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

Effective Gene Editing of Melanoma by Delivery of Cas9 mRNA with Highly Branched Poly(β-amino ester)

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Materials

Bisphenol A glycerolate diacrylate, N-methyl-1,3-propanediamine, 1,2-ethanedithiol, triacrylate trimethylolpropane triacrylate, 4-(2-aminoethyl)morpholine, 1,5-diamino-2-methylpentane were purchased from Sigma-Aldrich, TCI, Aladdin, and Meryer, and used as received without further purification. jetMESSENGER (jetM, catalog number 101000005, Polyplus), EGFP mRNA (TriLink BioTechnologies, San Diego, CA, USA), Fluc mRNA (RNAlfa Biotech, Hefei, Anhui, China), MTT working solution (Beyotime, ST316), T7 Endonuclease I (New England Biolabs, M0302S) were used according to manufacturers' protocols. Cell culture media and all other biological reagents were purchased from Biological Industries.

Gel permeation chromatography (GPC) measurements

The number-average molecular weight (M_n), weight-average molecular weight (M_w), and polydispersity index ($Đ$) of HPAE were analyzed using a GPC system (Agilent 1260 Infinity II) equipped with a refractive index (RI) detector. Samples were prepared by dissolving in 1 mL of dimethylformamide (DMF) containing 0.1% LiBr, followed by vigorous vortex mixing for 30 seconds. The resulting solutions were then filtered through a 0.22 μm filter. The GPC system utilized PolarGel-M Gard (50 mm × 7.5 mm) and two PolarGel-M columns (300 mm × 7.5 mm) connected in series, with DMF containing 0.1% Lithium bromide (LiBr) as the mobile phase at a flow rate of 1 mL/min. The column temperature was kept constant at 50 °C. Calibration of the columns was performed using linear poly(methyl methacrylate) (PMMA) standards.

NMR measurements

The purity and chemical structure of HPAE were confirmed through proton nuclear magnetic resonance (^{1}HNMR) analysis. Samples were dissolved in deuterated chloroform ($CDCl_3$) prior to measurement. ^{1}HNMR spectra were recorded using a Varian Inova spectrometer operating at 400 MHz (Bruker, Switzerland). Chemical shifts were reported in parts per million (ppm), with the $CDCl_3$ solvent peak (7.26 ppm) used as the internal reference.

mRNA binding affinity of HPAE

The mRNA binding affinity of HPAE was evaluated using the Quant-iT™

RiboGreen™ Kit (Invitrogen). Polyplexes were prepared at an HPAE/mRNA w/w ratio of 60:1 by adding 10 μL containing 50 ng of EGFP mRNA to a 96-well plate and adjusting the volume to 50 μL with 1X TE buffer. Then, 100 μL of RiboGreen reagent (diluted 1:200 in 1X TE buffer) was added, mixed thoroughly, and incubated for 5 minutes. Fluorescence was measured using a Synergy Hybrid H1 microplate reader (excitation/emission: 480/520 nm). mRNA alone was used as a control to calculate binding efficiency. The mRNA binding affinity of HPAE was determined as follows:

$$\text{mRNA binding affinity (\%)} = [1 - (F_{\text{Sample}} - F_{\text{Blank}})/(F_{\text{mRNA}} - F_{\text{Blank}})]\times 100\%$$

Where the F_{Sample}, F_{mRNA} and F_{Blank} are the fluorescence intensity of the sample, control and blank, respectively.

Size and Zeta potential measurements of polyplexes

Polyplexes were prepared at a w/w ratio of 60:1 using 0.1 μg of mRNA, then diluted to 1 mL with deionized water. The hydrodynamic size and zeta potential were measured using a Malvern Zetasizer Nano ZSE (wavelength: 632 nm, scattering angle: 90°). Measurements were conducted in triplicate, and only data meeting the software quality criteria were included.

Transmission electron microscopy (TEM) observation

Polyplexes were prepared at a w/w ratio of 60:1, washed twice with deionized water to remove salts, and re-suspended in 10 μL of deionized water. A 2.5 μL aliquot of the solution was deposited onto a carbon-coated copper grid and allowed to adsorb for 5 minutes. Excess liquid was removed using filter paper. The grid was then analyzed using a TEM (Thermo Fisher Scientific, Talos L120C) operating at an accelerating voltage of 120 kV. Images were captured to assess the size, shape, and internal structure of the polyplexes, with multiple areas imaged to ensure representative results.

Cell culture

All cell lines used in this study were sourced from Cell Bioscience, Inc. (Shanghai, China). Human embryonic kidney cells (HEK293T) and human melanoma cells (A375) were maintained in Dulbecco's Modified Eagle Medium (DMEM), mouse melanoma

cells (B16F10) in Roswell Park Memorial Institute Medium (RPMI), and human melanoma cells (A2058) cells in Minimum Essential Medium (MEM). Each culture medium was supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (P/S). Cells were incubated at 37°C in a humidified atmosphere with 5% CO_2 and were regularly tested to confirm the absence of mycoplasma contamination.

Repeated transfection of cells

HEK293T and B16F10 cells were seeded in 96-well plates at a density of 5×10^4 and 2×10^4 cells per well, respectively, and incubated overnight at 37°C prior to transfection. For each well, polyplexes containing 50 ng EGFP mRNA were prepared at a w/w ratio of 60:1 as previously described. For each transfection, the culture medium was replaced with 100 μL of fresh medium containing 1% P/S and 10% FBS, followed by the addition of the polyplexes. After 4 hours of incubation, the medium was replaced with 100 μL of fresh complete medium, and the cells were maintained under standard culture conditions. For repeated transfection (two or three rounds), cells were initially seeded at the same density. Before each transfection cycle, the culture medium was replaced with pre-warmed complete medium, followed by the addition of the polyplexes. After 4 h of incubation, the medium was replaced with fresh complete medium, and the cells were cultured for an additional 20 h. After the final transfection round, no further medium changes were made until the end of the experiment. EGFP expression was assessed by fluorescence microscopy and flow cytometry at 24, 48, 72, 96, and 120 hours after the initial transfection. Cell viability was evaluated simultaneously based on the percentage of live cells determined by flow cytometry.

Flow cytometry analysis

EGFP expression following transfection was analyzed by flow cytometry. 24 hours post-transfection, cells were harvested, washed, and resuspended in PBS containing 1% BSA. Single-cell suspensions were prepared by pipetting and filtering through a 70 μm cell strainer. Flow cytometry was performed using a BD FACSCanto flow cytometer (BD Biosciences) with BD FACSDiva software (version 8.0.1). Data from 10,000 cells were collected and analyzed using FlowJo software (version 10.8). Single cells were

gated based on forward and side scatter to exclude debris and doublets, and EGFP expression was evaluated within the live cell population.

Cell viability evaluation

Cell viability following transfection was assessed using the (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) (MTT) assay. 24 hours after transfection, the culture medium was removed, and cells were incubated with 100 μL of 0.5 mg/mL MTT solution for 4 hours. After incubation, the MTT solution was carefully removed, and the resulting formazan crystals were dissolved in 100 μL of DMSO. Absorbance was measured at 590 nm using a microplate reader.

Construction of HEK293T-EGFP and B16F10-EGFP

The plasmids pLVX-EGFP-Puro (P15212), pMD2.G (P0262), and psPAX2 (P0261) were obtained from Miaoling Plasmid (Wuhan, China). For lentiviral packaging, HEK293T cells were co-transfected with the transfer vector pLVX-EGFP-Puro, packaging plasmid psPAX2, and envelope plasmid pMD2.G at a 4:3:1 ratio using Lipofectamine™ 3000 (Thermo Fisher Scientific, L3000015) according to the manufacturer's instructions. Viral supernatants were collected at 48 and 72 hours post-transfection and filtered through 0.45 μm sterile filters. For stable cell line generation, HEK293T and B16F10 cells were transduced with the collected lentivirus in the presence of 8 μg/mL polybrene. After 24 hours, the medium was replaced with fresh complete growth medium. Stable cell lines were selected with 1 μg/mL puromycin for 7 days, with medium changes every 2–3 days to maintain selection pressure and remove dead cells.

Polymerase chain reaction and T7E1 analysis

Genomic DNA was extracted from cells 72 hours after transfection with Cas9 mRNA (L-7606-1000, polyadenylated, unmodified) and sgEGFP using the Genomic DNA Extraction Kit (TIANGEN, DP304-02). The regions flanking the on-target sites were amplified using 2X PCR Master Mix (Beyotime, D7228) and the following primers:

F: GACCACATGAAGCAGCACGA;

R: CGATGTTGTGGCGGATCTTG.

PCR products (400 ng) were denatured and hybridized in NEBuffer 2 (New England Biolabs) by heating to 95°C for 5 minutes, followed by a ramp down to 85°C at 2°C/second and then to 25°C at 0.1°C/second using an Applied Biosystems PCR system. The hybridized samples were digested with T7 Endonuclease I (New England Biolabs, M0302S) at 37°C for 15 minutes, then incubated at 65°C for 5 minutes to terminate the reaction. The digested products were purified and analyzed by agarose gel electrophoresis with SmArt Red Nucleic Acid Stain. Gel images were acquired using the Bio-Rad ChemiDoc™ Touch Imaging System.

Animal study

All animal procedures were approved by the Institutional Animal Care Committee of Xi'an Jiaotong University (Ethics Approval No. XJTUAE2023-1020) and conducted in accordance with institutional guidelines. Female C57BL/6 mice (6–8 weeks old) were maintained under standard housing conditions.

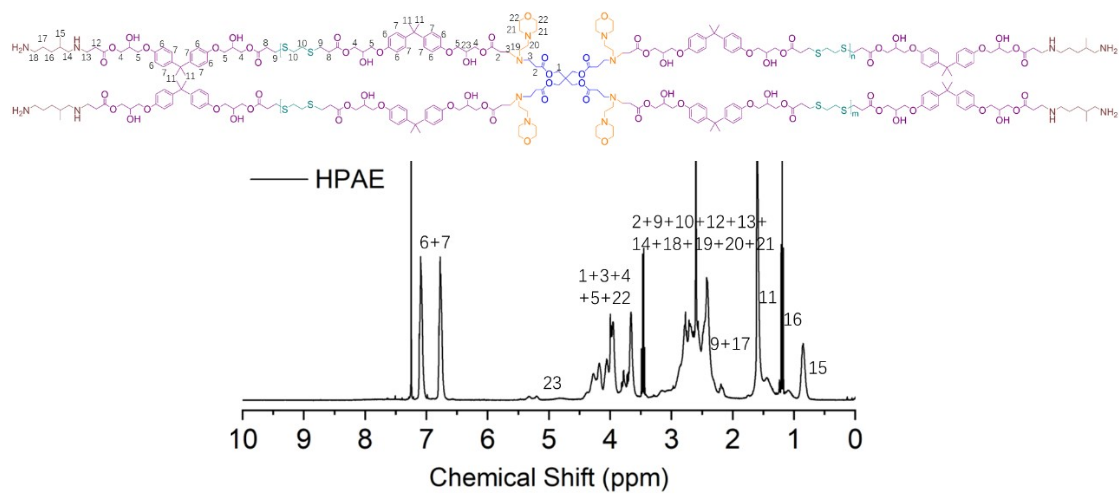

Figure S1. ^{1}HNMR analysis of HPAE. All characteristic signal peaks corresponding to the monomer units and the end-capping agent were clearly visible.

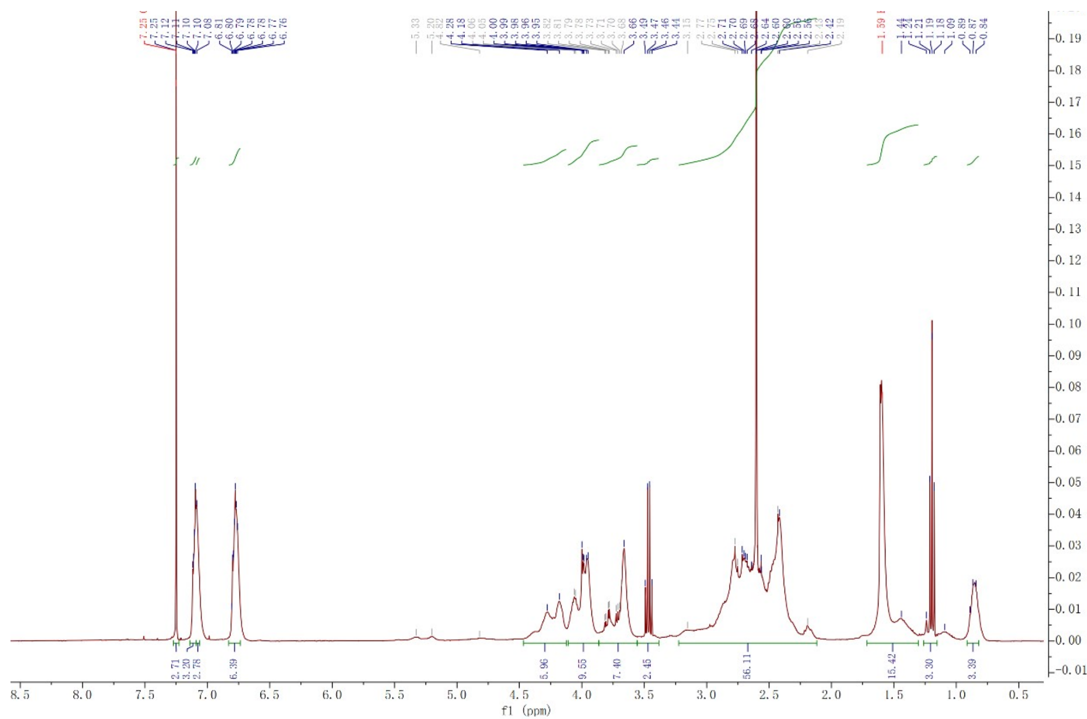

Figure S2. Calculation of the chemical composition of HPAE.

The chemical composition of BEDA, PETA, AMP, EDT and DMP in the HPAE was calculated as follows:

If [BEDA]:[PETA:[AMP]:[EDT]:[DMP]=a:b:c:d:e, according to NMR integral area:

$8a=12.44$ Eq. (1)

$3e=3.39$ Eq. (2)

$16b+8a+4c=22.91$ Eq. (3)

$20a+8c+4d+6e=56.11$ Eq. (4)

$2a+4b=2c+2d+e$ Eq. (5)

$4b+c=2.62$ Eq. (6)

Therefore, [a]:[b]:[c]:[d]:[d]=1.55:0.165:1.96:0.64:1.13

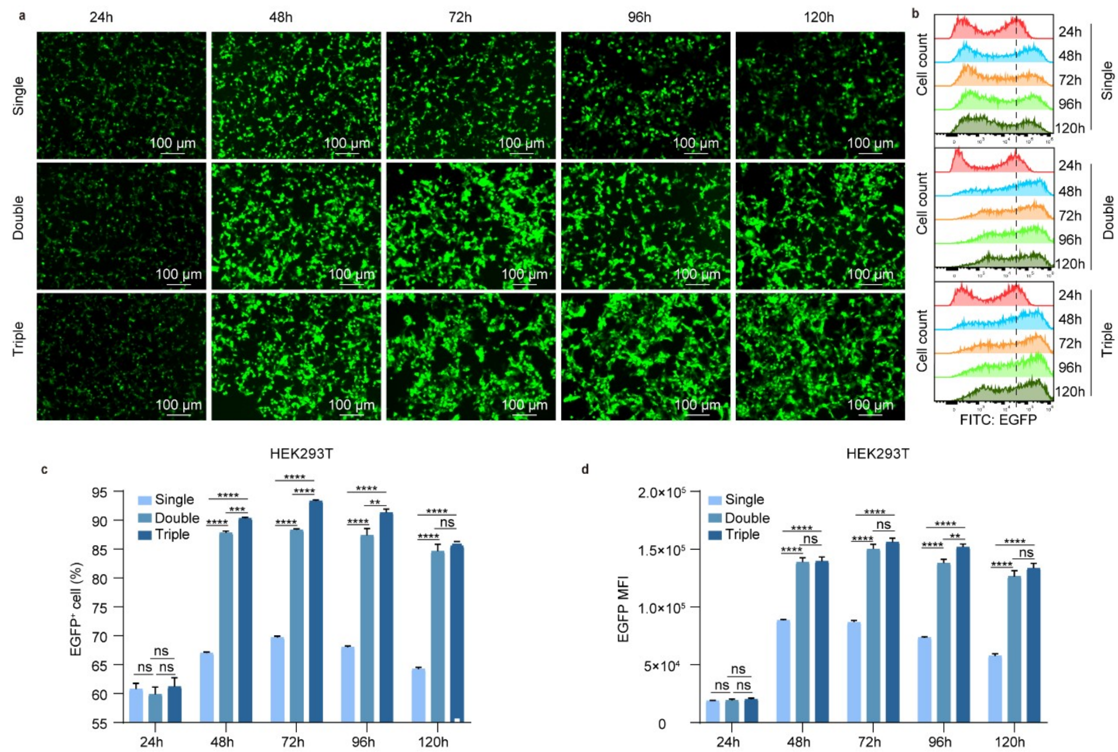

Figure S3. Repeat dosing enhances the mRNA transfection efficiency and duration mediated by HPAE in HEK293T cells. (a) Fluorescence microscopy images after single, double, and triple transfections, Scale bar: 100 μm; (b) Flow cytometry profiles; (c) Percentage of EGFP-positive cells; (d) MFI of EGFP in different groups. Data were analyzed using Student's t-test. $^{**}P < 0.01$, $^{***}P < 0.001$; ns, not significant. Data are presented as mean±SD.

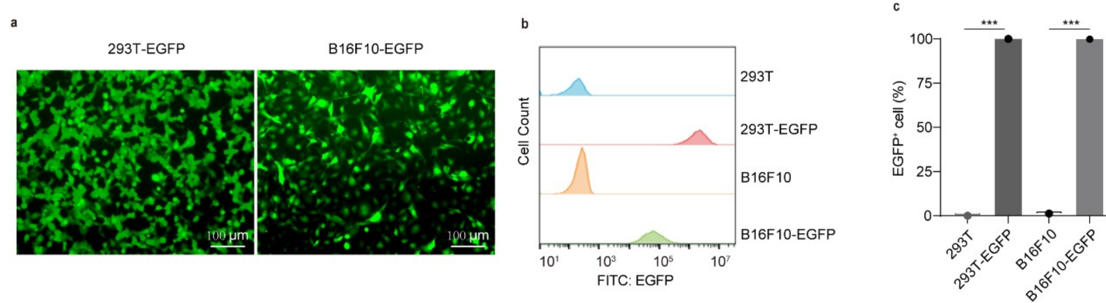

Figure S4. Establishment of HEK293T-EGFP and B16F10-EGFP stable cell lines. (a) Representative fluorescence images; scale bar: 100 μm. (b) Flow cytometry analysis; (c) EGFP-positive cell percentage. Significance was determined by using Student's t test. (***P< 0.001).

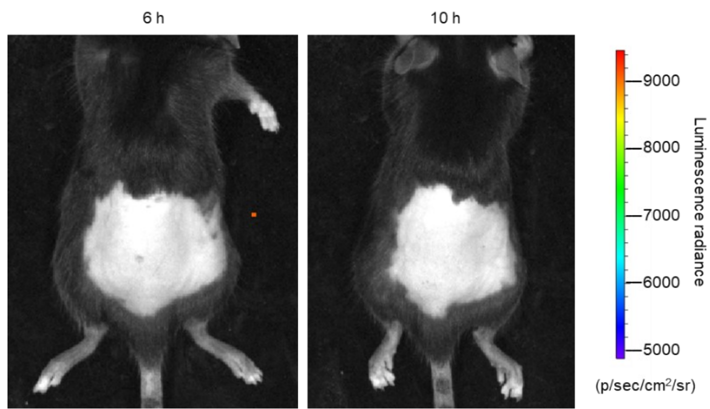

Figure S5. Luminescence images of mice at 6 and 10 hours after topical application of the control group with Fluc mRNA alone.