

Supplementary Materials for

Transdermal Delivery of CRISPR/Cas9-Mediated Melanoma Gene Therapy via Polyamines-Modified Thermosensitive Hydrogels

Methods

Materials

L-aspartic acid-4-N-carboxycyclic anhydride (NCA), N,N-Dimethylformamide (DMF), hexylamine, pentaethylenhexamine, and N-methylpyrrolidone (NMP) were purchased from Macklin (Shanghai, China). Poloxamer 407 (P407), Poloxamer 188 (P188), hyaluronic acid, DOX, Annexin V-FITC/PI Apoptosis Detection Kit and AM/PI Apoptosis Detection Kit were purchased from Meilunbio (Dalian, China). RNA extraction Kit and Cell Total RNA Isolation Kit were purchased from Foregene (Chengdu, China). Reverse transcription kit and Fastking RT Kit were purchased from Tiangen (Beijing china). TranscriptAid T7 High Yield Transcription Kit was purchased from Thermo Scientific (Shanghai, China). The Genome-Editing Mutation Detection Kit was purchased from Beyotime (Shanghai China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and Dul-becco's Modified Eagle's Medium (DMEM) were purchased from Sigma-Aldrich (St Louis, MO, USA). Fetal bovine serum (FBS) were obtained from Gibco (Grand Island, NY, USA).

Cell culture and animals

B16F10 and 293T cells were obtained from the Cell Bank of Shanghai, Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in DMEM with 10% FBS and 1% penicillin–streptomycin. Both cell lines were cultured in a humidified atmosphere incubator with 5% CO₂ at 37 °C.

6-8 weeks old healthy male C57BL/6 mice were obtained from Hubei Provincial Center for Disease Control and Prevention (Wuhan, China). All animal protocols complied with institutional and local ethical regulations and were approved by the Institutional Animal Care and Use Committee of Hubei University (No. 20240012). This study complies with the ARRIVE guidelines.

T7 Endonuclease 1 (T7E1) mutation detection

Gene editing potency of YB-1 by Psh delivered RNP complex was characterized in B16F10

cells. Briefly, cells were seeded into 6-well plates at a density of 2×10^5 per well and incubated for 24 hours. After transfected with Psh@RNP (Molar ratio of Cas9/sgRNA is 1:3) for 6 hours, the cells were incubated with DMEM containing 2% FBS for next 72 hours. The total genome of B16F10 was extracted by FastPure cell/tissue DNA isolation mini-kit (Vazyme, China). Genomic regions flanking the target sites were amplified with primers (details in Table S1) by PCR at an annealing temperature of 54 °C. 3 µL of the PCR product containing 300 µg of DNA was diluted with 2 µL of $10 \times$ Reaction buffer and 12 µL Nuclease water for the annealing process. Next, 1 µL of T7E1 was added to the system and incubated at 37 °C for 30 min. The cleavage products were evaluated by 1% agarose gel electrophoresis. The brightness of bands was measured by ImageJ software to calculate the gene-editing efficiency and off-targeted effect.

Quantitative RT-PCR (qRT-PCR)

The expression of YB-1 gene was quantified by qRT-PCR. Total RNA was extracted from B16F10 cells or tumor tissues by RNA extraction Kit and Cell Total RNA Isolation Kit (Foregene, Chengdu, China) according to the manufacturer's instructions. The total RNA was reverse transcribed into cDNA with reverse transcription kit and Fastking RT Kit (Tiangen, Beijing, China). The qPCR was performed using $2 \times$ Universal Blue SYBR Green qPCR Master Mix (Servicebio, China) on a Bio-Rad CFX 96 Touch Real Time PCR System (Thermo Fisher, Waltham, MA, USA) according to the manufacturer's protocols. Primers were listed in Table S1.

Expression and purification of Cas9

The plasmid pET28a-Cas9-2NLS-6×His was expressed and transformed using E. coli BL21 (DE3) cells. A single colony was inoculated into LB liquid medium containing kanamycin and cultured at 37°C with shaking at 220 r/min until the OD600 reached 0.6. Isopropylβ-D-1-thiogalactopyranoside (IPTG, 1 mM) was then added to induce protein expression, and the culture was incubated at 18°C with shaking at 200 r/min. Cells were harvested when the OD600 reached 0.8. The bacterial pellet was resuspended in PBS and lysed using a low-temperature ultra-high-pressure continuous-flow cell disruptor (JNBIO,

China). After centrifugation, the supernatant was collected and loaded onto a Ni-NTA affinity chromatography column. The mixture was gently stirred at 4°C for 1 hour, followed by washing with imidazole gradients of varying concentrations to elute the Cas9. The purity of the eluted fractions was analyzed by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The Cas9-containing imidazole eluate was concentrated using an ultrafiltration tube, flash-frozen in liquid nitrogen, and stored at -80°C for future use.

Design and synthesis of sgRNA

Three sgRNA sequences targeting the YB-1 gene were designed using CRISPOR (tefor.net). The transcription template was amplified using the lentiCRISPR V2 plasmid as a template, and sgRNA was synthesized using the TranscriptAid T7 High Yield Transcription Kit (Thermo Scientific). The sgRNA was purified with lithium chloride (final concentration: 2.5 M) and analyzed by 2% agarose gel electrophoresis to confirm size and integrity. The concentration was measured using a NanoDrop 8000 spectrophotometer (Thermo Scientific, Massachusetts, Waltham, USA). The sgRNA was aliquoted, flash-frozen in liquid nitrogen, and stored at -80°C for future experiments.

74 **Table S1** Relevant primers.

Target locus	Primer F	Primer R
YB-1 (RT-PCR)	AGGTCATCGCAACGAAG	CACCAGCTGAGAATTCGT
Cas9	TATACCATGGGCAGCAGC ATGGACAAGAAGTACAG CATCGG	GTCTCAGCTGGGAGGCGAC CACCACCACCACCACCAC
PET 28a	CACCACCACCACCACCA CTGAGAT	AGGAGATATACCATGGGCA GCAGC
Puc 18	CCAGCTGAGAATTCGTC CTGGGTAATGGAGCATTAC G	ATGATGCGGGTGACGCAGG TCATCGCAACGAAG
YB-1 (qPCR)	CAGACCGTAACCATTATAG ACGC	ATCCCTCGTTCTTTTCCCCAC
β-actin (qPCR)	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCAT GT

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77 **Table S2** Sequences and Primers of sgRNA(YB-1) and sgRNA(GFP).

Target locus	Sequence	Primer F	Primer R
SgRNA (YB-1)-1	TGGTGGAGTTCC AGTTCAAG	TTCTAATACGACTCA CTATAGTGGTGGAG T TCCAGTTCAAGGTTT TAGAGCTAGAAA	CGAGTCGGTGC
SgRNA (YB-1)-2	GAGAGTGGGGAA AAGAACGA	TTCTAATACGACTCA CTATAGGAGAGTGG GGAAAAGAACGAG TTTTAGAGCTAGAA A	CGAGTCGGTGC
sgRNA (YB-1)-3	GTCAGCAGCCAC CTCAACGT	TTCTAATACGACTCA CTATAGGTCAGCAG C CACCTCAACGTGTTT TAGAGCTAGAAA	CGAGTCGGTGC
SgRNA (GFP)	CAAGATCCGCCA CAACATCG	TTCTAATACGACTCA CTATAGCAAGATCC G CCACAACATCGGTT TTAGAGCTAGAAA	CGAGTCGGTGC

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80 **Table S3** Preparation scheme and properties of five kinds of gelation. Date was expressed
81 as mean $\pm$ SD (n=3).

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Sample	Mass ratio of P407	Mass ratio of P188	Gelation Time/s	Injectability
Nuh ₁	20%	1.2%	57 $\pm$ 5	Easy to inject
Nuh ₂	20%	2.5%	20 $\pm$ 5	Injectable
Nuh ₃	20%	5%	10 $\pm$ 5	Hard to inject
Nuh ₄	20.81%	3.46%	40 $\pm$ 5	Easy to inject
Nuh ₅	24%	5%	5 $\pm$ 5	Cannot inject

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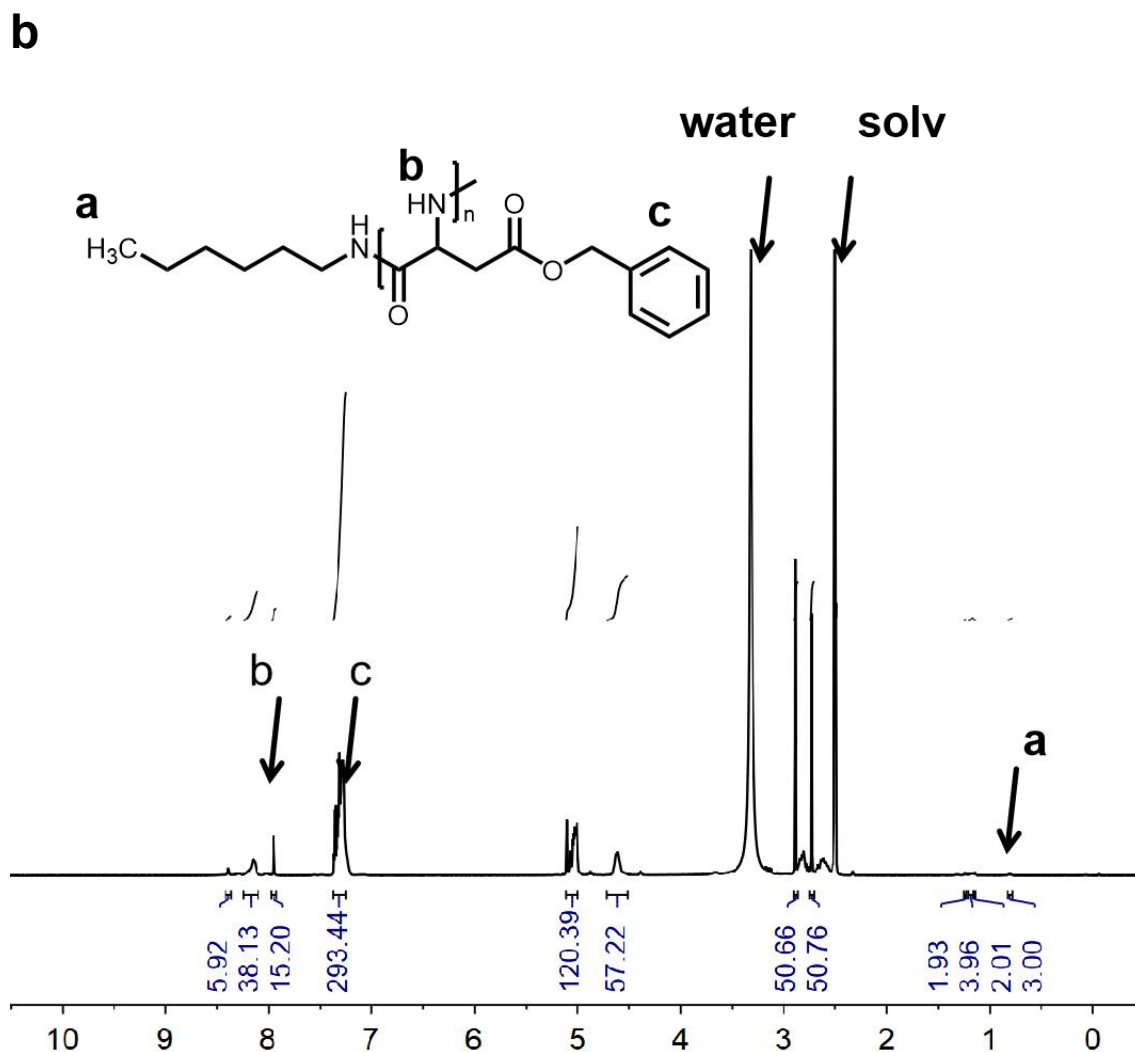
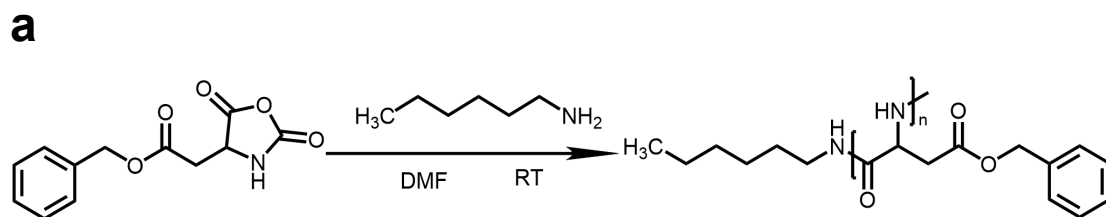
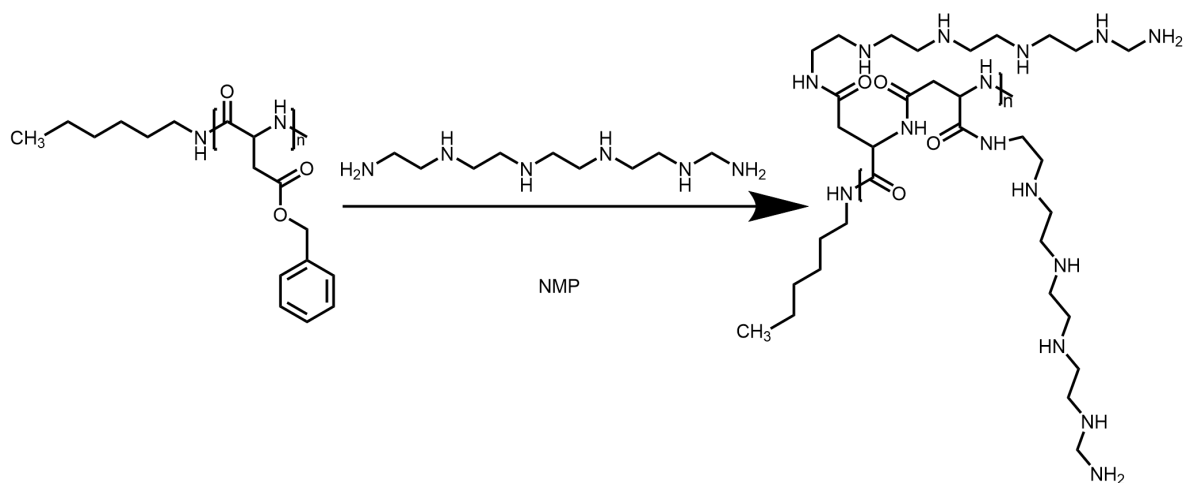


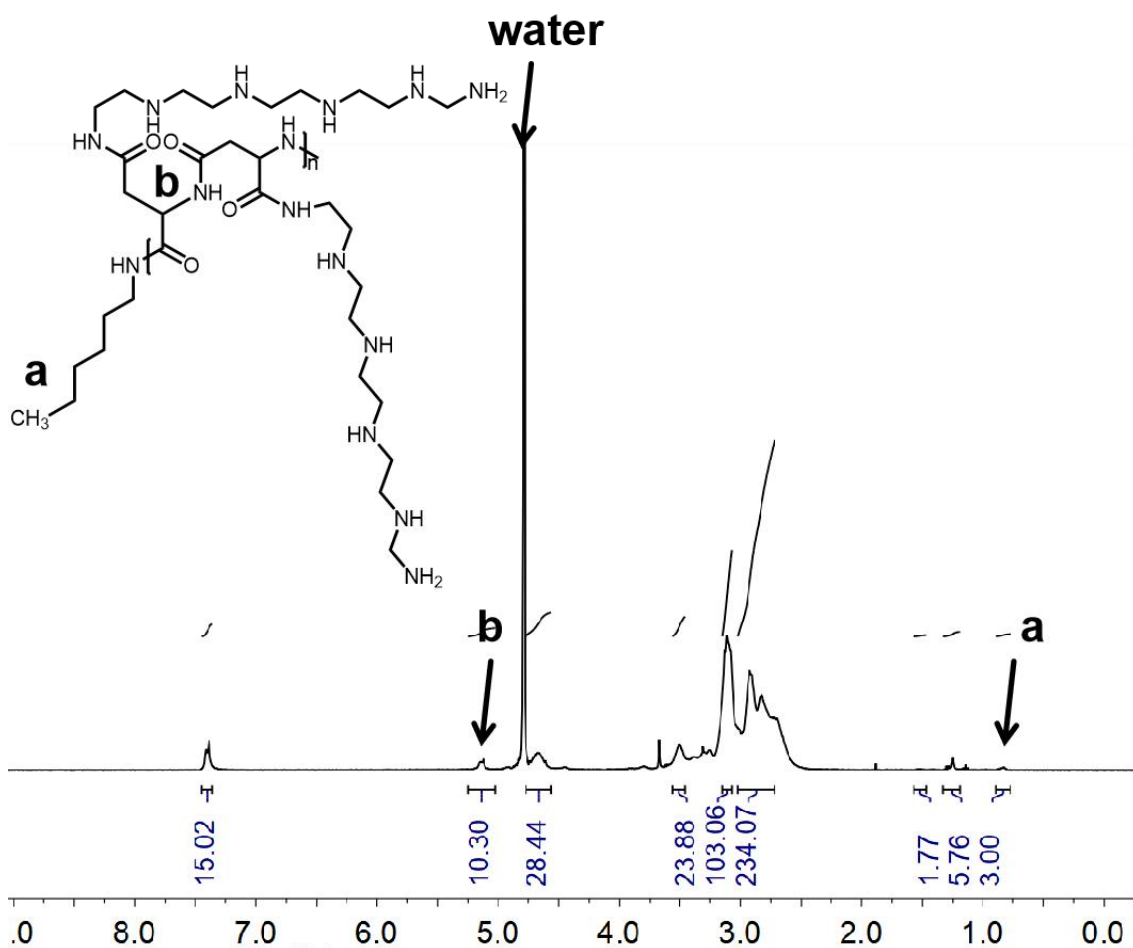
Fig. S1 Synthetic scheme and ¹H NMR spectra of PBLD. (a) Synthetic scheme of PBLD copolymer [1,2]. (b) ¹H NMR spectra of PBLD (solvent: DMSO- d_6).

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102 **b**



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104 **Fig. S2** Synthesis scheme and ¹H NMR spectra of polyamines. (a) Synthesis scheme of
 105 cationic polymers (polyamines) [1]. (b) ¹H NMR spectra of polyamines (solvent: D₂O).



Fig. S3 Psh@DOX has fluid injectability.

a

b

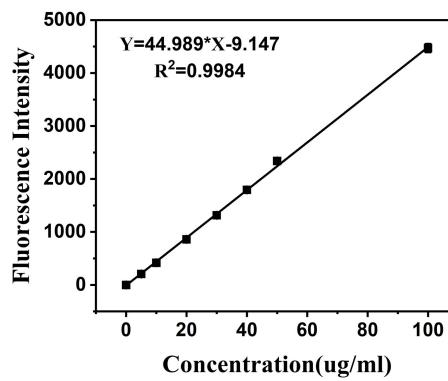
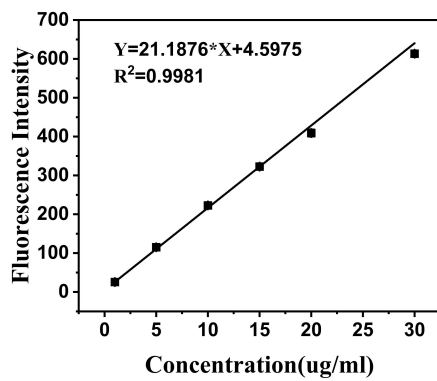


Fig. S4 Regression curve of DOX and FITC-BSA. (a) Regression curve of DOX. (b) Regression curve of FITC-BSA. Data was expressed as mean $\pm$ SD (n=3).

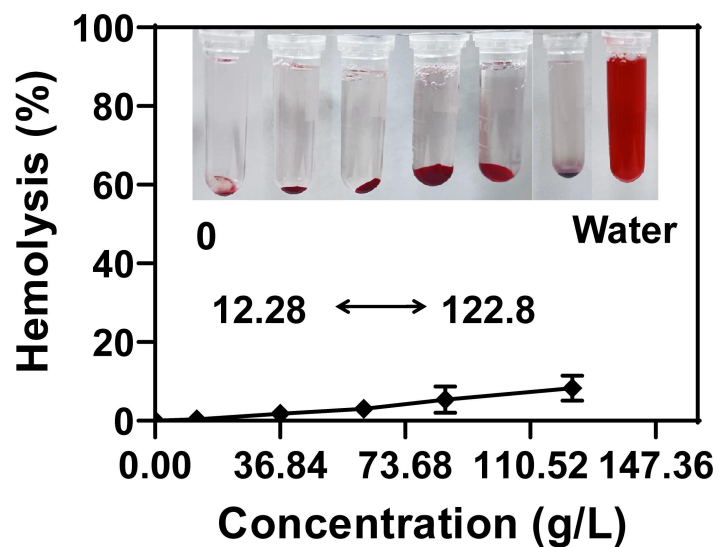


Fig. S5 In vitro hemolysis results of Ngh. Date was expressed as mean $\pm$ SD (n=3).

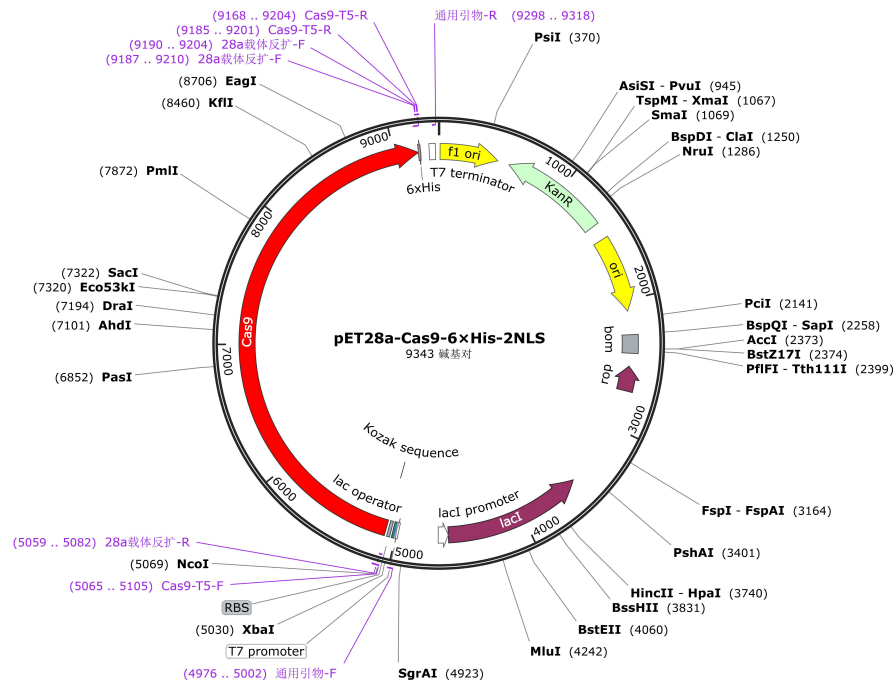


Fig. S6 Map of pET28a-Cas9-6xHis-2NLS plasmid.

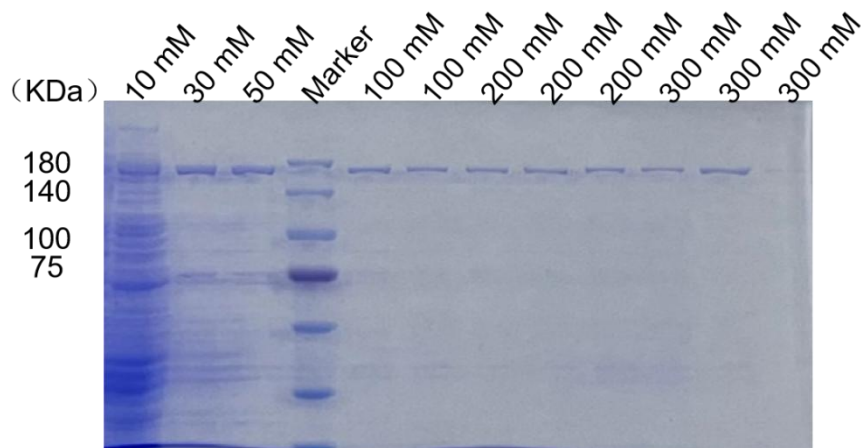


Fig. S7 SDS-PAGE results of Cas9 protein purification process (*Ni*-NTA spin columns).

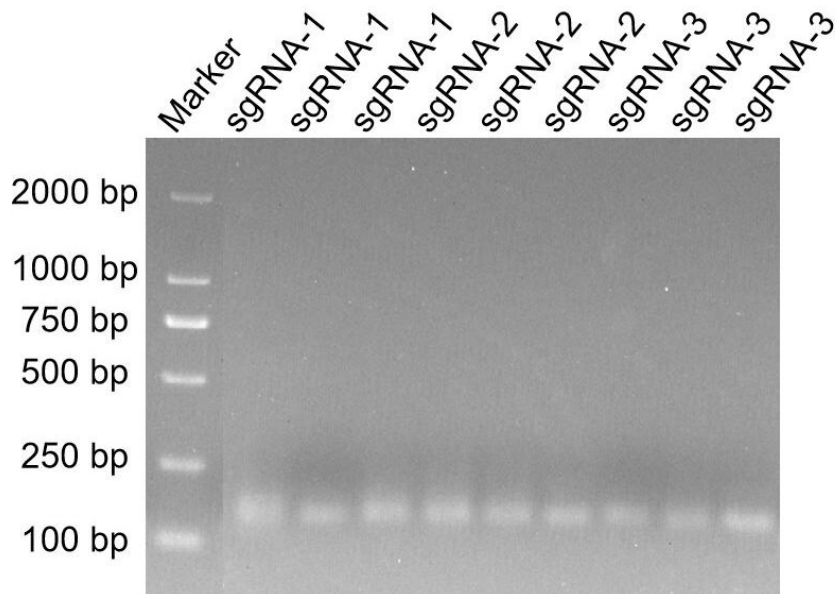


Fig. S8 Agarose electrophoresis results of sgRNA obtained by transcription.

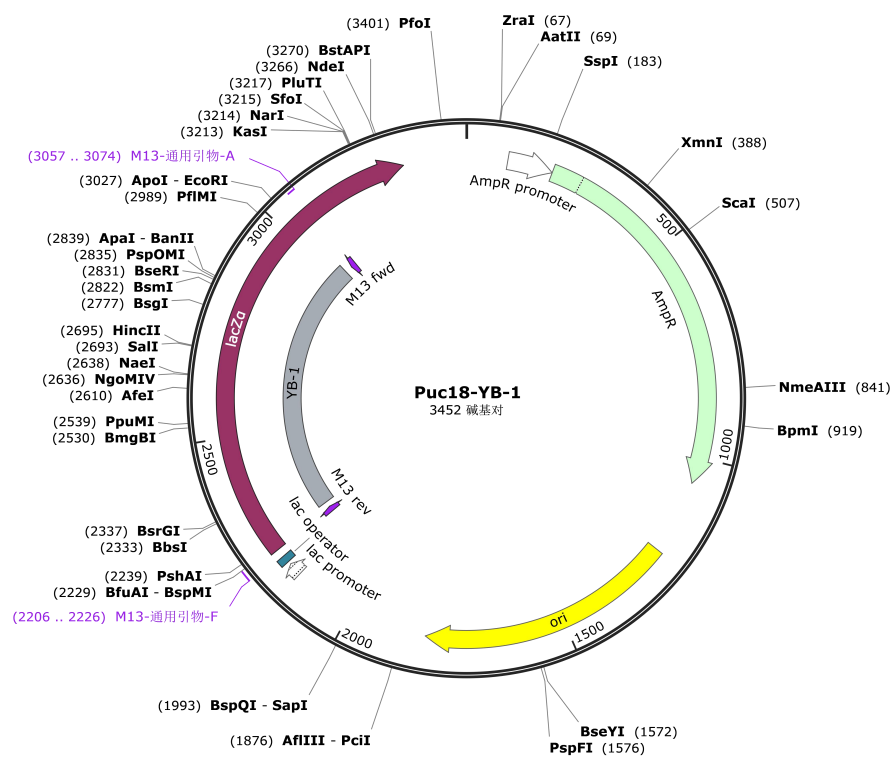


Fig. S9 Map of Puc18-YB-1 plasmid.

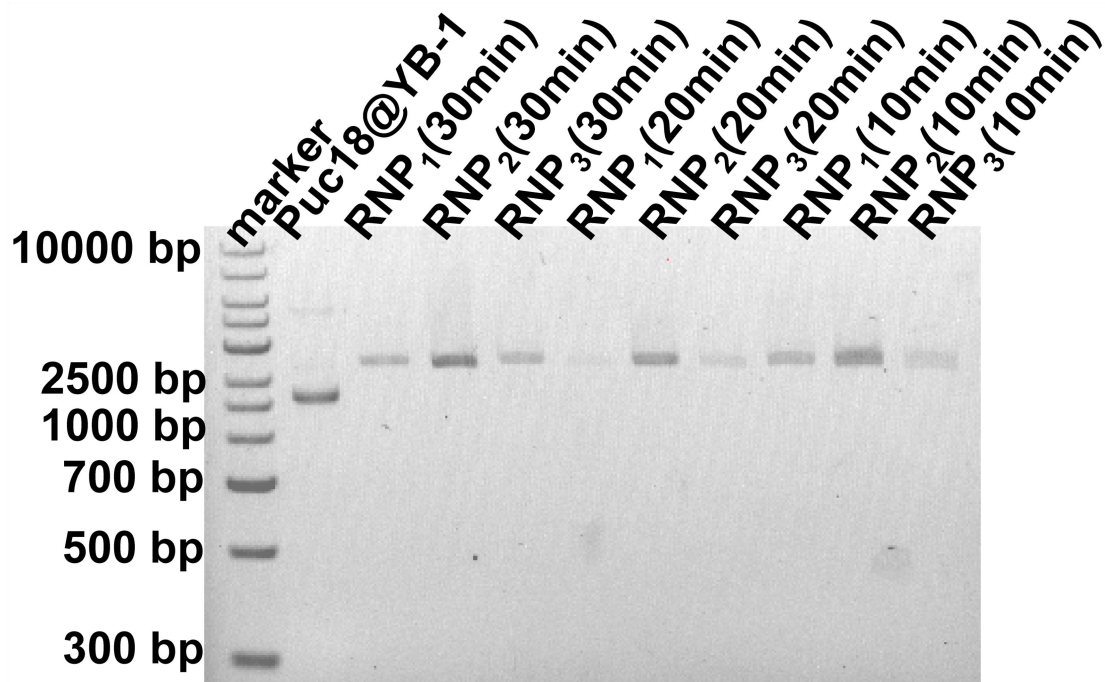


Fig. S10 In vitro cleavage of three sgRNAs at different cleavage times.

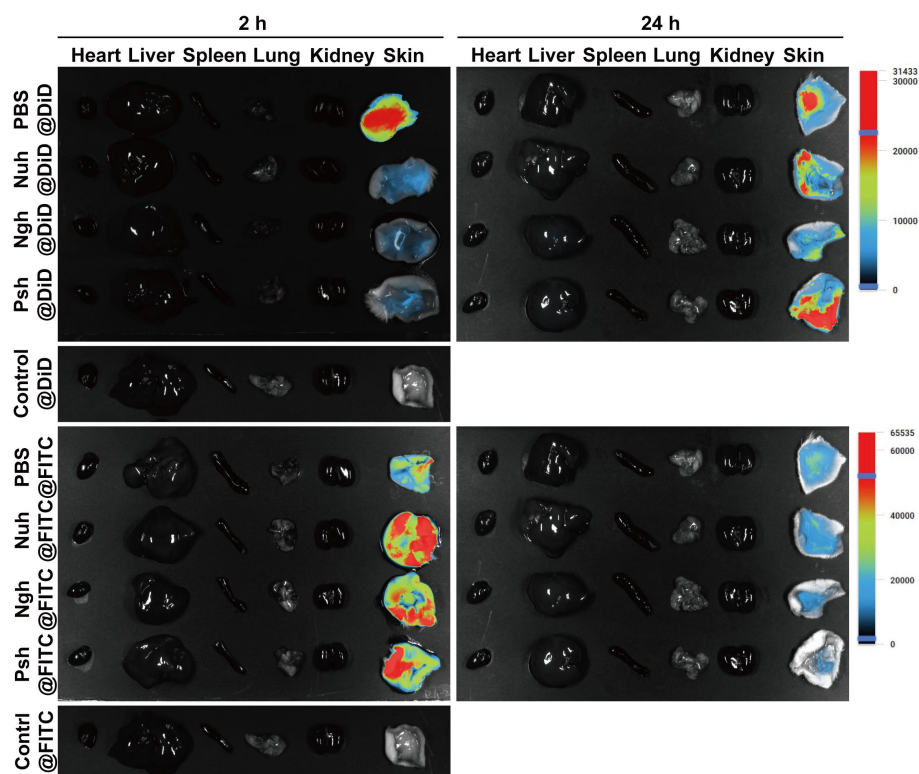


Fig. S11 In vivo fluorescence imaging revealed the biodistribution of DiD and FITC in mouse organs at different time points after injection of PBS@DiD@FITC-BSA, Nuh@DiD@FITC-BSA, Ngh@DiD@FITC-BSA, or Psh@DiD@FITC-BSA.

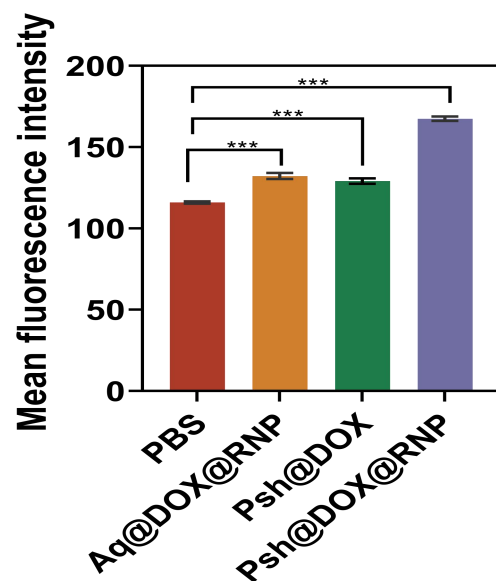


Fig. S12 Quantification of mean fluorescence intensity of TUNEL by Imagej. Data was expressed as mean $\pm$ SD (n=3).

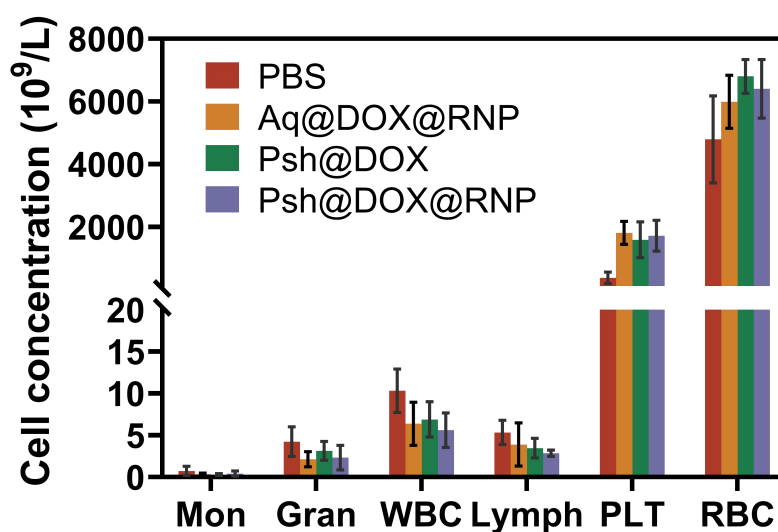


Fig. S13 C57BL/6 mice Hematologic analysis of mouse blood. Data was expressed as mean $\pm$ SD (n=6).

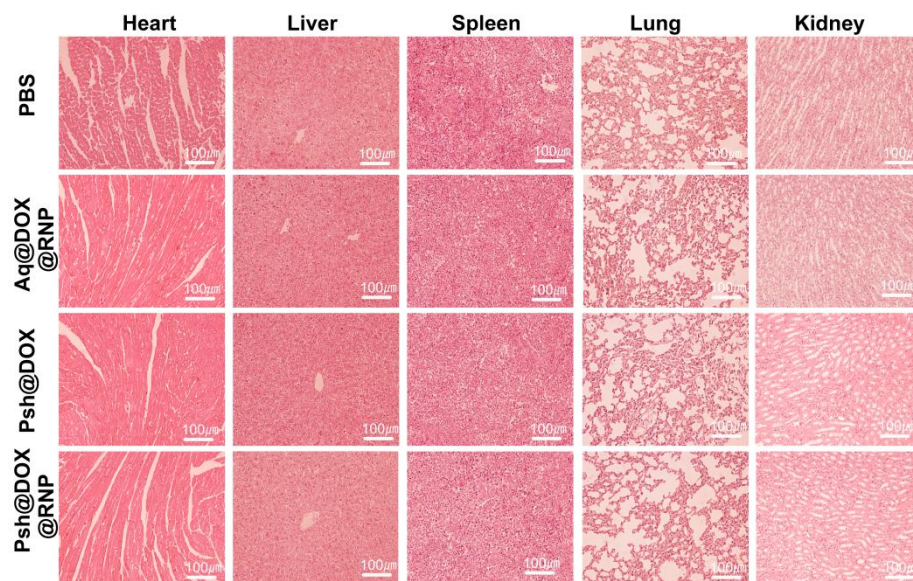


Fig. S14 H & E staining sections of organs from C57BL/6 mice after treatment.

References

1. Li J, Wu C, Wang W, He Y, Elkayam E, Joshua-Tor L, Hammond PT: **Structurally modulated codelivery of siRNA and Argonaute 2 for enhanced RNA interference.** *Proc Natl Acad Sci U S A* 2018, **115**:E2696-e2705.
2. Zong L, Li X, Wang H, Cao Y, Yin L, Li M, Wei Z, Chen D, Pu X, Han J: **Formulation and characterization of biocompatible and stable I.V. itraconazole nanosuspensions stabilized by a new stabilizer polyethylene glycol-poly(β -Benzyl-L-aspartate) (PEG-PBLA).** *Int J Pharm* 2017, **531**:108-117.