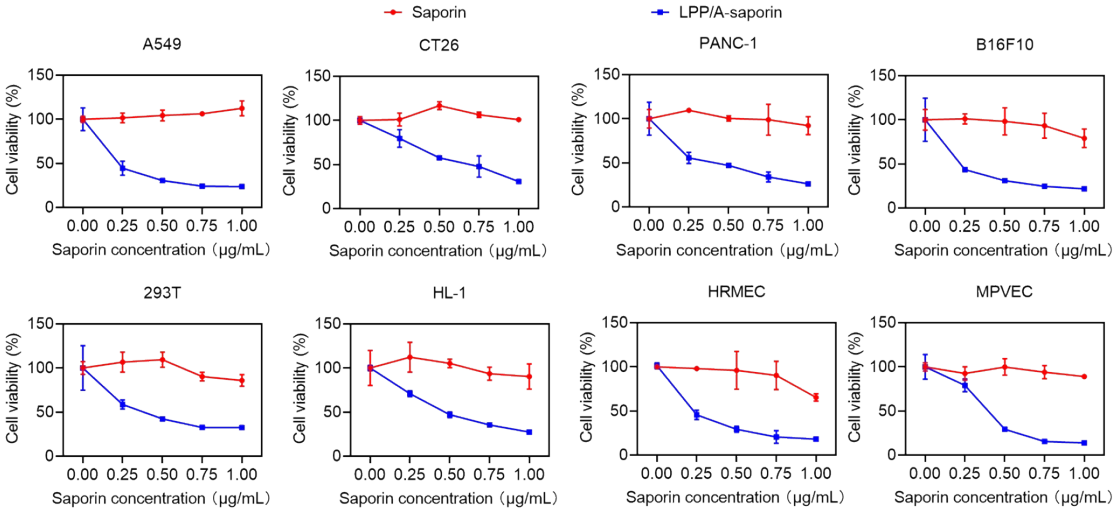

Figure S13. Viability of A549, CT26, PANC-1, B16F10, 293T, HL-1, HRMEC, and MPVEC cells following 4-h incubation with saporin or LPP/A-saporin NCs at different concentrations and additional 44-h incubation in fresh medium (n = 3).

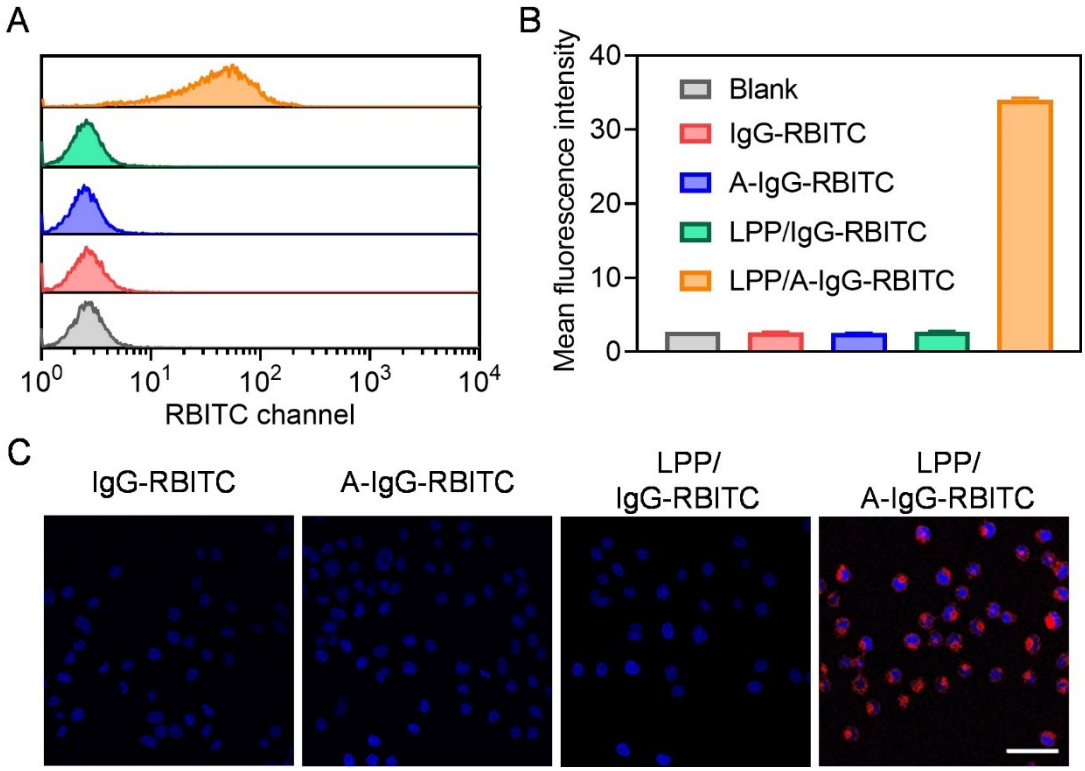

Figure S14. Flow cytometric diagrams (A), mean fluorescence intensity (B, n = 3), and CLSM images (C, scale bar = 50 μm) of HeLa after 4-h incubation with IgG-RBITC, A-IgG-RBITC, LPP/IgG-RBITC NCs, or LPP/A-IgG-RBITC NCs (2 μg IgG-RBITC/mL). Cell nuclei were stained with Hoechst 33258 (5 μg/mL).

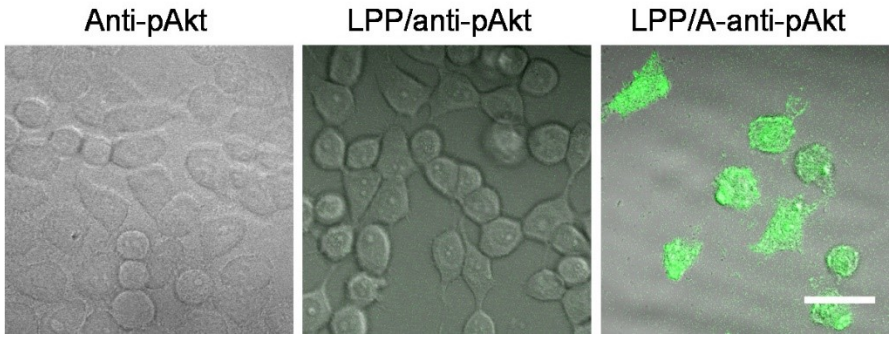

Figure S15. CLSM images of HeLa cells treated with anti-pAkt, LPP/anti-pAkt NCs, or LPP/A-anti-pAkt NCs (2 μg anti-pAkt/mL) for 4 h and additional 8 h followed by staining with caspase3/7 live cell fluorescence real-time detection kit (scale bar = 50 μm).

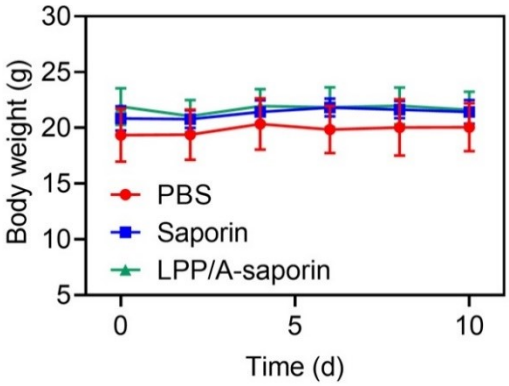

Figure S16. Body weight changes of HeLa tumor-bearing mice within the observation period of 11 d in the *in vivo* anti-tumor efficacy study (n = 6).

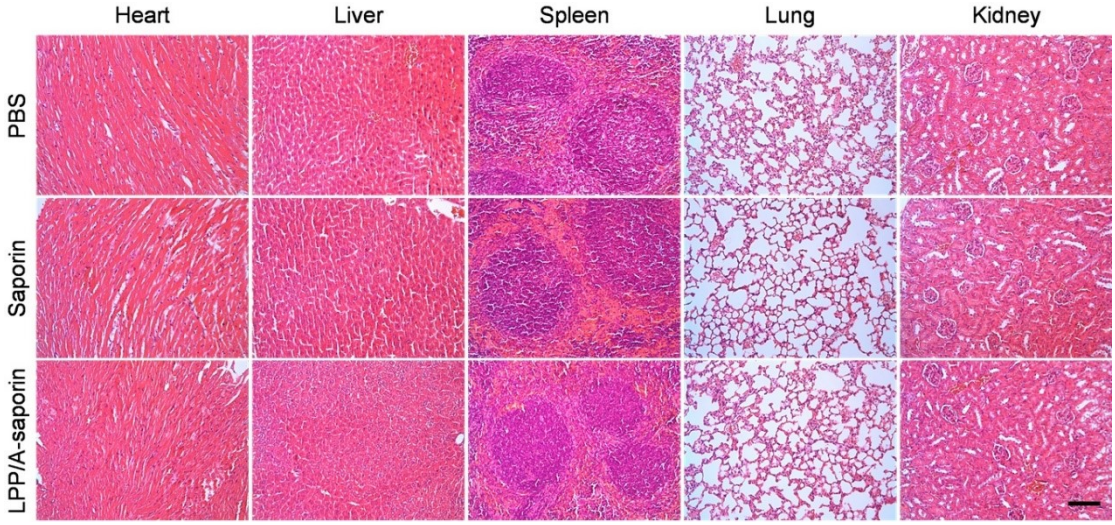

Figure S17. H&E-stained sections of major organs harvested on day 10 in the *in vivo* anti-tumor efficacy study (scale bar = 100 μm).

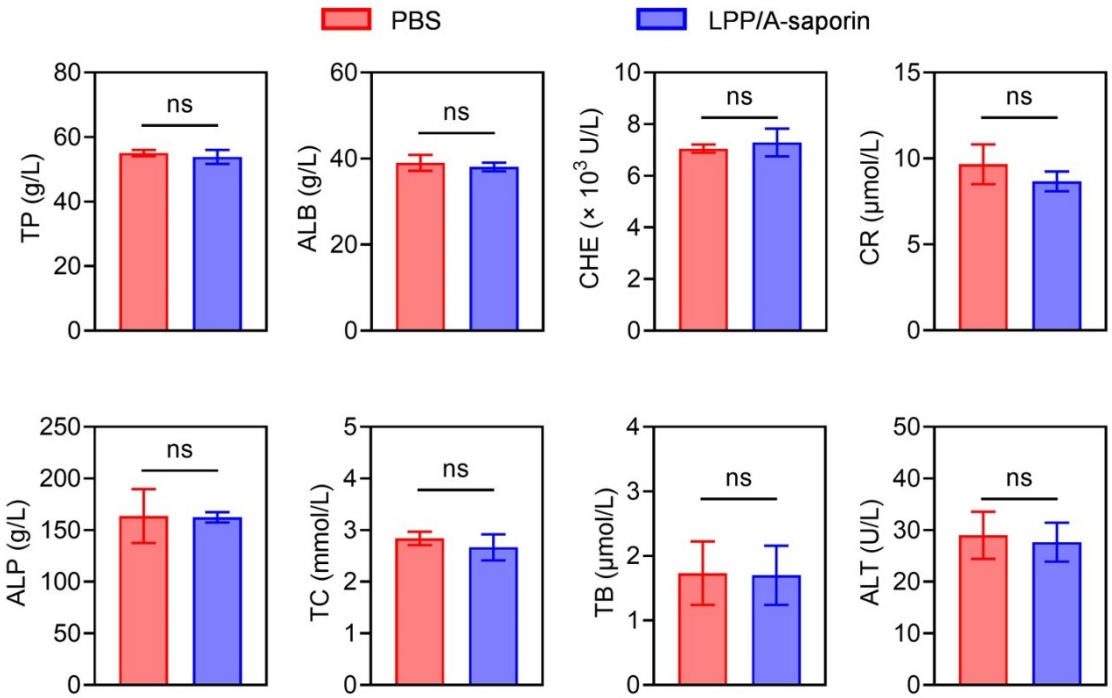

Figure S18. Blood biochemical levels of mice at 24 h post the second injection (n = 3). PBS or LPP/A-saporin NCs were intratumorally injected to HeLa tumor-bearing mice on day 0 and 2 at 0.1 mg saporin/kg.

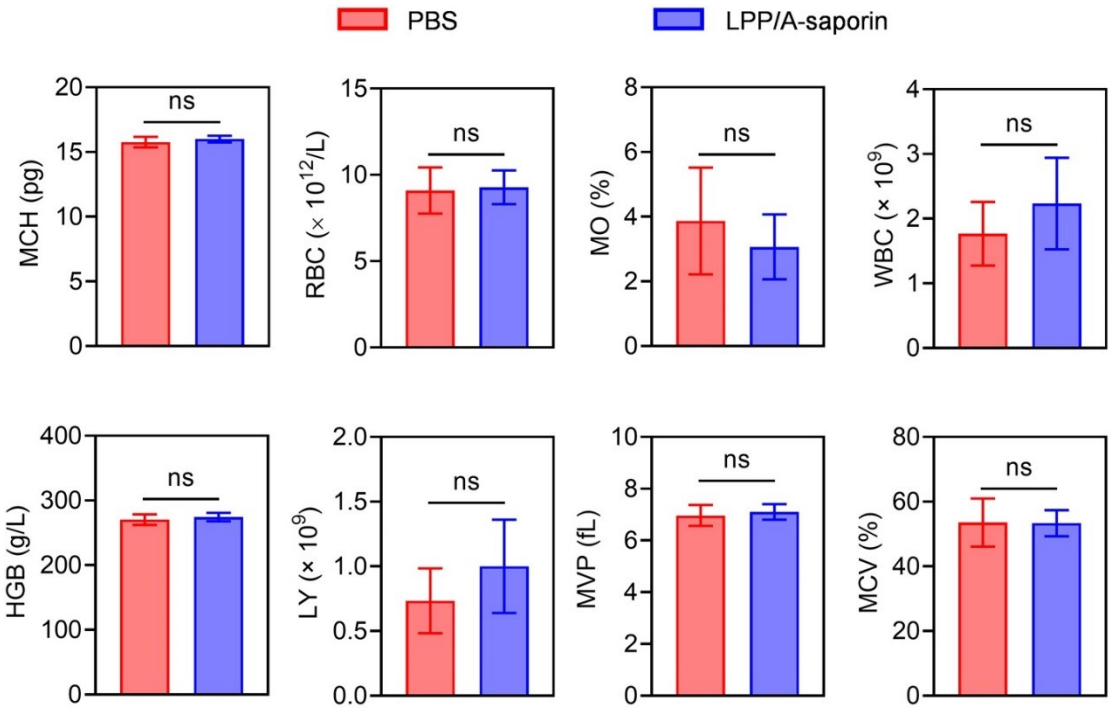

Figure S19. Hematological parameters of mice at 24 h post the second injection (n = 3). PBS or LPP/A-saporin NCs were intratumorally injected to HeLa tumor-bearing mice on day 0 and 2 at 0.1 mg saporin/kg.

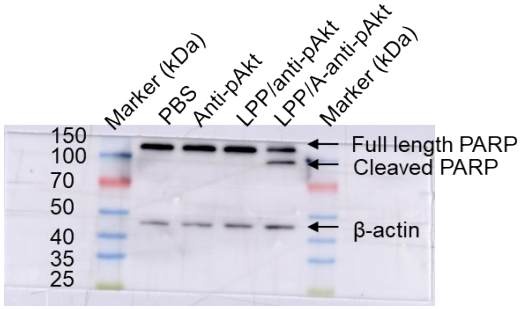

Figure S20. Uncropped blots for Figure 5C.

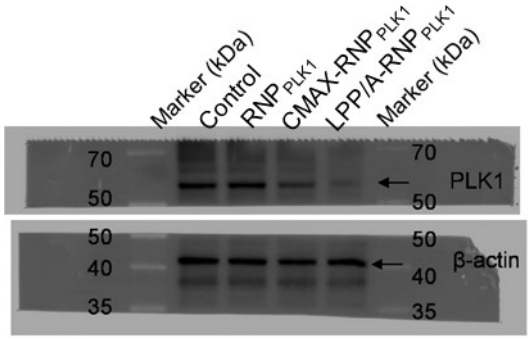

Figure S21. Uncropped blots for Figure 6E.

Table S1. Sequences of sgRNA used in this study.

Nucleic Acid ID	Sequence (5'-3')
$sgRNA_{GFP}$	GACAAGATGTCCTCGGCGAA
$sgRNA_{PLK1}$	CGGAGGCTCTGCTCGGATCG

Table S2. Primer sequences for the T7E1 assay.

Name	Sequence (5'-3')
PLK1-F	GCGTCCGTGTCAATCAGGTT
PLK1-R	ATCTCTTTCGCCGGTGGAG

Table S3. Primer sequences for real-time PCR assay.

Gene	Sequence (5'-3')
PLK1-F	GGCAACCTTTTCCTGAATGA
PLK1-R	AATGGACCACACATCCACCT
β-actin-F (Human)	TCTGGCACCACACCTTCTACAATG
β-actin-R (Human)	GGATAGCACAGCCTGGATAGCAA

References

1. X. Liu, Z. Y. Zhao, F. Wu, Y. B. Chen and L. C. Yin, *Adv. Mater.*, 2022, **34**, 2108116.
2. Z. Y. Zhao, X. Liu, M. Y. Hou, R. X. Zhou, F. Wu, J. Yan, W. Li, Y. J. Zheng, Q. M. Zhong, Y. B. Chen and L. C. Yin, *Adv. Mater.*, 2022, **34**, 2110560.