

Supporting Information

Attenuating Metabolic Competition of Tumor Cells for Favoring the Nutritional Demand of Immune Cells by a Branched Polymeric Drug Delivery System

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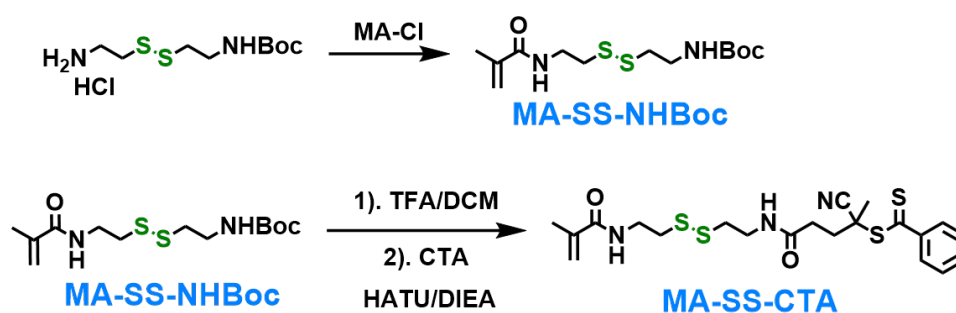
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Keywords: metabolic competition, branched polymeric prodrug conjugate, chemo-immunotherapy, tumor immune microenvironment, immunogenic cell death

Supplementary Text

S.1 Materials

VA044 (CAS No. 27776-21-2) and CTA (CAS No. 201611-92-9) were obtained from Sigma-Aldrich, and Trifluoroacetic acid (TFA) and Epirubicin hydrochloride from Aladdin Reagent Inc. (Shanghai, China). The 4T1 murine breast cancer cell line and Human Umbilical Vein Endothelial Cells (HUVECs) were acquired from Shanghai Institute for Biological Sciences (China). The Cell Counting Kit-8 kit was purchased from Suzhou Yuheng Biotechnology Co., Ltd. (China) and the AnnexinV-PI, DAPI, Lyso-Tracker Green markers from Beyotime Co., Ltd. (China). In addition, compounds of HPMA, MA-GG-NHNHBoc and MA-SS-MA were prepared via synthesis routes in previously reported studies [1]. Primary antibodies including β -actin (13E5), Bcl-2 (3498S), Bax (2772s), Cleaved-Caspase-3 (Asp175) (5A1E) (9664S), Caspase-3 (D3R6Y) (14220S), PARP (9542S), p21Waf1/Cip1 (E2R7A), and p53 (1C12) (2524S) were purchased from Cell Signaling Technology (CST, USA).

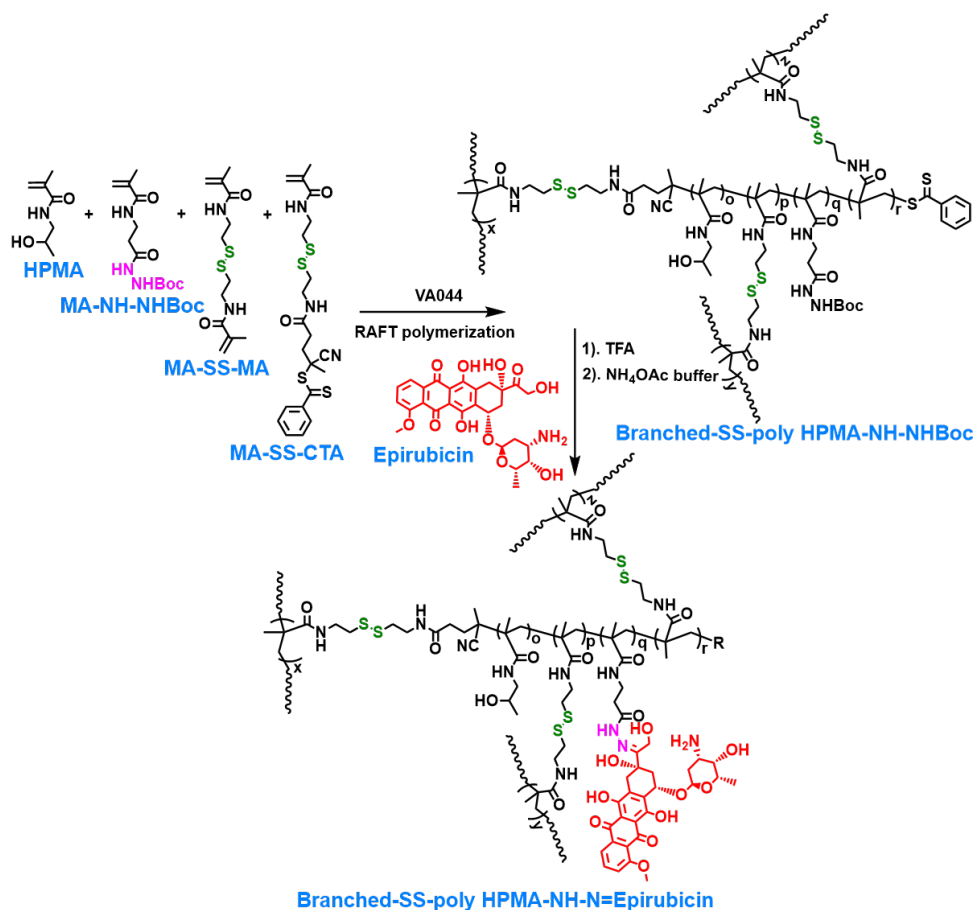
S.2. Synthesis of functional chain transfer agent MA-SS-CTA

Scheme S1. Structure and synthetic route for the functional monomer MA-SS-CTA.

MA-SS-NHBoc was synthesized according to the reported literature [2]. At 0 °C, to a solution of Boc-Cystamine·HCl (500 mg, 1.73 mmol, 1 eq), triethylamine (Et₃N, 526 mg, 5.19 mmol, 3 eq) and 0.05 eq 4-Methoxyphenol as an inhibitor in Dichloromethane (DCM), Methacryloyl chloride (0.36 mL, 2.42 mmol, 1.4 eq) was dropwise added. The mixture reacted at room temperature for 2 h. After completing the reaction, 50 mL of sat.aq. NaHCO₃ and 30 mL of DCM were added to this solution, and the aqueous layer was extracted with DCM (30 mL × 2). The organic layers were pooled, washed with sat.aq. NaHCO₃ (100 mL × 2), brine (100 mL × 2) successively, dried over Na₂SO₄, filtered and concentrated to give a crude mixture. The mixture was purified by column chromatography (silica gel, DCM/CH₃OH = 20: 1), giving the product MA-SS-NHBoc as a powder with a yield of 82.4% (457 mg), HR-MS: m/z = 321.1324 [M + H]⁺, m/z = 343.1426 [M + Na]⁺.

At 0 °C, 10 mL DCM and 10 mL TFA was added to a solution of MA-SS-NHBoc (420 mg, 1.31 mmol, 1 eq) sequentially. After reaction overnight, the solvents were removed and the residue was treated with diethyl ether to obtain a solid. The obtained solid, 4-Cyano-4-(phenylcarbonothioylthio)pentanoic acid (CTA, 439 mg, 1.57 mmol, 1.2 eq), 4-Methoxyphenol (0.05 eq), and HATU (747 mg, 1.97 mmol, 1.5 eq) in DMF were added to DIEA (508 mg, 3.93 mmol, 3 eq). The mixture reacted at room temperature for 2 h. The solvent was removed and the residue was purified by column chromatography (silica gel, DCM/CH₃OH = 30: 1 to 20: 1), giving the product MA-SS-CTA as a red oily substance with a yield of 74.6% (471 mg). ¹H NMR (400 MHz, d₆-DMSO, δ in ppm) (Figure S1): 1.85 (m, 3H, -C(**CH**₃)(CN)-S-(C=S)-), 1.91 (m, 3H, **CH**₃-C(=CH₂)-), 2.36-2.48 (m, 4H, -C(CH₃)(CN)-**CH**₂-**CH**₂-CONH-), 3.35-3.45 (m, 4H, -**CH**₂-CH₂-SS-CH₂-**CH**₂-), 5.30-5.68 (m, 2H, CH₃-C(=CH₂)-), 7.47-7.56 (m, 2H, -S-(C=S)-Ar-**H**_{ortho}), 7.64-7.72 (m, 1H, -S-(C=S)-Ar-**H**_{para}), 7.88-7.94 (m, 2H, -S-(C=S)-Ar-**H**_{meta}), 8.05-8.28 (m, 2H, -CONH-). ¹³C NMR (100 MHz, d₆-DMSO, δ in ppm) (Figure S2): δ 223.74, 144.12, 139.74, 128.97, 126.38, 119.27, 118.69, 46.32, 38.48, 37.98, 37.14, 36.93, 30.55, 23.12, 18.57. LC-MS (ES⁺): m/z = 482.0 [M + H]⁺ for peak at 6.549 min (Figure S3). HR-MS: m/z = 482.1054 [M + H]⁺, m/z = 504.0870 [M + Na]⁺ and m/z = 520.0622 [M + K]⁺ (Figure S4).

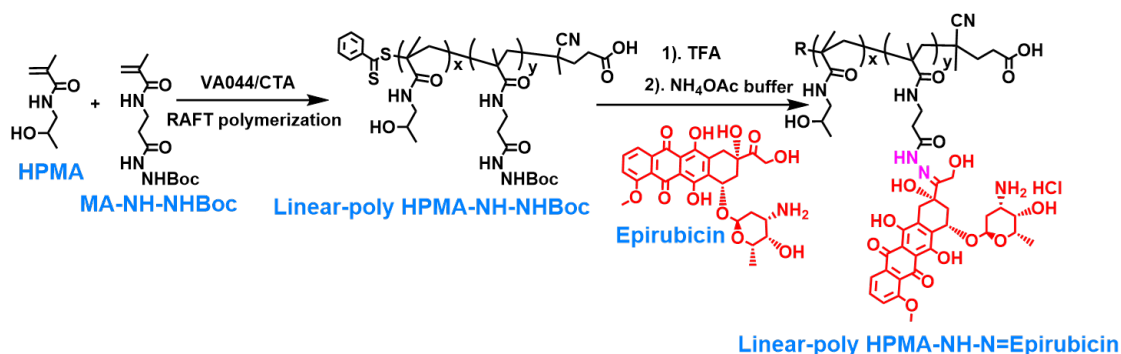
S.3. Synthesis of Branched-SS-poly HPMA-NH-N=Epirubicin and Linear-poly HPMA-NH-N=Epirubicin



Scheme S2. Structure and synthetic route for Branched-SS-poly HPMA-NH-N=Epirubicin.

The HPMA monomer (558.5 mg, 3.90 mmol), MA-NH-NHBoc (66.1 mg, 0.24 mmol), MA-SS-CTA (12.2 mg, 25.3 μ mol) as a functional chain transfer agent, MA-SS-MA (4.2 mg, 14.6 μ mol) as a functional crosslinker, and VA044 (2.4 mg, 8.4 μ mol) were dissolved in a deionized water/methanol mixture (2.9 mL, 1: 4). The mixture was bubbled with nitrogen for over 50 min at 0 °C, and stirred at 46 °C for 8 h and then quenched with liquid nitrogen. After dialysis against water in the dark (4 °C, MWCO 2 KDa) and subsequent filtration and freeze drying, the pink solid product was treated with excessive V501 for 2 h to remove the benzodithioate group and purified via dialysis against water. Branched-SS-poly HPMA-NH-NHBoc was obtained (358.4 mg, yield: 56.0%, 76.2 KDa, PDI = 2.86). 350 mg of Branched-SS-poly HPMA-NH-NHBoc was treated with 4 mL of TFA in a reaction bottle for 12 h at room temperature. The solvent was removed via a rotary evaporator and the residue was dissolved in 10 mL of RO water via constant stirring. The obtained solution was treated with dialysis against water in the dark (4 °C, MWCO 2 KDa), filtration and freeze drying. A polymer precursor, Branched-SS-poly HPMA-NH-NH₂ (74.8 KDa, PDI = 2.92, 320 mg), was obtained. Branched-SS-poly HPMA-NH-NH₂ (300 mg) was dissolved in 15 mL of 0.1 M PBS buffer (pH 5.7) and Epirubicin

hydrochloride (70 mg) was introduced. The mixture reacted for 24 h. The product stream was treated with dialysis in the dark (4 °C, MWCO 2 KDa), filtration and freeze drying. The final red product, Branched-SS-poly HPMA-NH-N=Epirubicin (320 mg, 79.2 KDa, PDI = 2.94, 8.74 wt% Epi), was obtained.



Scheme S3. Structure and synthetic route of Linear-poly HPMA-NH-N=Epirubicin.

The synthesis method for Linear poly HPMA-NH-N=Epirubicin was similar to that for the branched one. Briefly, the HPMA monomer (698.1 mg, 4.88 mmol), MA-NH-NHBoc (82.7 mg, 3.05 mmol), CTA (4.9 mg, 17.5 μ mol) as a chain transfer agent, and VA044 (1.7 mg, 5.8 μ mol) were dissolved in a deionized water/methanol mixture (3.5 mL, 1: 4). The mixture was bubbled with nitrogen for over 50 min at 0 °C. The solution was stirred at 46 °C for 8 h and then quenched with liquid nitrogen. After dialysis in the dark (4 °C, MWCO 2 KDa), subsequent filtration and freeze drying, the pink solid product was treated with excessive V501 for 2 h to remove the benzodithioate group and purified via dialysis against water. Linear-poly HPMA-NH-NHBoc was obtained (425.9 mg, yield: 54.2%, 41.6 KDa, PDI = 1.20). Linear-poly HPMA-NH-NHBoc (400 mg) was treated with TFA and purified to produce 360 mg of a polymer precursor, Linear-poly HPMA-NH-NH₂ (40.2 KDa, PDI = 1.18). Three-hundred mg of Linear-poly HPMA-NH-NH₂ reacted with 70 mg of Epirubicin hydrochloride to produce the final product, Linear-poly HPMA-NH-N=Epi (316 mg, 42.6 KDa, PDI = 1.28, 8.29 wt% Epi). Successful preparation of the responsive polymers was verified via ¹H NMR.

The drug load ratio in the polymeric drug conjugates was calculated from UV absorbance values via a UV-visible spectrophotometer (Shimadzu UV-1800, Japan). The average molecular weight was determined by Gel Permeation Chromatography (GPC, Prominence system, RID-20A, SHIMADZU, Japan). A Shodex Asahipak GF-510 HQ GPC column (7.5 mm ID $\times$ 300 mmL) was used. The injection volume was 50 μ L of 2 mg/mL sample solutions. The mobile phase was 0.2 M lithium chloride sodium solution at a flow rate of 0.6 mL/min (40 °C). The molecular weight and polydispersity (M_w, M_n and PDI) were measured using Pullulan as

a standard.

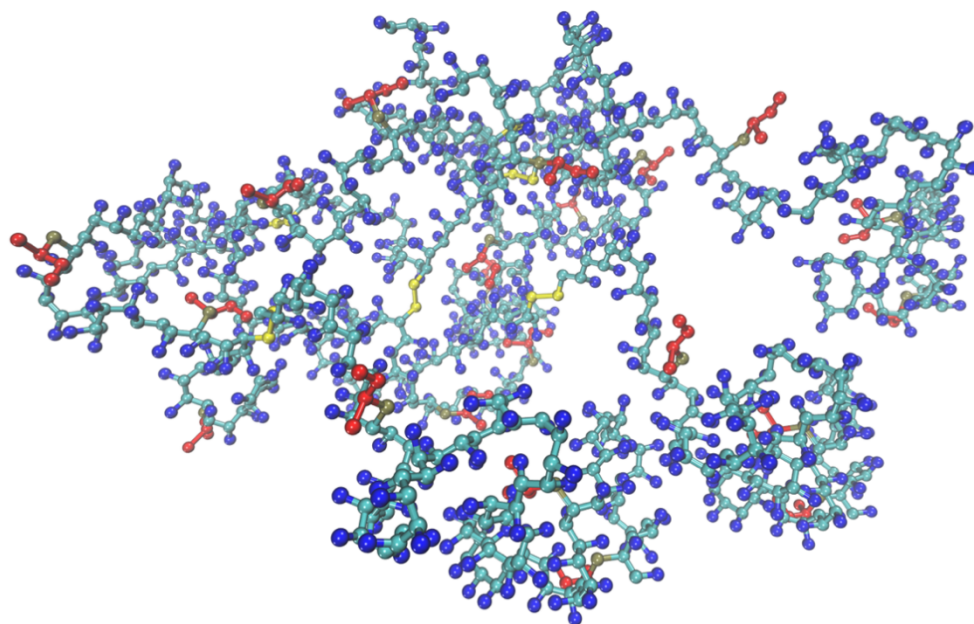
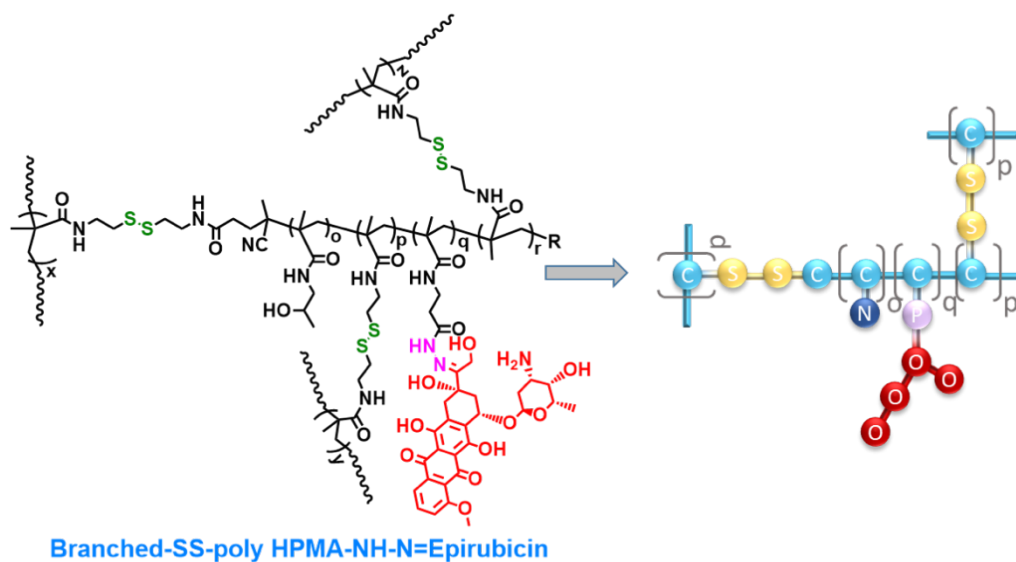
S.4. Computer simulation

The coarse-grained (CG) models for dissipative particle dynamics (DPD) simulation were constructed to describe the self-assembly process of the nanoparticles and the drug release from the nanoparticles [3]. There were six bead types for self-assembly simulations: 1 light blue bead for Methacrylate (“C”), 1 dark blue bead for Amino-2-propanol (“N”), 1 purple bead for hydrazone (“P”), 2 yellow beads for disulfide bond (“S”), 3 red beads for Epi (“O”) and five beads for the molecules of H₂O (W). After the self-assembly simulation, we ionized the drug release site of the nanoparticles. “OH” and “PH” beads were used to replace “O” and “P” beads after protonation, respectively. All these bead masses were reduced to 1. The interaction parameters α_{ij} for the DPD simulation were obtained by the Flory- Huggins parameter χ and calculated through the equation $\Delta\alpha_{ij} = 3.27\chi_{ij}$ (Table S1). The Flory-Huggins parameters were estimated from the equation:

$$\chi_{ij} = \frac{V_{ij}}{RT}(\delta_i - \delta_j)^2$$

Where V_{ij} is the molar volume of the bead, R is the gas constant, T is the absolute temperature. The solubility parameter for bead i and j were obtained by the Materials Studio.

In the DPD simulation, a box size of 30×30×30 with a three-dimensional periodic boundary was constructed. The bead number density of the box was 3. The time step (pf) for the simulation was 0.04. According to the polymer design and computational results, the branched polymer structure (1400 beads) had 7 chains, each with a molecular weight of 20 KDa at the ratio of $n_{\text{prodrug}}: n_{\text{polymer monomer}}: n_{\text{linker}} = 3: 90: 1$. The molecular weight of the linear polymer structure (528 beads) was 40 KDa at the ratio of $n_{\text{prodrug}}: n_{\text{polymer monomer}} = 8: 240$. There were 9 branched polymeric prodrugs or 24 linear polymeric prodrugs in each box (30 × 30 × 30) to allow the same number of beads for both branched and linear systems in the box.



Scheme S4. Schematic illustration of the molecular structure and the corresponding coarse-grained model of Branched-SS-poly HPMA-NH-N=Epi.

S.5. Results and discussion of polymeric prodrug synthesis

8

Linear-poly HPMA-NH-NHBoc and Branched-SS-poly HPMA-NH-NHBoc. After treatment with TFA, the -Boc group was removed since its characteristic peak disappeared and there were significant changes in the peaks for -NH-NHBoc. After conjugation with Epi, characteristic peaks of Epi at around 7.5-7.8, 5.0-5.5, and 4.0-4.5 ppm appeared in the ^1H NMR spectrum. Besides, evident changes were found for the peaks of -NH-NH₂. Thus, ^1H NMR spectra confirmed that both the linear and branched polymeric prodrug conjugates were successfully prepared with a high purity. Since the Epi content in both linear and branched polymeric prodrug conjugates could not be directly estimated via the ^1H NMR spectra, it was measured via UV-vis spectrophotometry and quantified from a standard curve (Figure S10c). Characterizations of both linear and branched polymeric prodrug conjugates including MW, molecular weight distribution (PDI), zeta potential and hydrodynamic size were shown in Table S1. The MW of the linear polymeric prodrug conjugate was 42.6 KDa with a PDI of 1.28, while the MW of the branched one was 79.2 KDa with a PDI of 2.94.

S.6. Physicochemical characterization.

The hydrodynamic size and zeta potential of the NPs prepared from the prodrug conjugates were characterized via DLS (Brookhaven Omni, USA). The linear and branched prodrug conjugates (1.0 mg/mL) were observed under a transmission electron microscopy (TEM, FEI Tecnai GF20STWIN, USA) after sample preparation on a silicon chip. The linear and branched prodrug conjugates were dissolved in DMSO, and the UV spectra of these solutions were detected and recorded via a UV-visible spectrophotometer. The detection wavelength was swept from 200 to 800 nm in a high-speed scanning mode.

S.7. Drug release profiles, stability and degradability.

Release of Epi from the prodrug conjugates in vitro was determined through dynamic dialysis of the NPs against a PBS solution at pH 7.4, a PBS solution at pH 5.4, a PBS solution containing 20 mM GSH, or a PBS solution at pH 5.4 containing 20 mM GSH. Five mg of the linear or branched NPs was dispersed in a release buffer shaken at 37 °C. At the designated time interval, 500 μL of the buffer solution was withdrawn and 500 μL of the corresponding PBS solution was added into the system. The released amount of Epi was detected via a UV/visible spectrophotometer. The changes in the particle size and zeta potential of the linear and branched NPs in ddH₂O (pH 7.4) at 1.0 mg/mL were monitored within 48 h via DLS to simulate the stability of the branched prodrug conjugates. To evaluate the degradability of the branched polymeric prodrug conjugate, it was incubated in a PBS solution at pH 5.4 containing 20 mM

GSH, and the size changes were monitored via DLS after 12 h. The product was collected for TEM and GPC analysis.

S.8. In vitro cytotoxicity.

The cytotoxicity of free Epi, the linear or branched NPs was assessed via CCK-8 assays. 4T1 cells were seeded in a 96-well plate (3000 cells per well) for 24 h incubation. Then, the spent medium was replenished with fresh medium charged with free Epi, the linear or branched NPs at different concentrations. Cell culture in the medium without drugs was utilized as a control. Cells were washed by PBS twice after 48 h incubation, and stained by the 10% CCK-8 reagent (MCE) in the serum-free medium for 1 h. A microplate reader was used to measure the absorbance of each well. The IC₅₀ values were estimated for free Epi, the branched and linear NPs. We also evaluated the cytotoxicity of polymeric nanocarriers without Epi against normal HUVECs.

S.9. Cellular uptake.

In a 12-well plate, 1×10^5 of 4T1 cells planted on each well were incubated in the RPMI 1640 medium for 48 h. The branched or linear NPs, or free Epi were added into each well plate at an equivalent concentration of Epi (5 $\mu\text{g/mL}$) for 0.5 h, 2 h and 4 h. After rinsing with PBS, Hoechst 33342 (Beyotime) at a final concentration of 10 $\mu\text{g/mL}$ was added for staining. Images were obtained by confocal laser scanning microscopy (Leica TCS SP2, Germany) with excitation at 561 nm to detect the fluorescence signal of Epi. In addition, after cells were treated by the same method, 4T1 cells were collected after digestion by trypsin and washed with PBS, and the quantitative uptake efficiency was assessed by flow cytometry (BD FACSCanto™ Flow Cytometer, USA).

S.10. Intracellular trafficking.

To observe the entry pathway of the NPs into the cell, 1×10^5 4T1 cells per well in a 12-well plate were incubated for 48 h. The branched or linear NPs, or free Epi were added into the plate at an equivalent Epi concentration at 5 $\mu\text{g/mL}$ for another 4 h incubation. Lysosomes, late endosomes and other acidic compartments were stained with Lyso-Tracker™ Green DND-26 (250 nmoL/L, Yeasen) for 20 min. Subsequently Hoechst 33342 (10 $\mu\text{g/mL}$) was introduced to counterstain the nuclei of 4T1 cells for another 15 min. The fluorescence signals of acidic compartments, Epi and the nuclei of 4T1 cells were simultaneously detected under a CLSM (NiKON, Japan) after cells were washed with PBS.

S.11. Cell apoptosis assay.

To detect the degree of apoptosis induced by the NPs on 4T1 cells, 1×10^5 4T1 cells planted on each well were incubated for 24 h. Branched or linear NPs, or free Epi were added into the well plate at an equivalent Epi concentration of 1 $\mu\text{g/mL}$ for another 24 h incubation. After these treatments, cells were harvested for cytometry analysis. Briefly, cells were detached by 100 μL trypsin after the spent medium was withdrawn. After digestion, the cells were centrifuged, washed twice with cold PBS, and subsequently suspended in 200 μL of the binding solution (1×10^6 cells/mL). 5 μL DAPI (Yeasen) and 5 μL Annexin V (Yeasen) were added to stain cells for 15 min in darkness. Finally, the degree of apoptosis was detected by flow cytometry (FACS Calibur, USA).

S.12. Cell cycle assay.

2×10^5 4T1 cells per well in a 6-well plate were planted for 24 h incubation. Branched or linear NPs, or free Epi at 1.0 $\mu\text{g Epi/mL}$ were added for another 24 h incubation. After these treatments, cells were re-suspended and rinsed twice with sterilized PBS before they were permeabilized with 70% ethanol overnight. 10 $\mu\text{g/mL}$ DAPI containing 0.1% Triton was used to dye the nucleus in the dark for about 15 min. After staining and washing with PBS, the cell cycle changes were investigated by a flow cytometry. The different cell cycle stages from G0/G1 phase to G2/M phase in each group were analyzed by flowjo and these changes were presented in a histogram.

S.13. Western blotting analysis.

After the same treatment in the cell cycle assay, the total cell protein was extracted with a RIPA lysate buffer (Beyotime). 40 μg protein of each sample was separated by SDS-polyacrylamide gel (12%) electrophoresis, and transferred to a PVDF membrane. The sealed membrane was incubated with primary antibodies (1: 1,000 dilution) against Bcl-2, PARP, caspase 3, cleaved caspase 3, SLC7A11, SLC6A14, MCT, Akt and pAkt. The β -Actin antibody (1: 5,000) was used for reference. The primary antibody-treated membrane was then incubated with secondary antibodies (1: 10,000) in TBS-T. Finally, a luminescent solution was dropped on the membrane and the membrane was exposed to X-ray films (Tanon, CN).

S.14. Detection of ATP secretion, CRT exposure and HMGB1 release.

To detect CRT exposure after treatment, 1×10^5 /well 4T1 cells planted into 12-well plates were cultured to reached 70% confluence. Epi, the linear or branched NPs were added and incubated for 24 h. After harvest and staining with eFluor 647-anti-CRT (Abcam), the treated cells were subjected to flow cytometric analysis. To detect ATP secretion and HMGB1 release, the treated cells and the supernatant were collected to detect the ATP content according to the

manual instruction of the ATP detection Kit (Yeaston), and the released HMGB1 by 4T1 cells in supernatant were detected by ELISA Kit (Elabscience) according to the manual instruction.

S.15. Tumor spheroids.

4T1 cells (700 cells/well) were cultured in a Perfecta 3D 96-well suspension drop culture plate. The medium was changed every three days. After 7 days, uniform spheroids were selected for the cellular uptake study. The tumor spheroids were incubated with free Epi, the linear or branched NPs for 3 h, and then stained with Hoechst 33342 and fixed with 4% paraformaldehyde, respectively. The fluorescence intensity was detected under a confocal microscope (Leica, Germany).

S.16. Ex vivo imaging and pharmacokinetic study.

Tumor-bearing mice were injected with saline, free Epi, the linear or branched NPs intravenously with an equivalent Epi concentration of 5 mg/Kg. The main organs and tissues (heart, liver, spleen, lung, kidney, and tumor) were collected at 24 h after *i.v.* injection for fluorescence imaging. The blood was collected at a pre-determined time interval and mixed with deionized water and acetonitrile. The blood samples were subjected to ultrasound treatment and centrifugation to obtain liquid supernatants for detection via a microplate reader. The pharmacokinetic data were processed using PKsolver 2.0 software.

S.17. In vivo anti-tumor efficacy.

To establish the tumor model, 1×10^6 4T1 cells were implanted into the mice. When the tumor volume reached about 50 mm³, the mice were randomly divided into seven groups: (i) control (PBS), (ii) Epi, (iii) α -PD-1, (iv) Linear NPs, (v) Branched NPs, (vi) linear NPs + α -PD-1 (Linear + P) and (vii) Branched NPs + α -PD-1 (Branched + P) ($n = 7$). Two hundred μ L of the linear or branched NPs, or Epi at a dose of 5 mg/kg (equivalent Epi) were administrated through tail vein injection on day 7, 11, 15 and 19, while α -PD-1 (5 mg/kg) was administrated into the mice in the α -PD-1, Linear + P, and Branched + P groups via intraperitoneal injection on the same days. The tumor size and weight were recorded every two days from the first day of treatment. The tumor volume was estimated from the equation:

$$\text{Tumor volume} = (\text{Tumor width} \times \text{Tumor width} \times \text{Tumor length})/2.$$

Each mouse was sacrificed after 15 days treatment. The tumor of each mouse was carefully dissected from adjacent tissues and weighed to calculate the tumor growth inhibition (TGI) by the equation:

$$TGI (\%) = (1 - \bar{m}_{\text{treatment}}/\bar{m}_{\text{control}}) \times 100$$

$\bar{m}$ represents the average mass of the tumor tissues. The kidney, heart, spleen, lung, liver and tumor were harvested for H&E staining analysis. Briefly, the organs or tissues were collected and then embedded in paraffin, followed by physical sectioning at a thickness of 4 μm . The samples were subjected to H&E, TUNEL and HMGB1 immunohistochemical staining, and CD3 and PD-1 immunofluorescent staining.

S.18. Immune cell population analysis.

The mice were subjected to the same treatment as the above *in vivo* anti-tumor efficacy study. Mice were killed on day 4 after the last injection. The harvested tumor tissue was digested with DNase I (15 U/mL, Absin), hyaluronidase (1.6 U/mL, Absin), and collagenase IV (200 U/mL, Absin). To obtain the single-cell suspensions, the digested tissues were filtered through a 70 μm strainer. T cells were detected after staining with APC-Cy7-anti-CD45 (dilution of 1: 1000), Alexa Fluor 700-CD3 (dilution of 1: 300), BV510-anti-CD8a (dilution of 1: 200) and eFluor 450-anti-CD4 (dilution of 1: 200). To detect CD3⁺ CD4⁺ Foxp 3⁺ and CD3⁺ CD8⁺ Foxp 3⁺ IFN- γ ⁺ T-cells, cells were fixed, permeated and stained with PE-anti-Foxp 3 (dilution of 1:1000) and APC-anti-IFN- γ (dilution of 1: 1,000) in accordance with the manufacturer's instructions. FACS analysis of the stained cells was conducted through the logic gates in Figure S34. DCs were detected after staining of APC-Cy7-anti-CD45 (dilution of 1: 1,000), APC-anti-CD11c (dilution of 1: 300), eFluor 450-anti-CD86 (dilution of 1: 200) and FITC-anti-MHCII (dilution of 1: 200). Flow cytometry analysis of the stained DCs was conducted through the logic gates in Figure S36.

S.19. Metabolomics study.

After 24 h of treatment with the branched NPs, the linear NPs and free Epi, respectively, cells at a density of 2×10^6 via a cell counter (Countstar, CN) in each treatment group were washed and scraped into a centrifuge tube, and they were treated with 80% CH₃OH containing an internal standard. Untreated cells were used as a control. The cell mixture was stored at -80 °C for 30 min and then subjected to ultrasonication under an ice bath. The mixture was centrifugated (14,000 g for 10 min, 4 °C), and the cell supernatants were collected and dried (30 °C for 3 h). The samples were redissolved in 0.5 mL solvents, and then subjected to ultrasonication under an ice bath. The mixtures were centrifuged (14,000 g for 10 min) at 4 °C to collect the supernatants for further measurements. Thirty microliters of each Quality control (QC) sample were also prepared. The experimental and QC samples were analyzed by LC-MS (AB SCIEX, CN). All these samples were randomly injected for LC-MS analysis. The retention times, characteristic chromatograms, and mass spectra of essential metabolites were recorded.

These raw data were imported into the commercial software Analyst (version 1.6.3) and MultiQuant (version 3.0.2) for peak extraction. Metabolites were identified through a local database established from standards of essential metabolites. The preprocessed data were further processed with R (V3.5.1) to preprocess the mass spectral data, including correcting the sample quality deviation, removing low-quality ions and unstable ions, filling of missing values, standardization, and PCA display quality control. Then, Wilcoxon rank sum test ($p < 0.1$) and OPLS-DA ($VIP \geq 1$) were used to identify differential metabolites, and metabolanalyst were used for KEGG analysis.

S.20. Determination of the extracellular concentration of glucose and lactic acid.

To detect glucose and lactic acid in the extracellular medium following the linear or branched NPs treatments, 4T1 cells were seeded into 96-well plates (5,000/well) and incubated overnight. Free Epi, the linear or branched NPs at an equivalent Epi concentration of 2.0 $\mu\text{g/mL}$ were added for 24 h incubation. The extracellular medium was collected for glucose and lactic acid detection according to the corresponding kit instructions.

S.21. DC maturation.

BMDCs were isolated from the bone marrow of Balb/c mice. In brief, the femur and tibia were removed and soaked in 70% alcohol for 2 min. The two ends of the bone were cut. A syringe needle was inserted into the bone marrow cavity and a cold RPMI 1640 intact culture solution was slowly injected to flush the cells. The cells were collected in a dish. Small fragments and the muscle tissue were filtered and the filtrate was centrifuged. After red blood cell lysed, the obtained cells were cultured in the RPMI 1640 medium containing 20 ng/mL GM-CSF (Absin) and 10 ng/mL IL-4 (Absin). The spent medium was then replenished every three days with new medium containing both cytokines at the same concentration. BMDCs were harvested on day 9, and the phenotype for immature DCs was determined by measuring the CD11c expression shown in Figure S37.

Immature BMDCs were co-incubated with 4T1 cells that were subjected to the treatment with 2 $\mu\text{g/mL}$ of the linear or branched NPs. DCs were stained with APC-anti-CD11c (dilution of 1: 300), Cy5-APC-anti-CD86 (dilution of 1: 200) and BV421-anti-CD80 (dilution of 1: 200) after 24 h incubation for flow cytometry analysis. To study the glucose effect on DCs maturation, DCs were stimulated with LPS (10 ng/mL, Sigma), cultured with media containing varying concentrations of glucose, labeled with the same antibodies, and analyzed by flow cytometry using the logic gates shown in Figure S38.

S.22. Culture of splenocytes with nutrients at different concentrations.

Splenocytes were mechanically isolated from the spleen of Balb/c mice. In brief, mouse spleens were cut with scissors, ground and filtered through a 40 μm cell filter to obtain single cell suspensions including T cells and myeloid cells. The spleen cells were co-incubated with 4T1 cells that were subjected to the treatment with 2 $\mu\text{g/mL}$ of the linear or branched NPs for 24 h. Nutrients including glucose (Sigma), glutamine (Boster), lactic acid (Sigma), and amino acids (Procell) at different concentrations were incubated with the spleen cells for 24 h. Then, DCs were stained with APC-anti-CD11c (dilution of 1: 300), Cy5-APC-anti-CD86 (dilution of 1: 200), and BV421-anti-CD80 (dilution of 1: 200), while T cells with APC-Cy7-anti-CD45 (dilution of 1: 1000), Alexa Fluor 700-CD3 (dilution of 1: 300), BV510-anti-CD8a (dilution of 1: 200), and eFluor 450-anti-CD4 (dilution of 1: 200). Cells were fixed, permeated and stained with PE-anti-Foxp 3 (dilution of 1: 1,000) and APC-anti-IFN- γ (dilution of 1: 1,000) for detection of CD3⁺ CD4⁺ Foxp 3⁺ and CD3⁺ CD8⁺ Foxp 3⁺ IFN- γ T cells in accordance with the manufacturer's instructions. Analysis of the stained cells were conducted through the logic gates in Figure S35 and S38.

Supplementary Figures

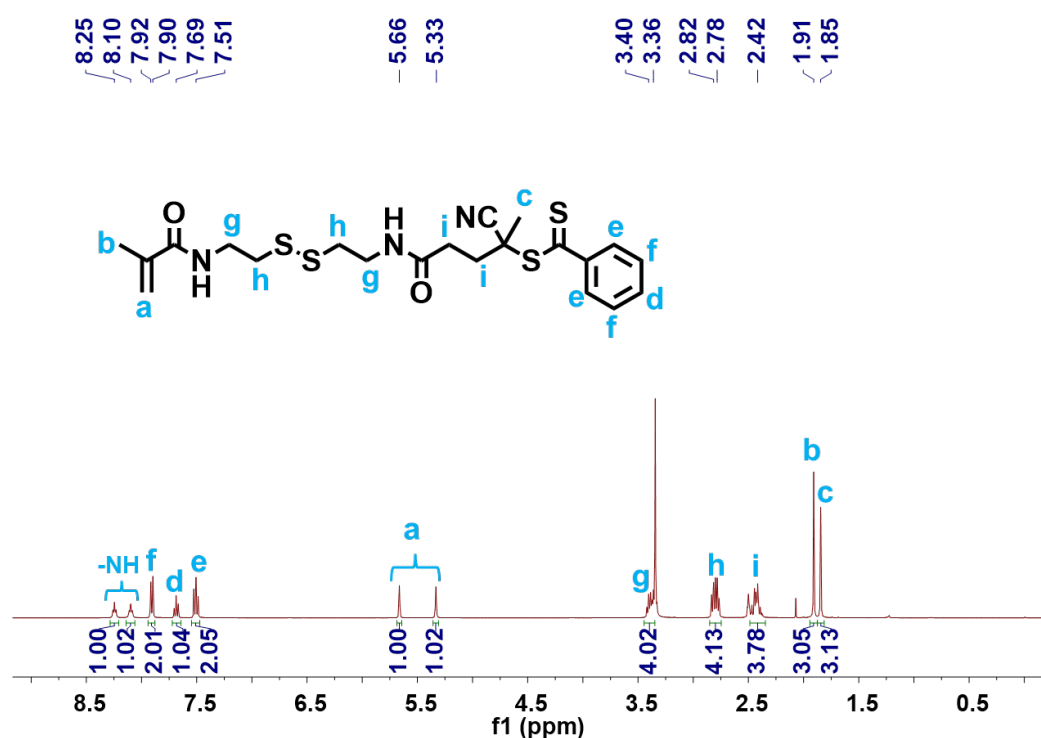


Figure S1. ¹H NMR spectrum of the functional chain transfer agent MA-SS-CTA in DMSO-d₆.

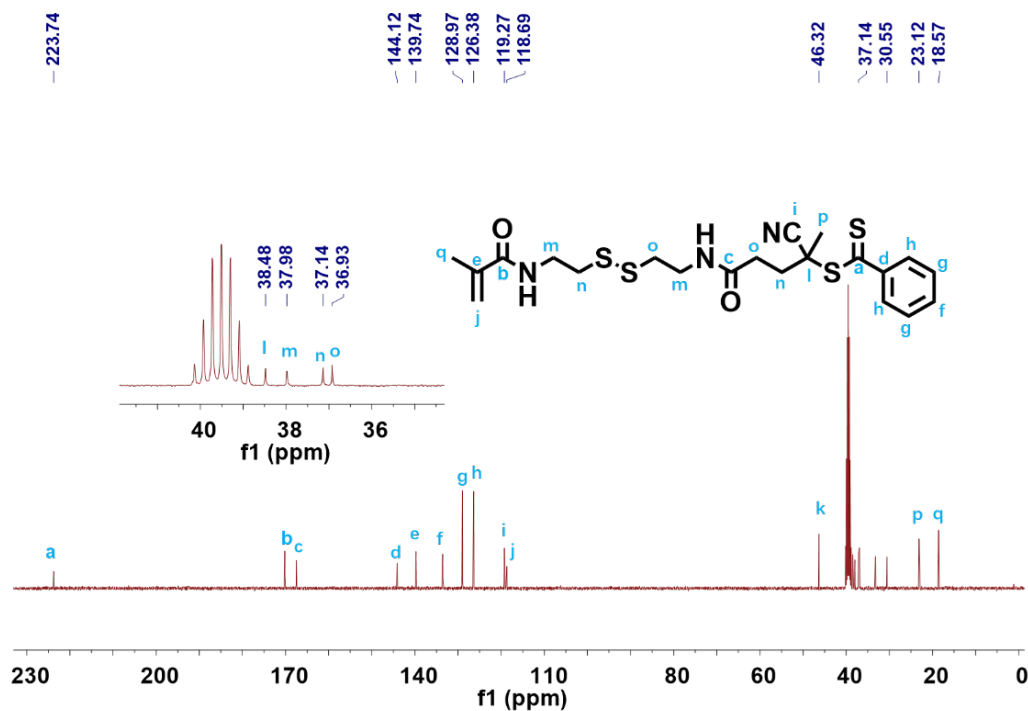


Figure S2. ¹³C NMR spectrum of the functional chain transfer agent MA-SS-CTA in DMSO-d₆.

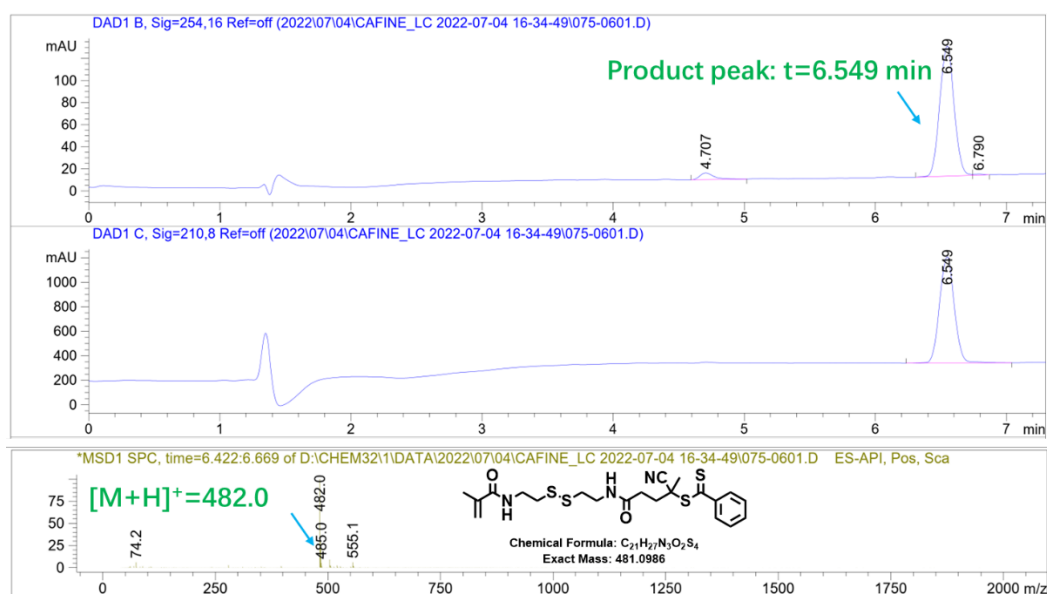


Figure S3. LC-MS result of the functional chain transfer agent MA-SS-CTA.

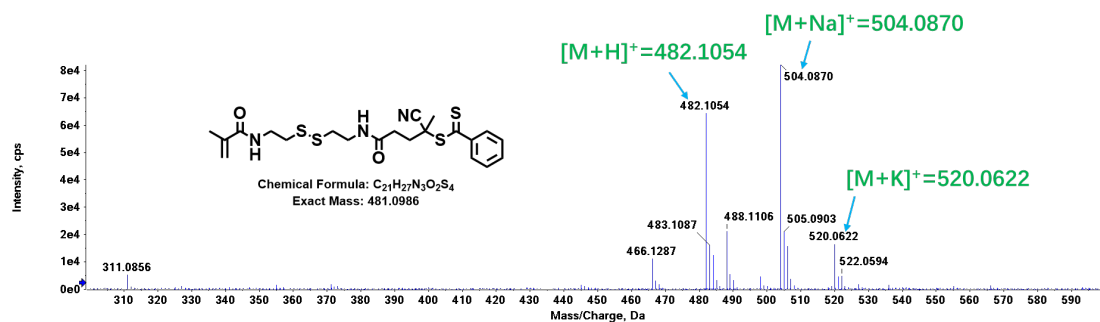


Figure S4. HRMS result of the functional chain transfer agent MA-SS-CTA.

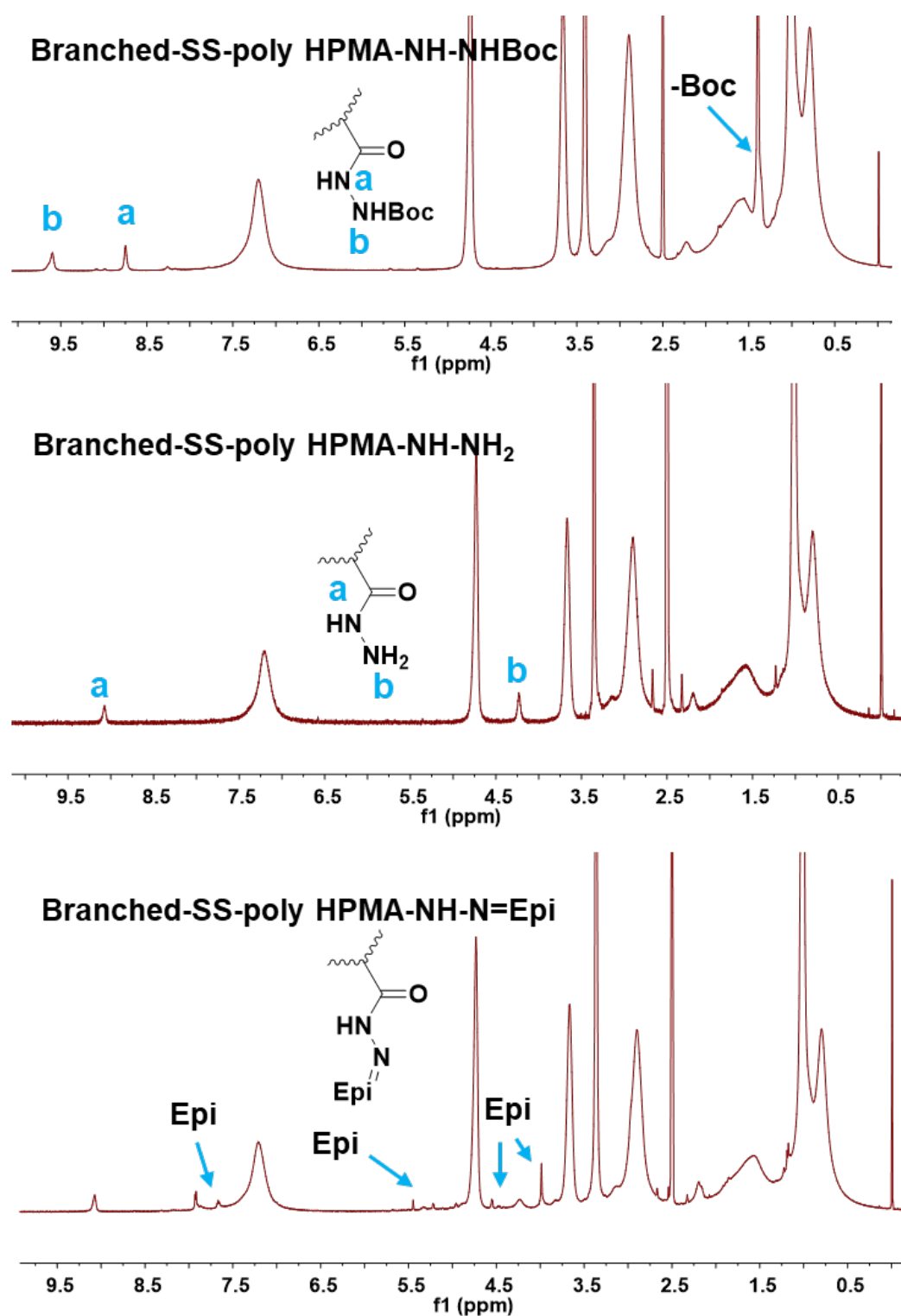


Figure S5. ^1H NMR spectra of Branched-SS-poly HPMA-NH-N=Epi and its polymer precursors in DMSO- d_6 .

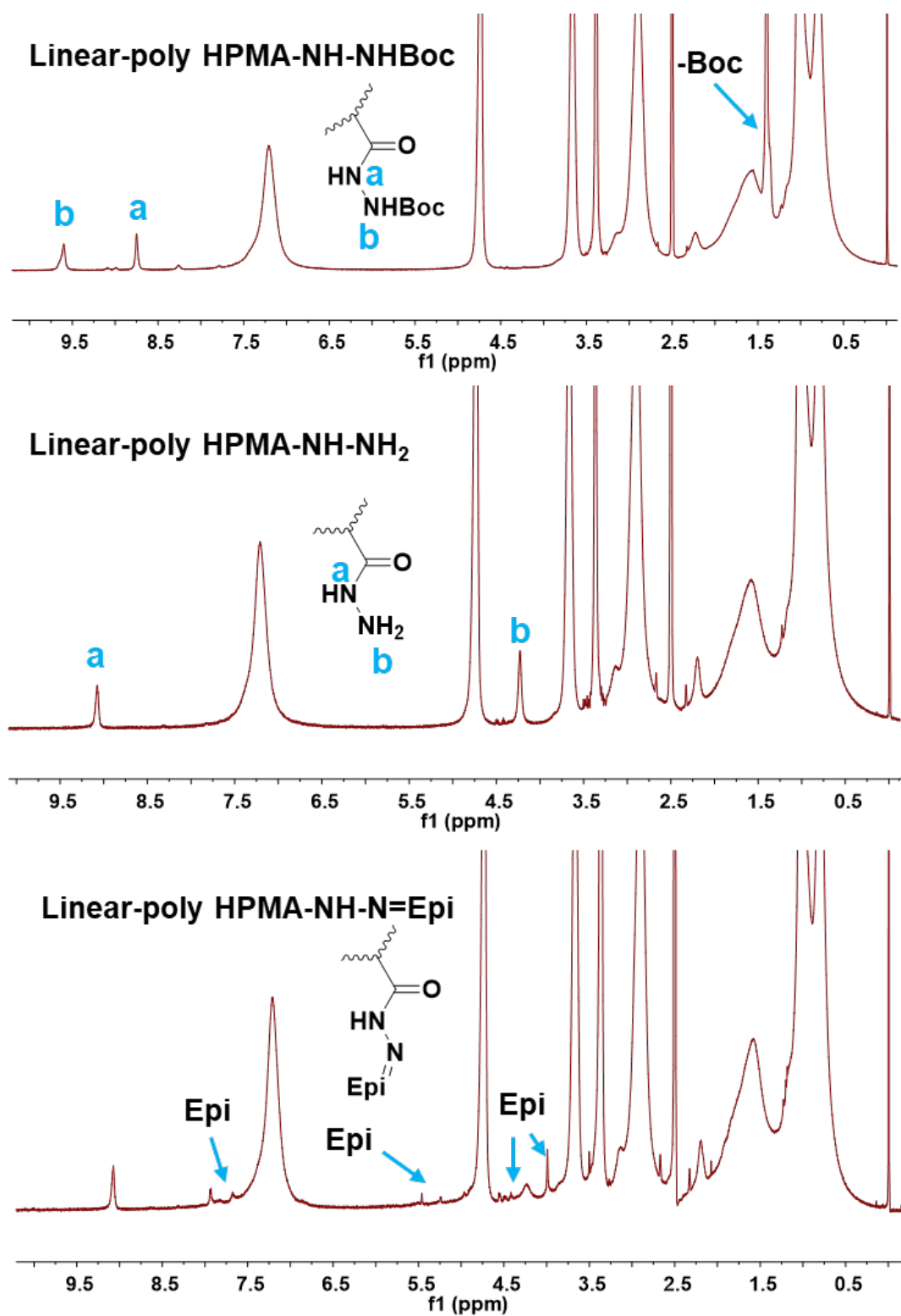


Figure S6. ^1H NMR spectra of Linear-poly HPMA-NH-N=Epi and its polymer precursors in DMSO- d_6 .

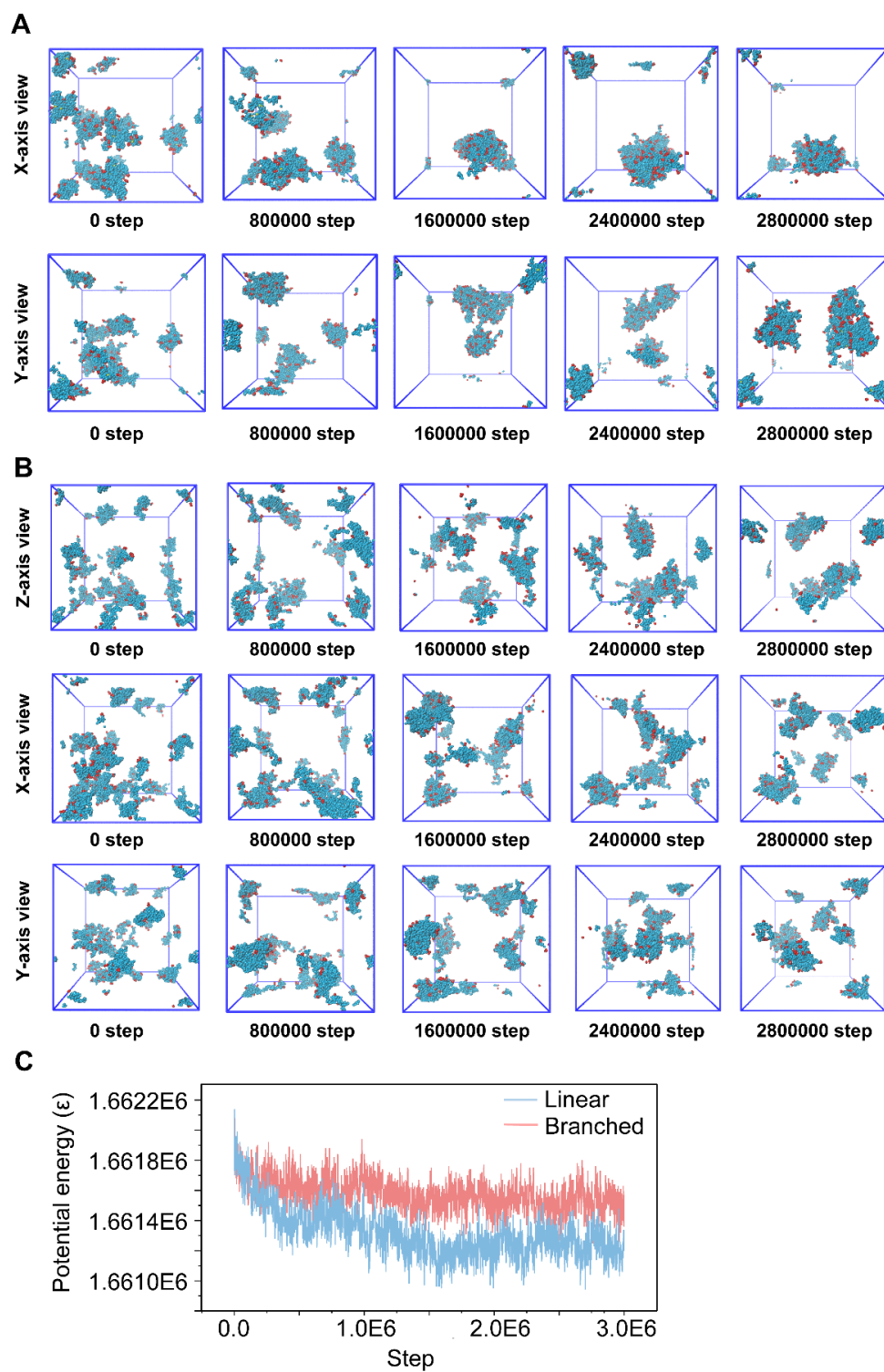


Figure S7. DPD simulations of the self-assembly process for the branched and linear structures. (A) DPD simulations of the self-assembly process for the branched structure. The structure represented by coarse-grained beads was shown in Scheme S4. The top row of each structure was obtained from the X axis view, while the bottom row from the Y axis view. (B) DPD simulations of the self-assembly process for the linear structure. The structure represented by coarse-grained beads was shown in Scheme S5. The top row of each step was obtained from the Z axis view, while the middle row was obtained from the X axis view, and the bottom row

was obtained from the Y axis view. (C) Temporal evolution of total potential energy of the linear and branched polymeric prodrugs. The total potential energy decreased during the self-assembly process and reached a relatively steady state.

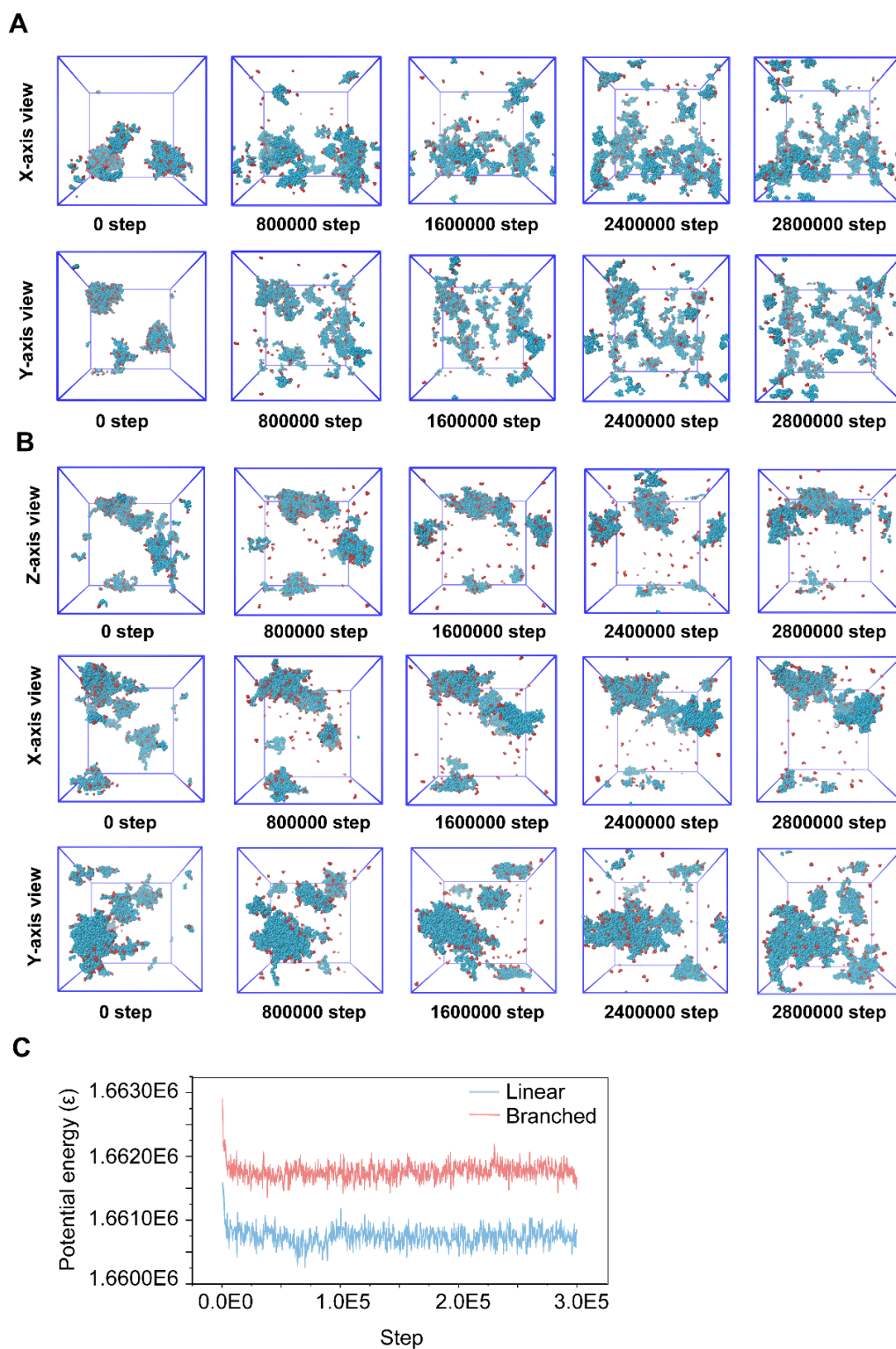


Figure S8. DPD simulations of the drug release and disassembly process for the linear and branched structures. (A) DPD simulations of the drug release and disassembly process for the branched structure. The top row of each step was obtained from the X axis view, while the

bottom row was obtained from the Y axis view. **(B)** DPD simulations of the drug release and disassembly process for the linear one. The top row of each structure was obtained from the Z axis view, while the middle row was obtained from the X axis view, and the bottom row was obtained from the Y axis view. **(C)** Temporal evolution of total potential energy of the linear and branched polymeric conjugates during the drug release and disassembly process. The total potential energy decreased during the process, and reached a relatively stable value after 2,800,000 simulation steps.

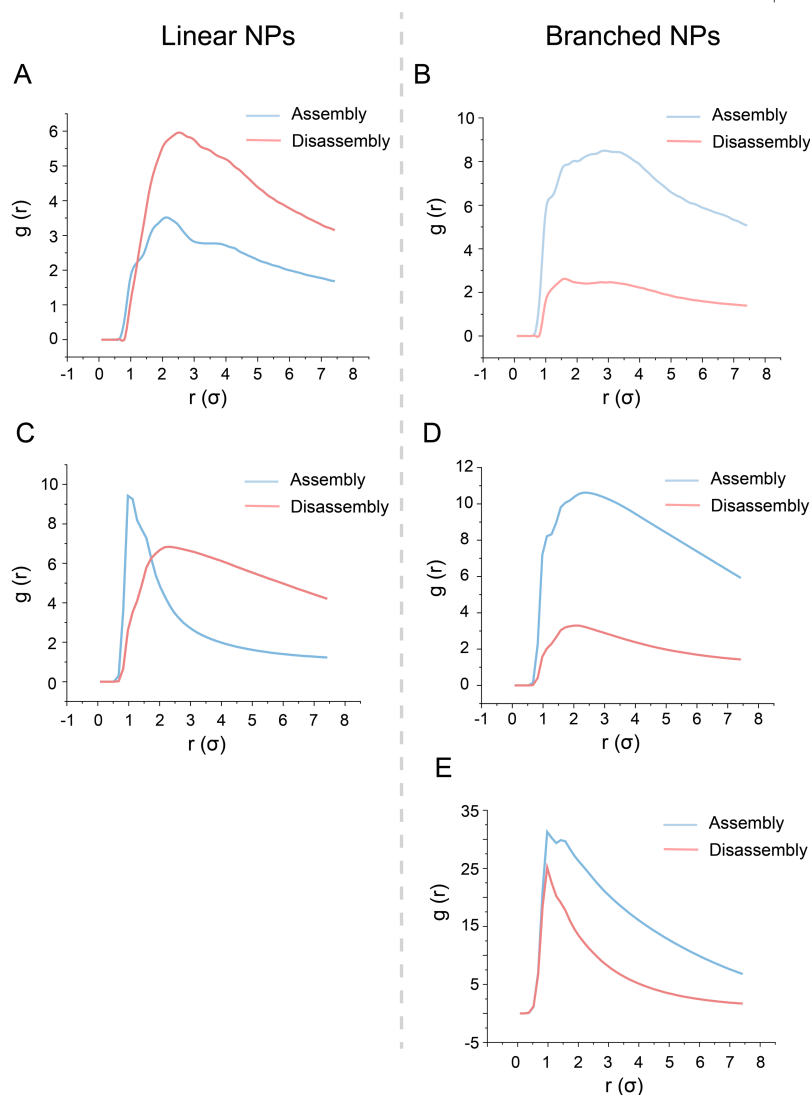


Figure S9. Relative Radial distribution functions (RDFs). (A and B) RDFs of the interaction between Epi and the hydrazone bond in the linear NPs (A) and branched NPs (B) in a stable assembly state and disassembly state from DPD simulations. (C and D) RDFs of the interaction between the HPMA polymer backbone and Epi in the linear NPs (C) and branched NPs (D) in a stable assembly state and disassembly state from DPD simulations. (E) RDFs of the interaction between the HPMA polymer backbone and the disulfide bond (or sulfydryl) in the branched NPs in a stable assembly state and disassembly state from DPD simulations. The

different RDF value changes between the assembly and disassembly processes were owing to the drug release and the protonation of the nanostructures in a mimic acidic condition.

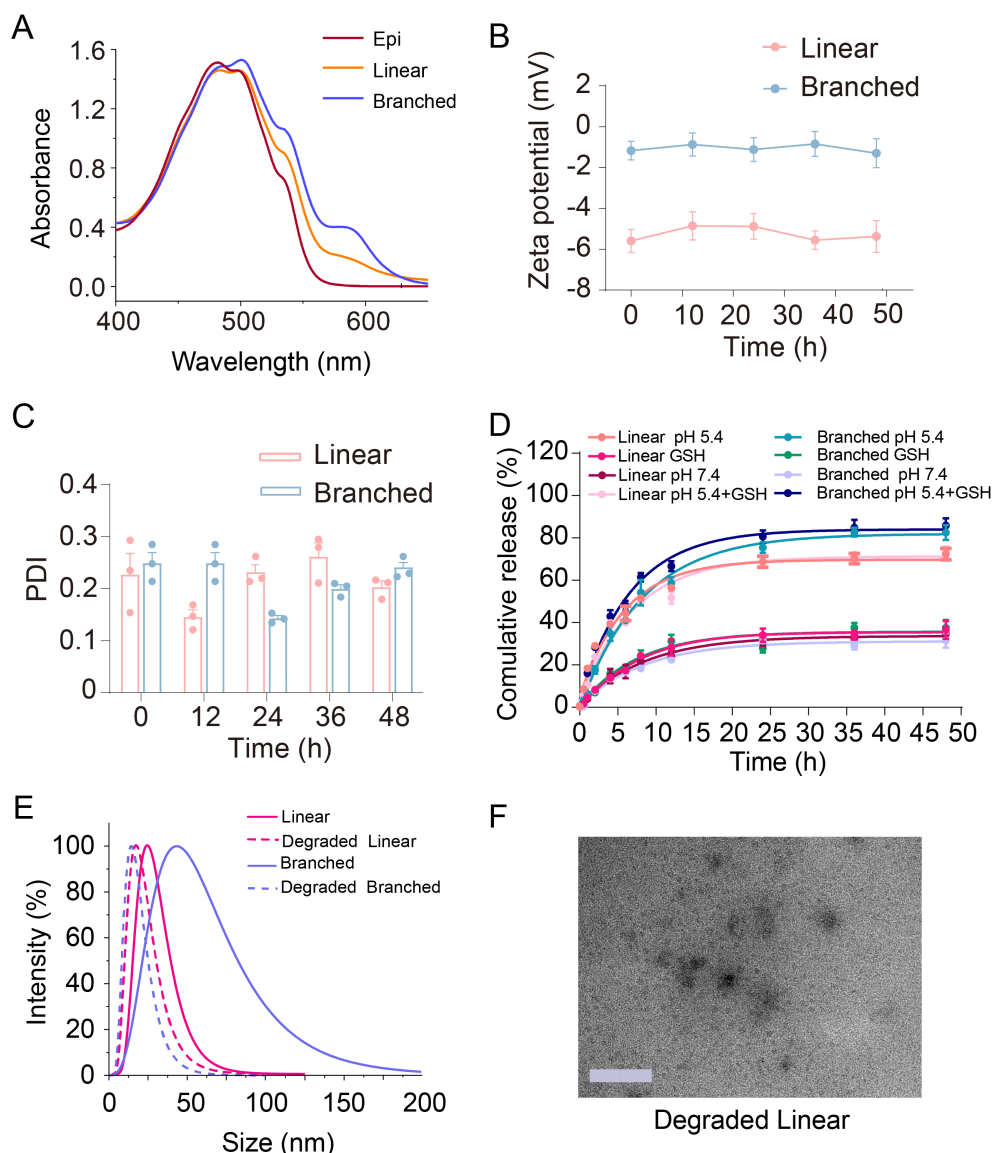


Figure S10. Physical characterization of Epi, the Linear NPs and Branched NPs. (A) UV spectra of Epi, the linear and branched NPs in DMSO. (B and C) Changes in the zeta potential (B) and PDI (C) of the linear and branched NPs incubated in pH = 7.4 PBS for two days. (D) *In vitro* drug release curves from the linear and branched polymeric prodrug conjugates under different mimicking release conditions, including a pH 7.4 neutral environment, a pH 7.4 neutral environment containing GSH, a pH 5.4 acidic environment, and a pH 5.4 acidic environment containing GSH. (E) The sizes of the linear and branched NPs and their degraded products via DLS after incubation with GSH (20 mM) in a pH 5.4 buffer for 12 h. (F) TEM results of degraded Linear NPs.

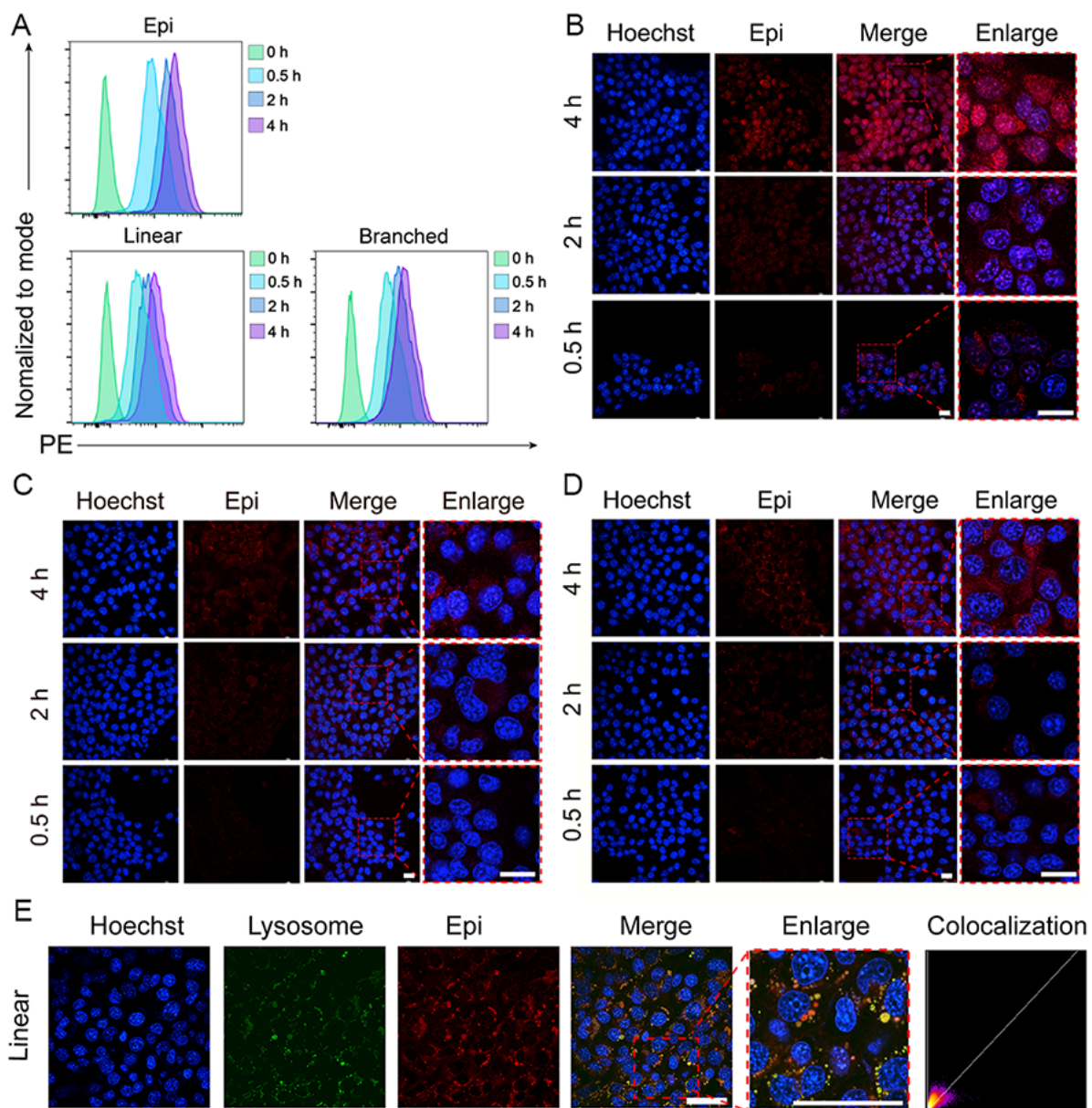


Figure S11. Intracellular uptake efficiency and intracellular localization of the linear and branched NPs. (A) Representative flow cytometric images for cellular uptake of free Epi, the linear and branched NPs at different time-points. (B, C and D) CLSM images for cellular uptake of free Epi (B), the linear NPs (C), and the branched NPs (D), respectively, at different time-points. Scale bar: 20 μ m. (E) CLSM images for subcellular localization of the linear NPs. Scale bar: 100 μ m.

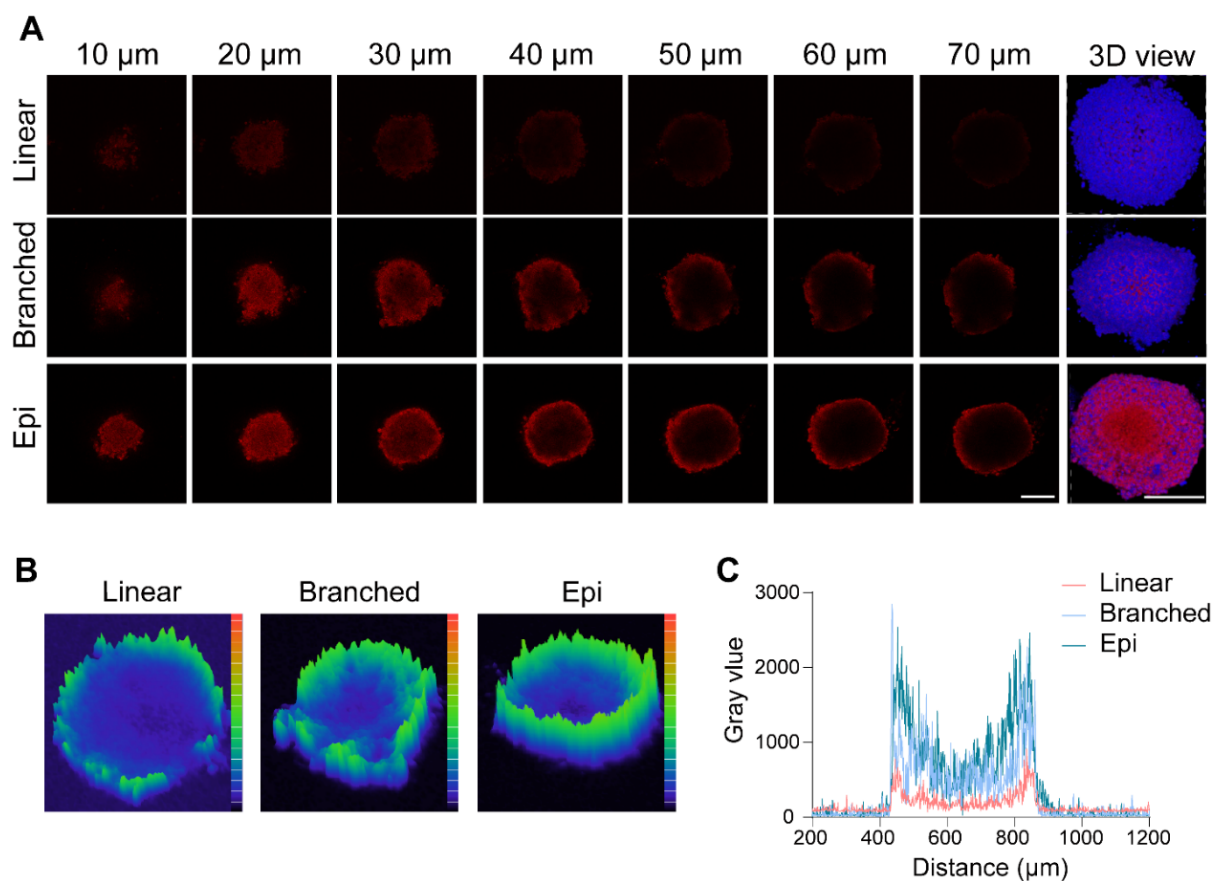


Figure S12. Tumor spheroids penetration and pharmacokinetic characteristics of the linear and branched NPs. (A) CLSM images for free Epi, the linear and branched NPs in multicellular tumor spheroids (MTSs) after co-incubation for 6 h at various depths (Epi: red, Hoechst: blue, Scale bar: 100 μm). (B) 3D topological images of Epi, the linear and branched in MTSs at a depth of 60 μm . (C) The fluorescence signal distribution along the horizontal line of the maximum cross section in the fluorescence images at a depth of 60 μm .

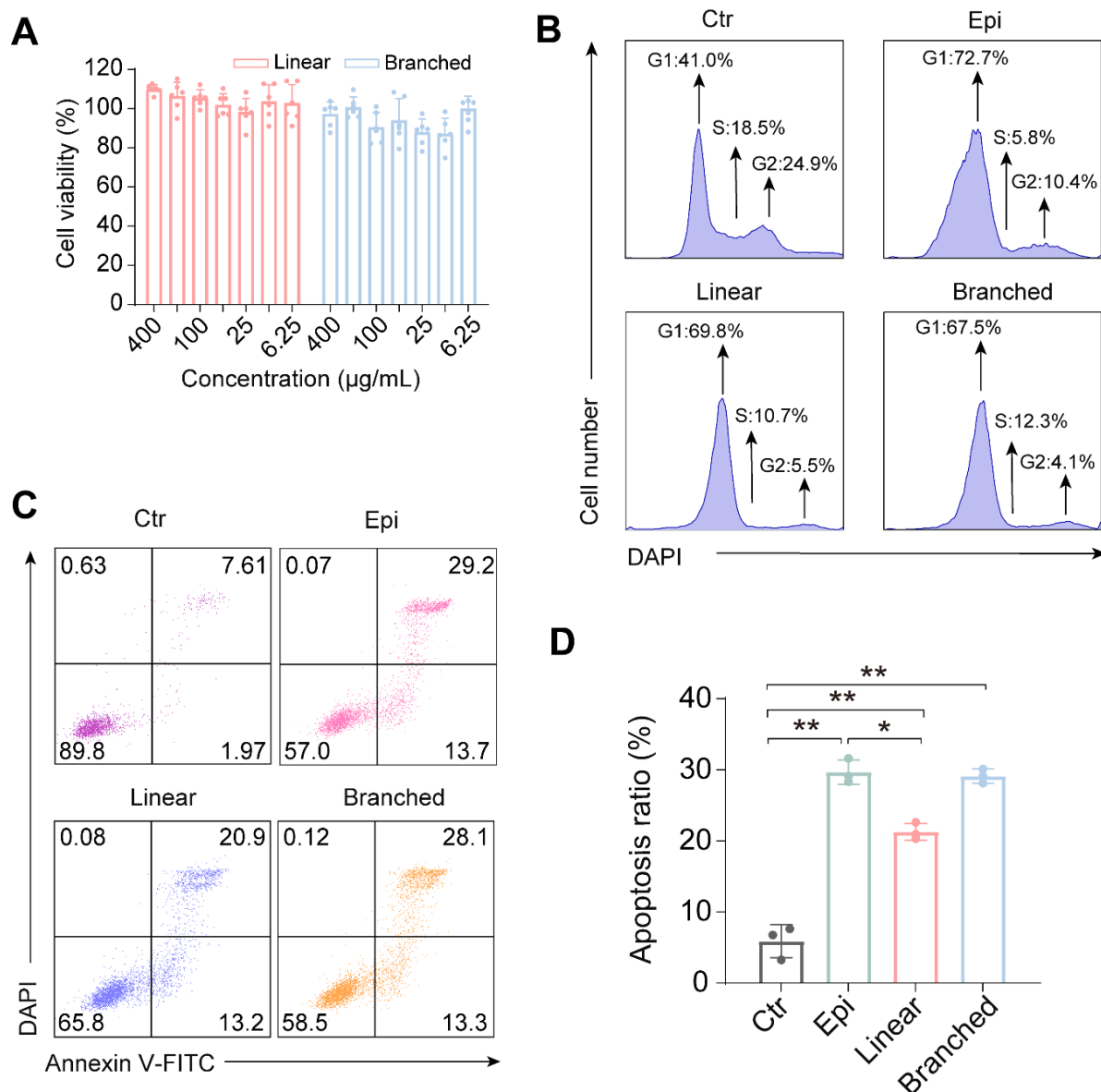


Figure S13. Cytotoxic characteristics of the linear and branched NPs. (A) The CCK-8 results of the drug-free linear and branched polymeric precursors against HUVECs. These drug carriers showed no obvious toxicity to HUVECs. (B) Representative flow cytometric images for cell cycle arrest at the phase G1, S and G2 in 4T1 cells after treatment with free Epi, the linear and branched NPs. (C and D) Representative flow cytometric images (C) and the quantitative level (D) of apoptosis in 4T1 cells induced by free Epi, the linear and branched NPs.

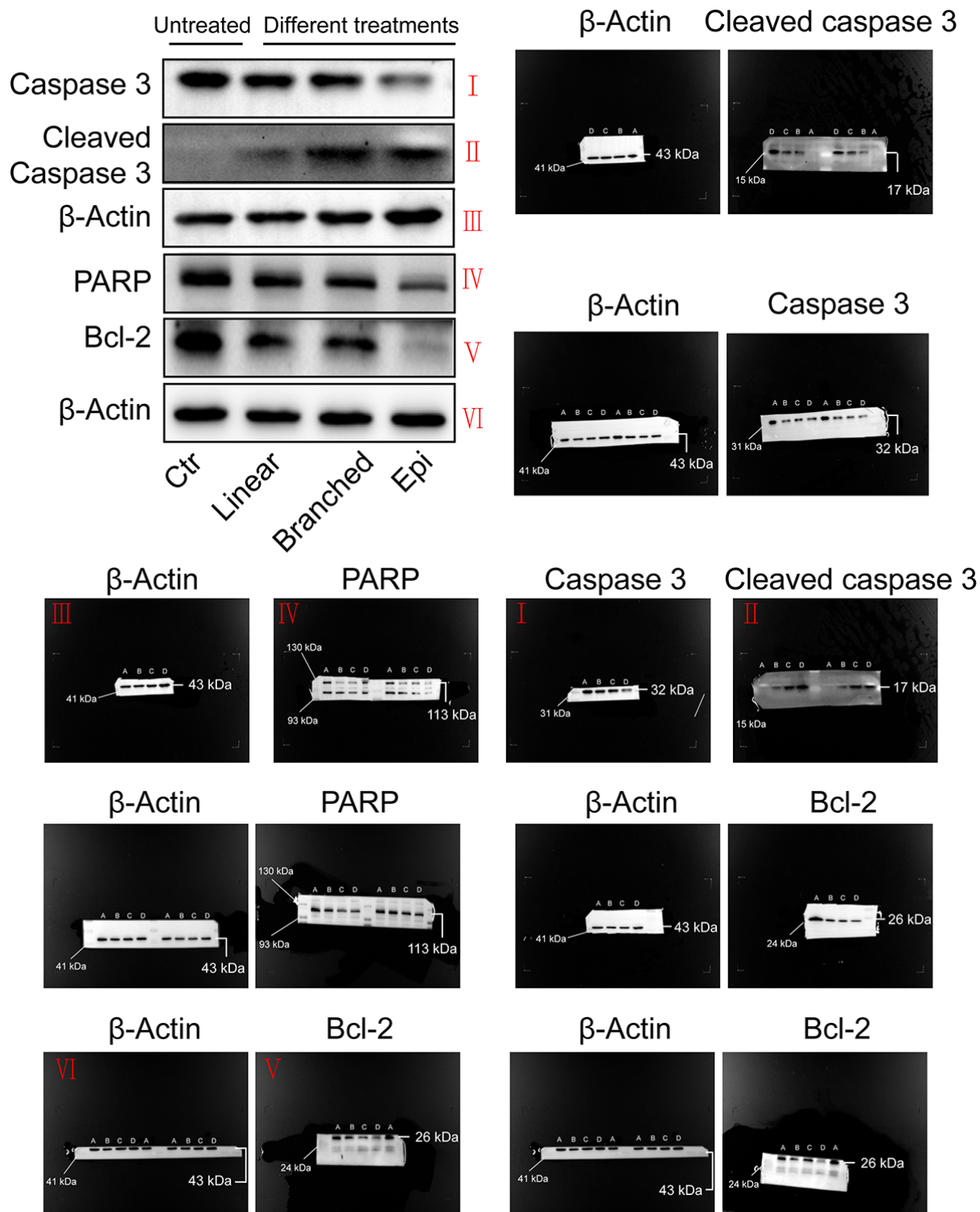


Figure S14. The expression of caspase-3, cleaved caspase 3, Bcl-2, and PARP was analyzed via western blotting. Raw images of western blotting for apoptosis-related protein expression in cells treated with the linear and branched NPs, respectively, while the protein expression in cells treated with free Epi or PBS was used as a control (A for control, B for the linear NPs, C for the branched NPs, D for free Epi).

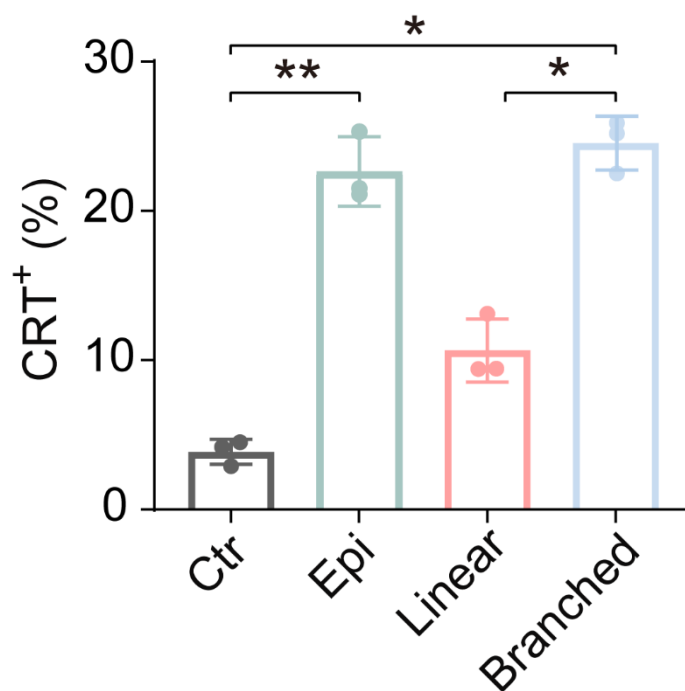


Figure S15. CRT exposure on the 4T1 cell surface induced by free Epi, the linear and branched NPs.

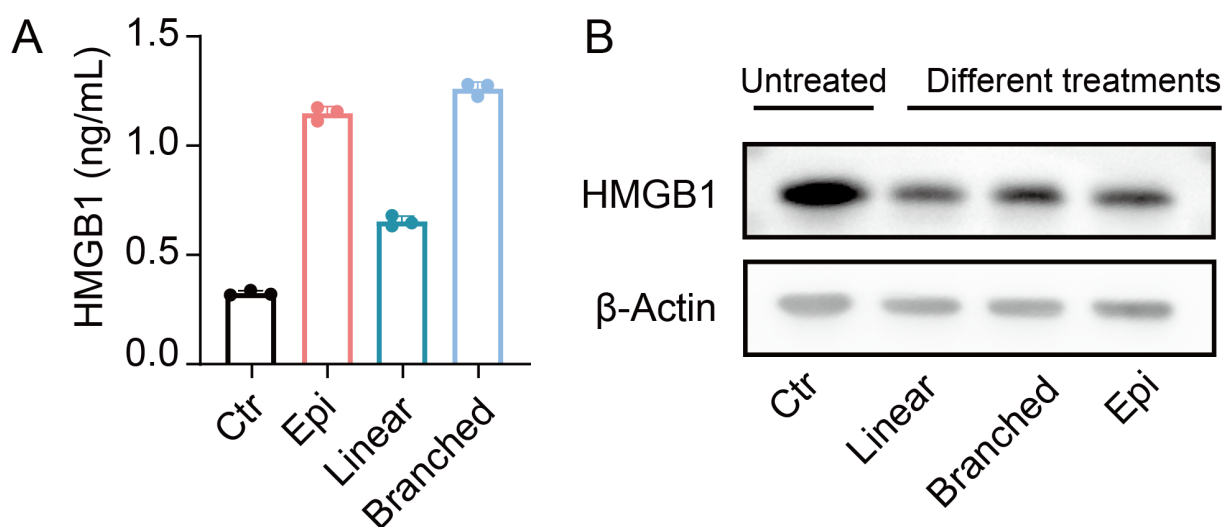


Figure S16. Detection of released HMGB1 after drug treatment. (A) Released HMGB1 in the supernatant of 4T1 cell measured by ELISA after treatment with free Epi, the linear and branched NPs. (B) Released HMGB1 in 4T1 cell measured by Western blot after treatment with free Epi, the linear and branched NPs.

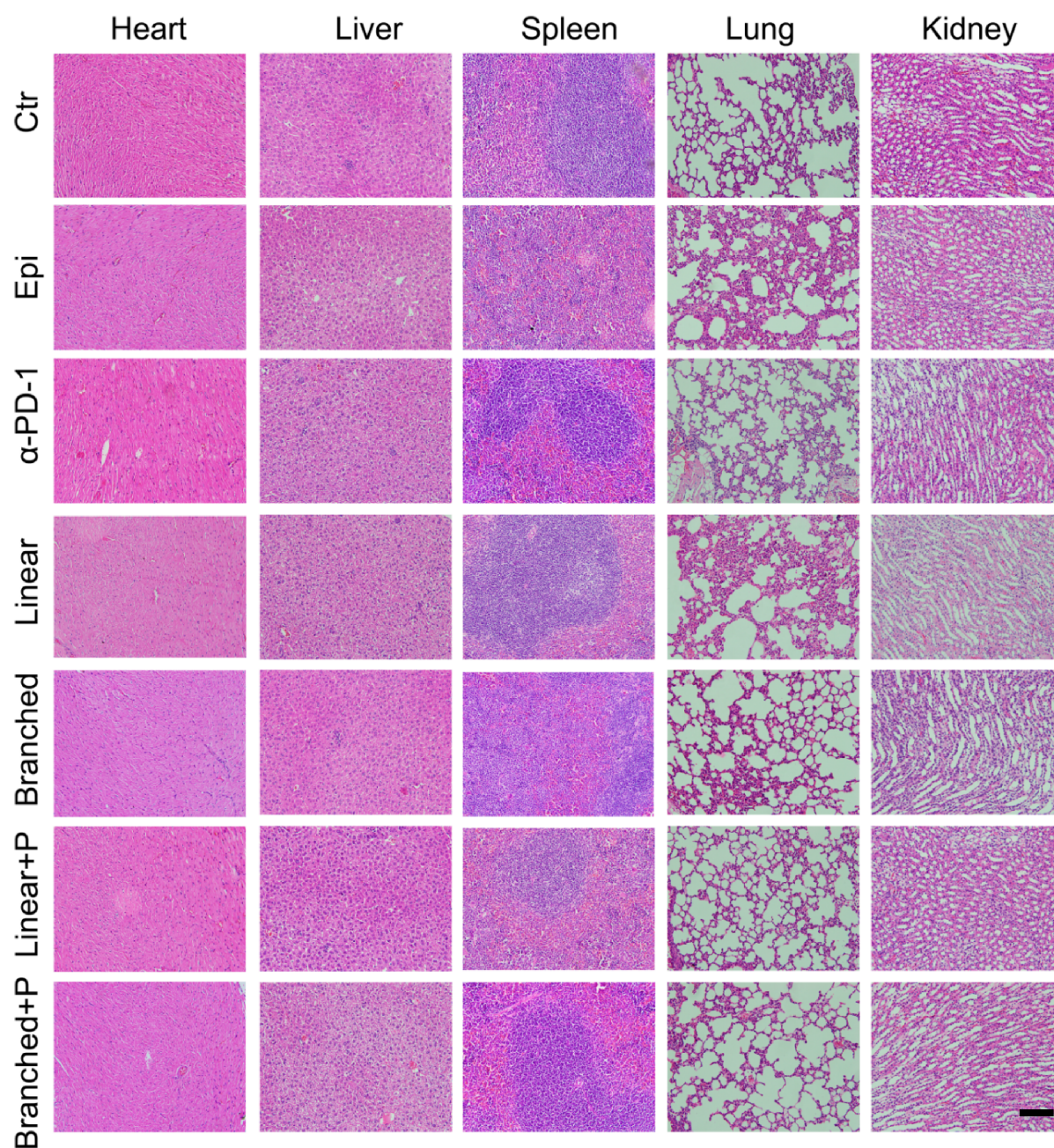


Figure S17. H&E staining analysis of the heart, liver, spleen, lung, and kidney from the mice treated with different formulations (P: α -PD-1, Linear: the linear NPs, Branched: the branched NPs). Scale bar: 200 μ m.

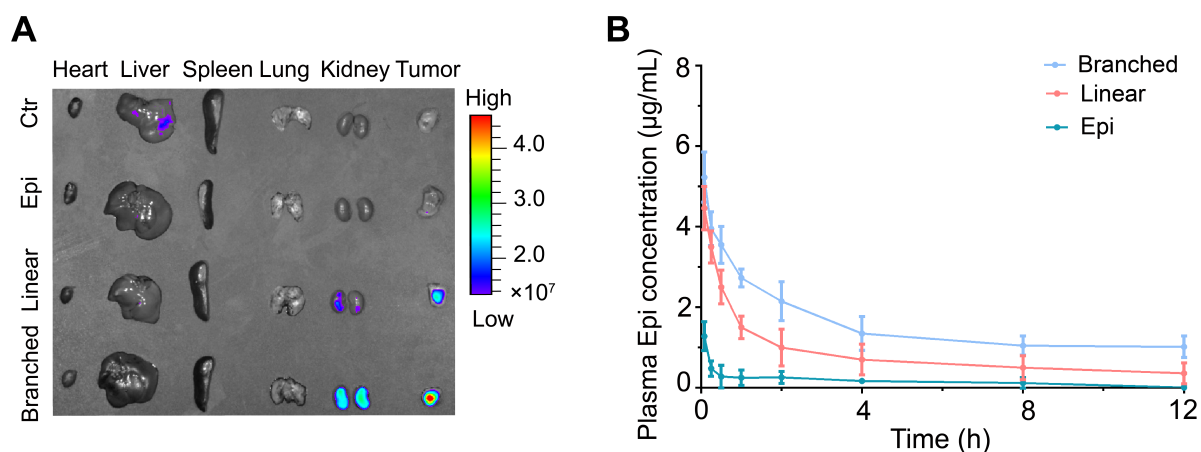


Figure S18. (A) Representative IVIS images of hearts, livers, spleens, lungs, kidneys, and tumors, collected at 24 h after injection of the PBS (Ctr), free Epi (Epi), and the linear NPs (linear) and branched NPs (branched). (B) Pharmacokinetic profiles of the linear and branched NPs and free Epi in the plasma of the mice receiving a single intravenous injection ($n = 5$).

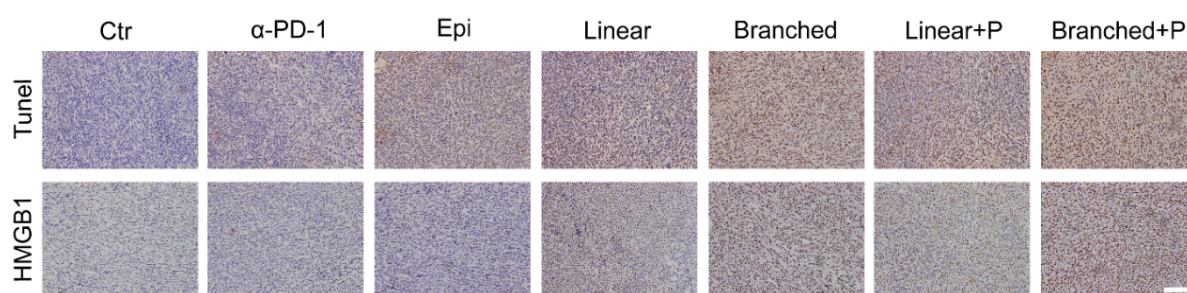


Figure S19. Representative images of TUNEL (top) and HMGB1 (bottom) immunohistochemistry staining of tumor tissues in the mice treated with PBS (Ctr), anti-PD-1 antibody (α -PD-1), free Epi (Epi), the linear NPs (linear), the branched NPs (branched), the linear NPs combined with anti-PD-1 antibody (linear + P), and the branched NPs combined with anti-PD-1 antibody (branched + P). Scale bar = 200 μm .

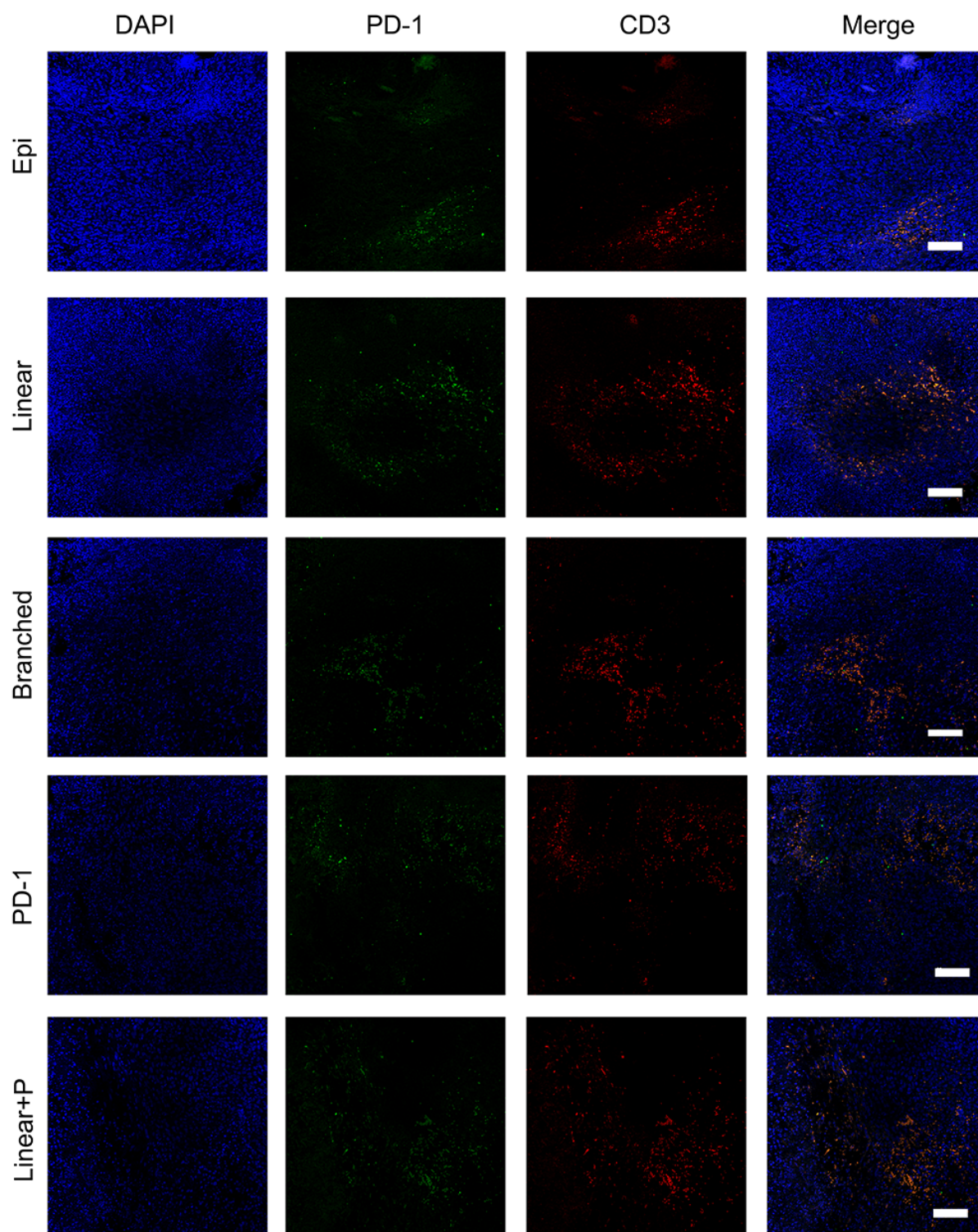


Figure S20. Immunofluorescence staining of CD3 and PD-1 in tumor tissues after treatment with different formulations (P: α -PD-1, Linear: the linear NPs, Branched: the branched NPs). Scale bar: 200 μ m.

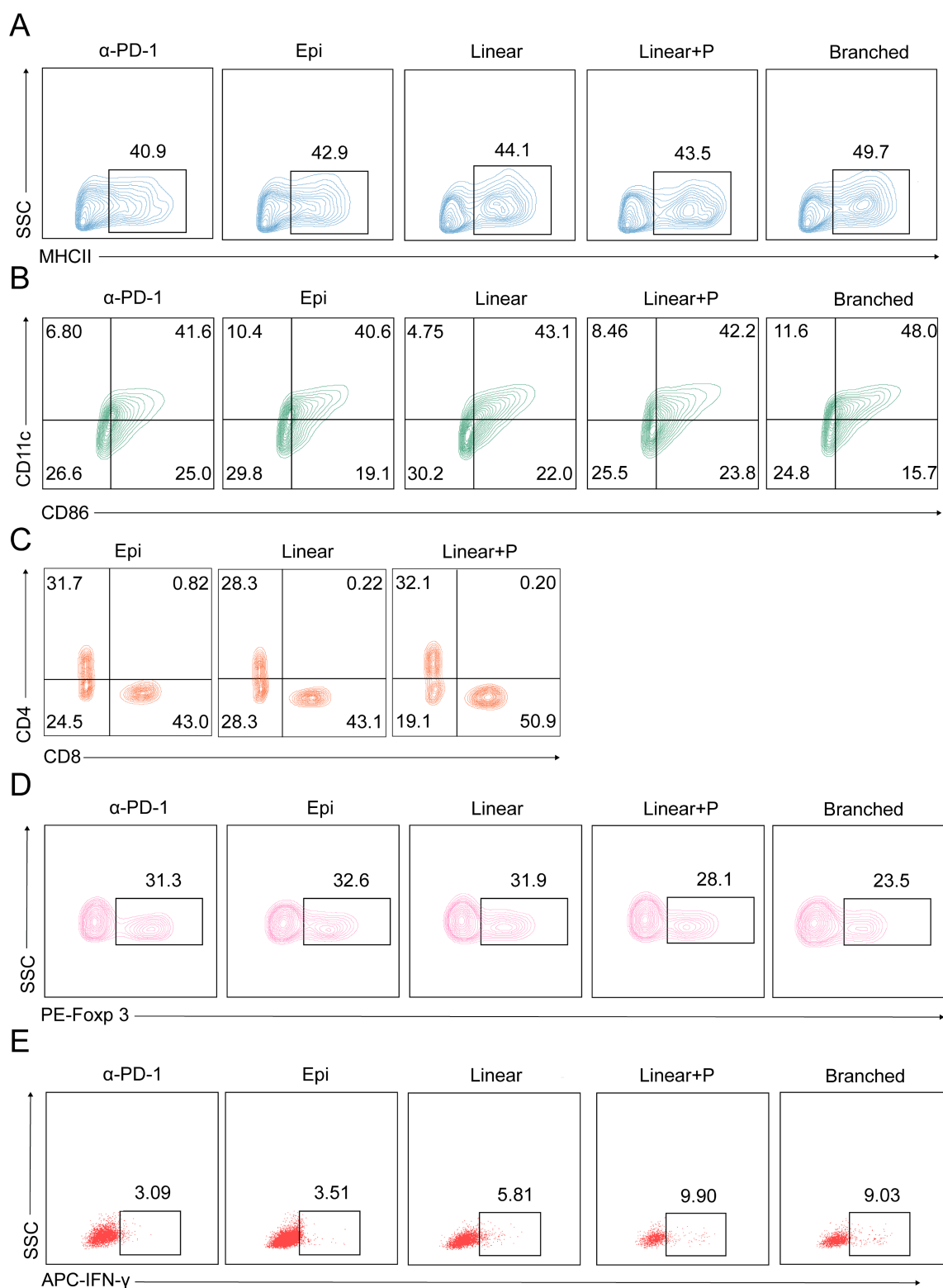


Figure S21. Flow cytometry analysis of tumor immune cells in mice after different treatments. (A) Representative flow cytometric images of MHCII⁺ DCs in CD11c⁺ cells. (B) Representative flow cytometric images of CD86⁺ DCs in CD11c⁺ cells, (C) Representative flow cytometric images of CD4⁺ and CD8⁺ T cells in CD3⁺ cells. (D) Representative flow cytometric images of T_{reg} cells (CD4⁺ Foxp 3⁺ T cells) in CD3⁺ CD4⁺ cells, and (E) Representative flow cytometric images of T_{eff} cells (IFN γ ⁺ CD8⁺ T cells) in CD3⁺ CD8⁺ cells.

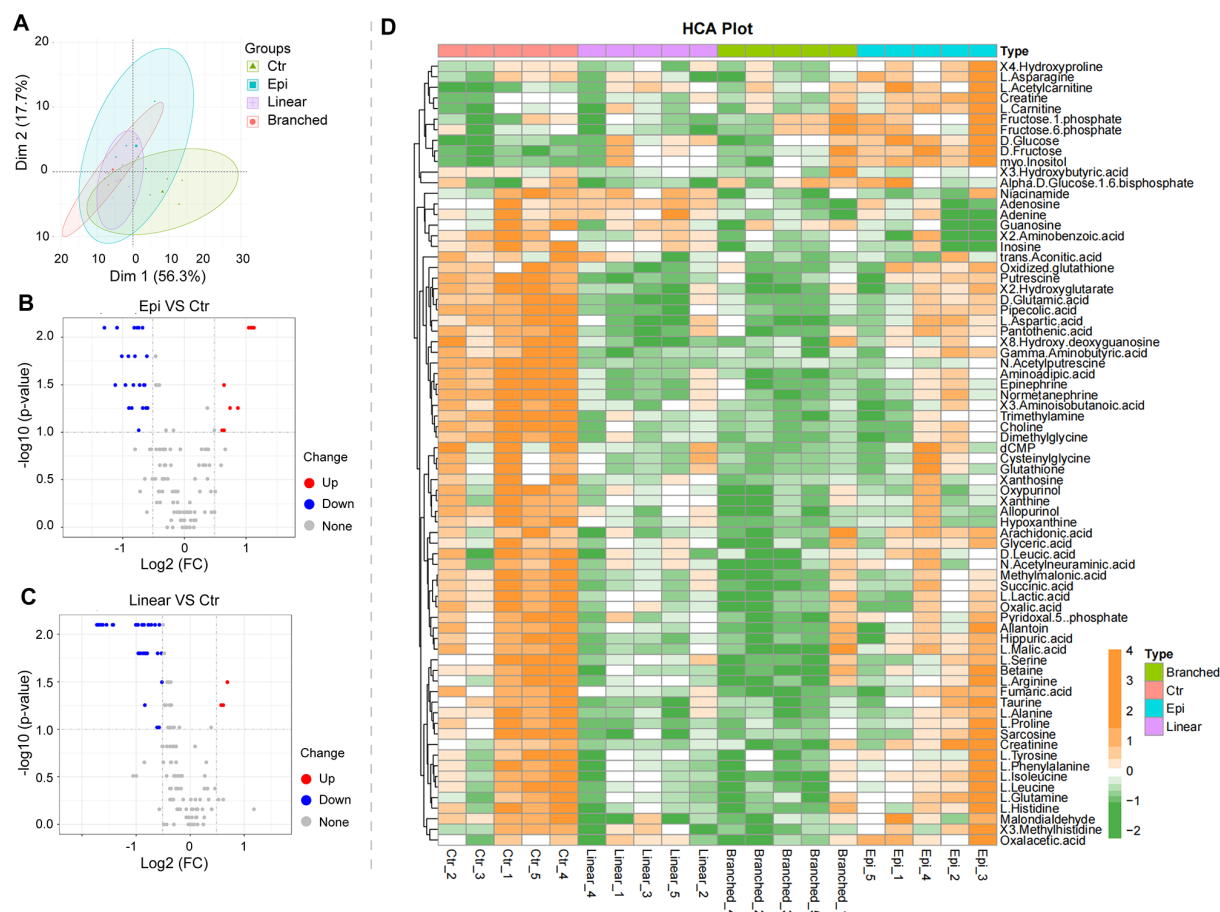


Figure S22. Metabonomic analysis of the intracellular metabolites in 4T1 cells after co-culture with the linear and branched NPs. (A) Principal components analysis (PCA) of intracellular normalized metabolomic data after treatment with the linear and branched NPs, free Epi and the control. (B) Volcano plots of differentially abundant intracellular metabolites in 4T1 cells treated with free Epi, compared with the control. (C) Volcano plots of differentially abundant intracellular metabolites in 4T1 cells treated with the linear NPs, compared with the control. (D) Heatmap for z-scores of intracellular metabolites in the treatment groups by the linear and branched NPs, free Epi and the control.

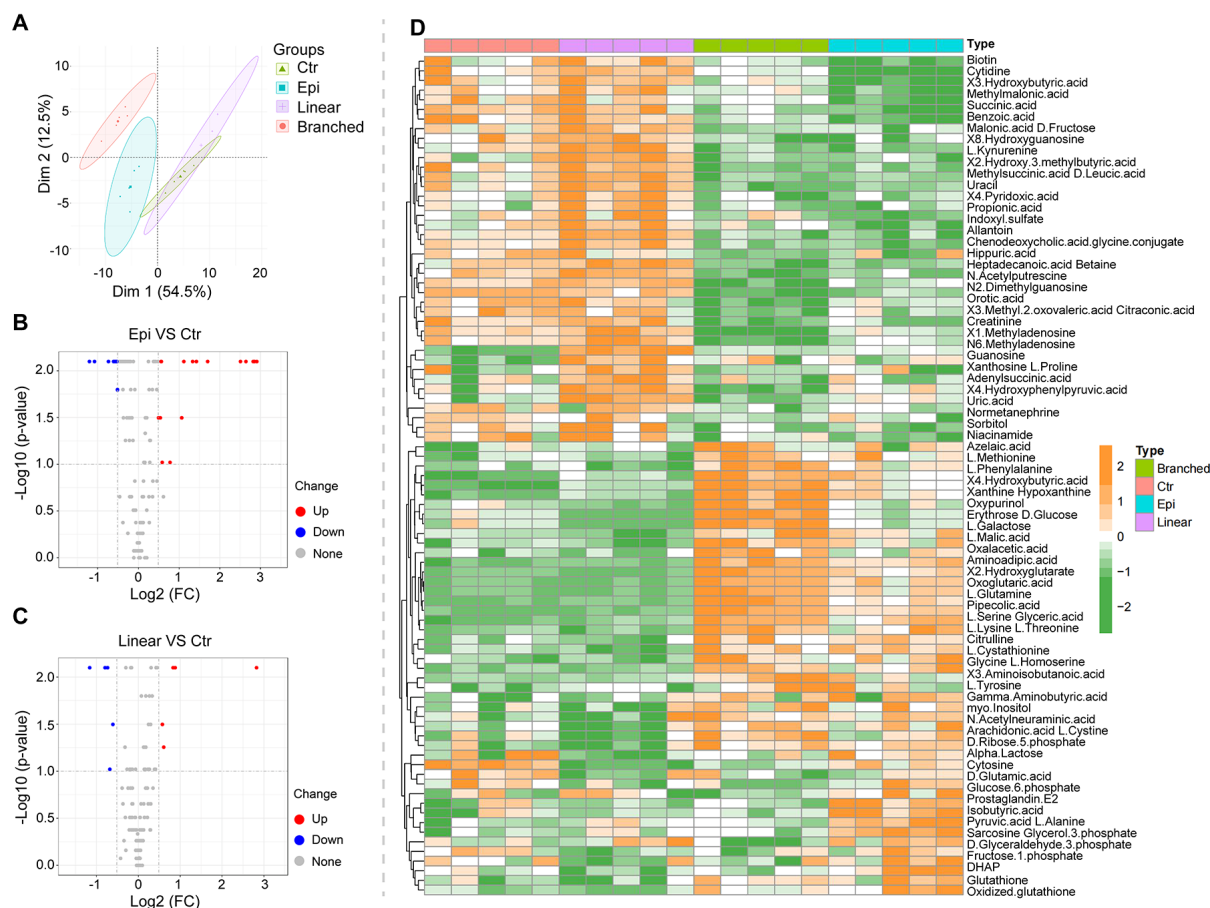


Figure S23. Metabonomic analysis of the extracellular metabolites in the co-culture system after incubated with the linear and branched NPs. (A) Principal components analysis (PCA) of extracellular normalized metabolomic data after treatment with the linear and branched NPs, free Epi and the control. **(B)** Volcano plots of differentially abundant metabolites in the supernatant of 4T1 cells after treatment with free Epi, compared with the control. **(C)** Volcano plots of differentially abundant metabolites in the supernatant of 4T1 cells after treatment with the linear NPs, compared with the control. **(D)** Heatmap for z-scores of extracellular metabolites in the groups treated with the linear and branched NPs, free Epi and the control.

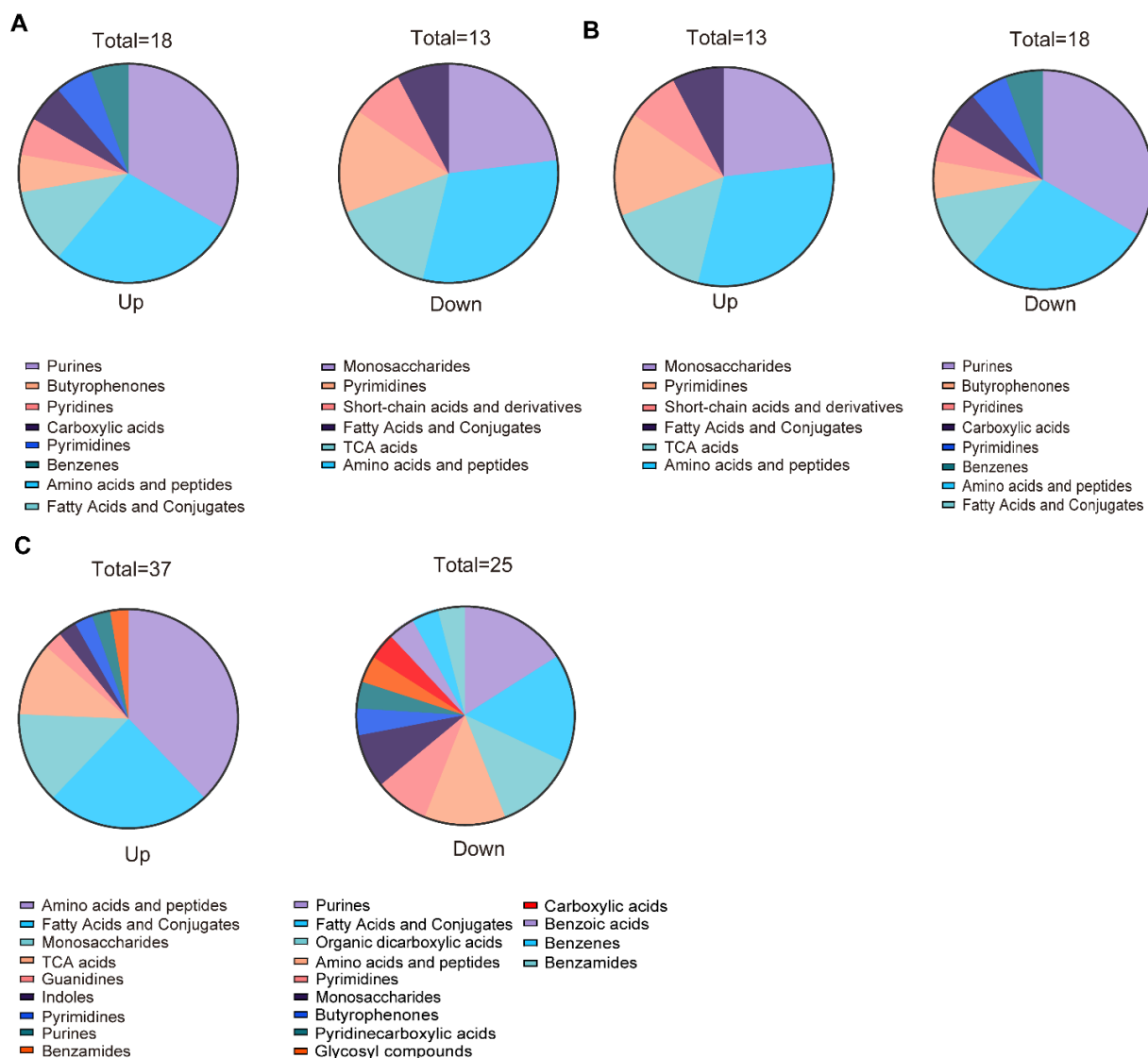


Figure S24. Metabolite profiles in the supernatant of 4T1 cells after co-culture with the linear and branched NPs. (A-C) Differentially abundant metabolites in the supernatant of the group treated with (A) free Epi, (B) the linear NPs, and (C) the branched NPs.

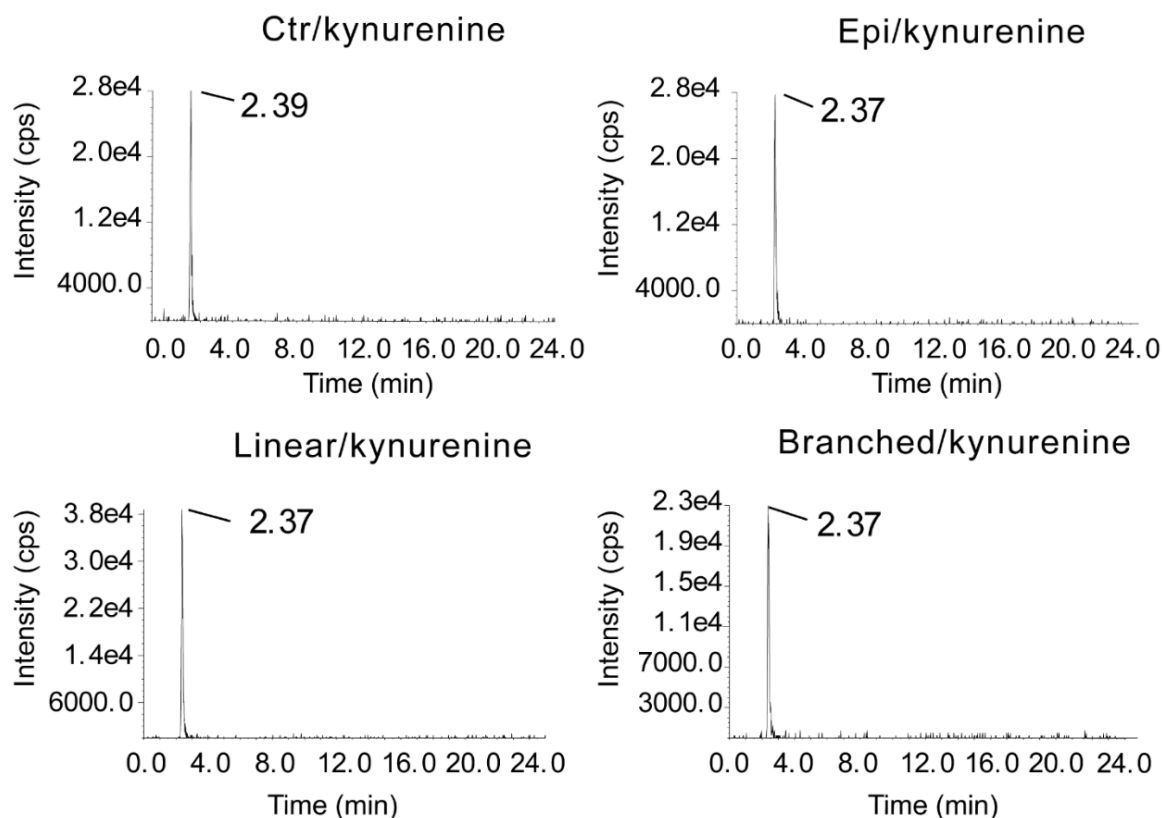


Figure S25. LC-MS spectra of kynurenine in the cell supernatant of the groups treated with the linear and branched NPs, free Epi and the control.

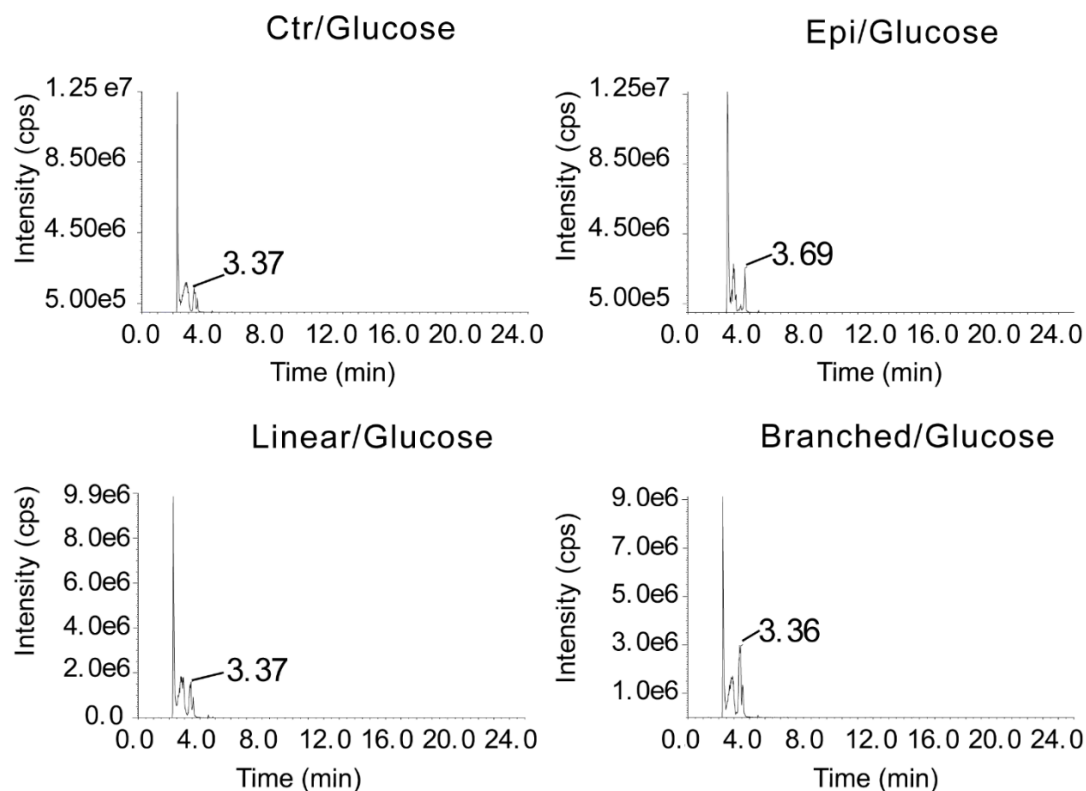


Figure S26. LC-MS spectra of glucose in the cell supernatant of the groups treated with the linear and branched NPs, free Epi and the control.

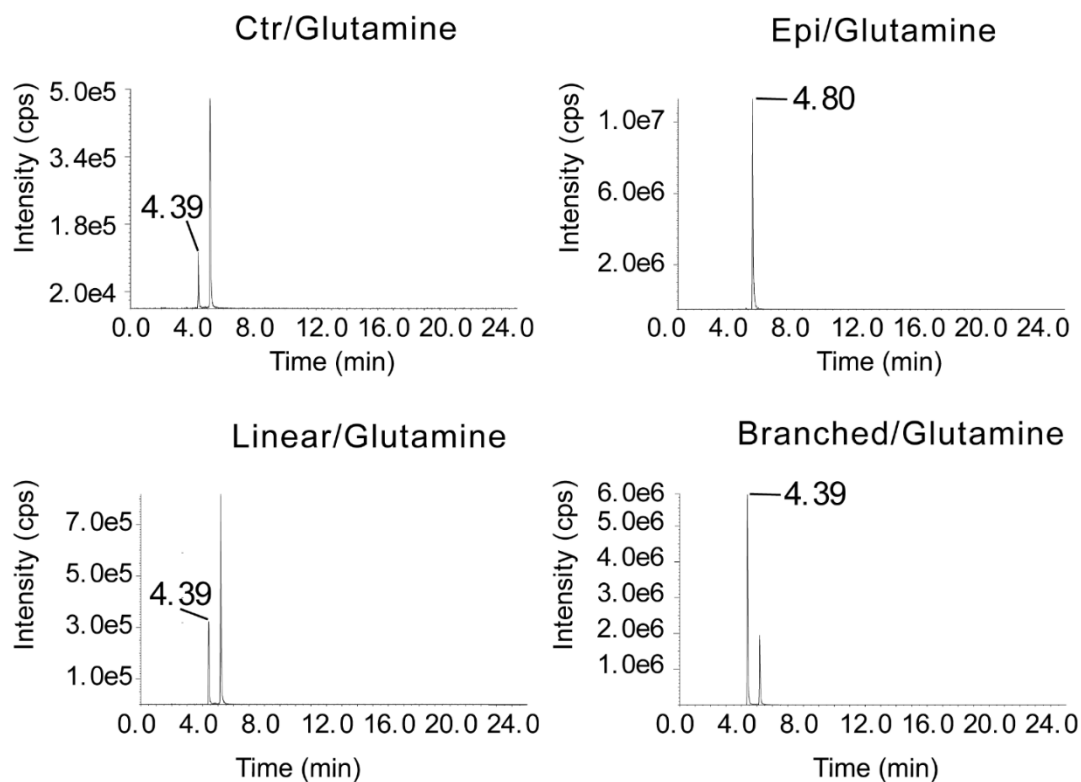


Figure S27. LC-MS spectra of glutamine in the cell supernatant of the groups treated with the linear and branched NPs, free Epi and the control.

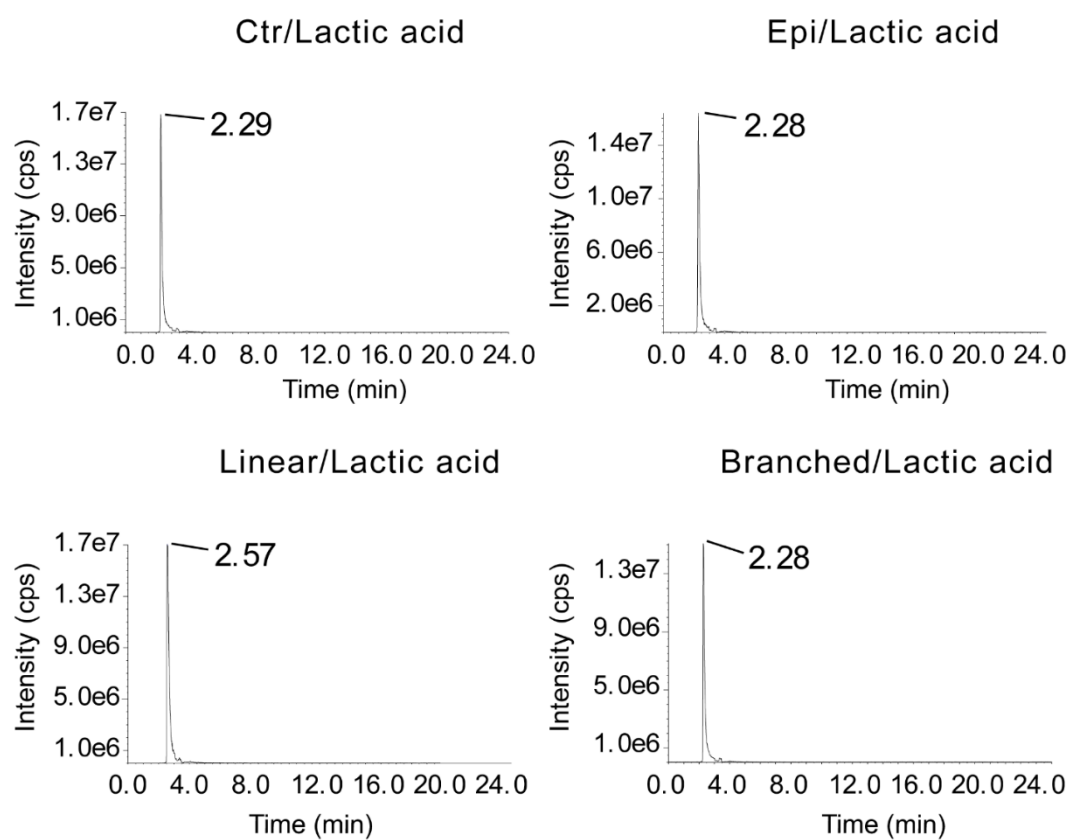


Figure S28. LC-MS spectra of lactic acid in the cell supernatant of the groups treated with the linear and branched NPs, free Epi and the control.

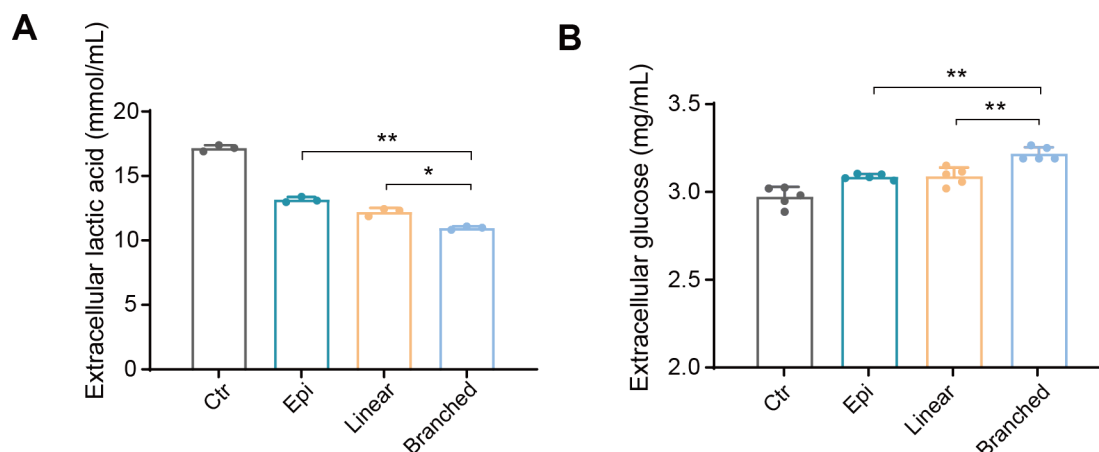


Figure S29. (A and B) The lactic acid concentration in the cell supernatant (A) measured by a lactic acid testing kit and the glucose concentration in the cell supernatant (B) detected by a glucose testing kit after treatment with the linear and branched NPs, free Epi and the control.

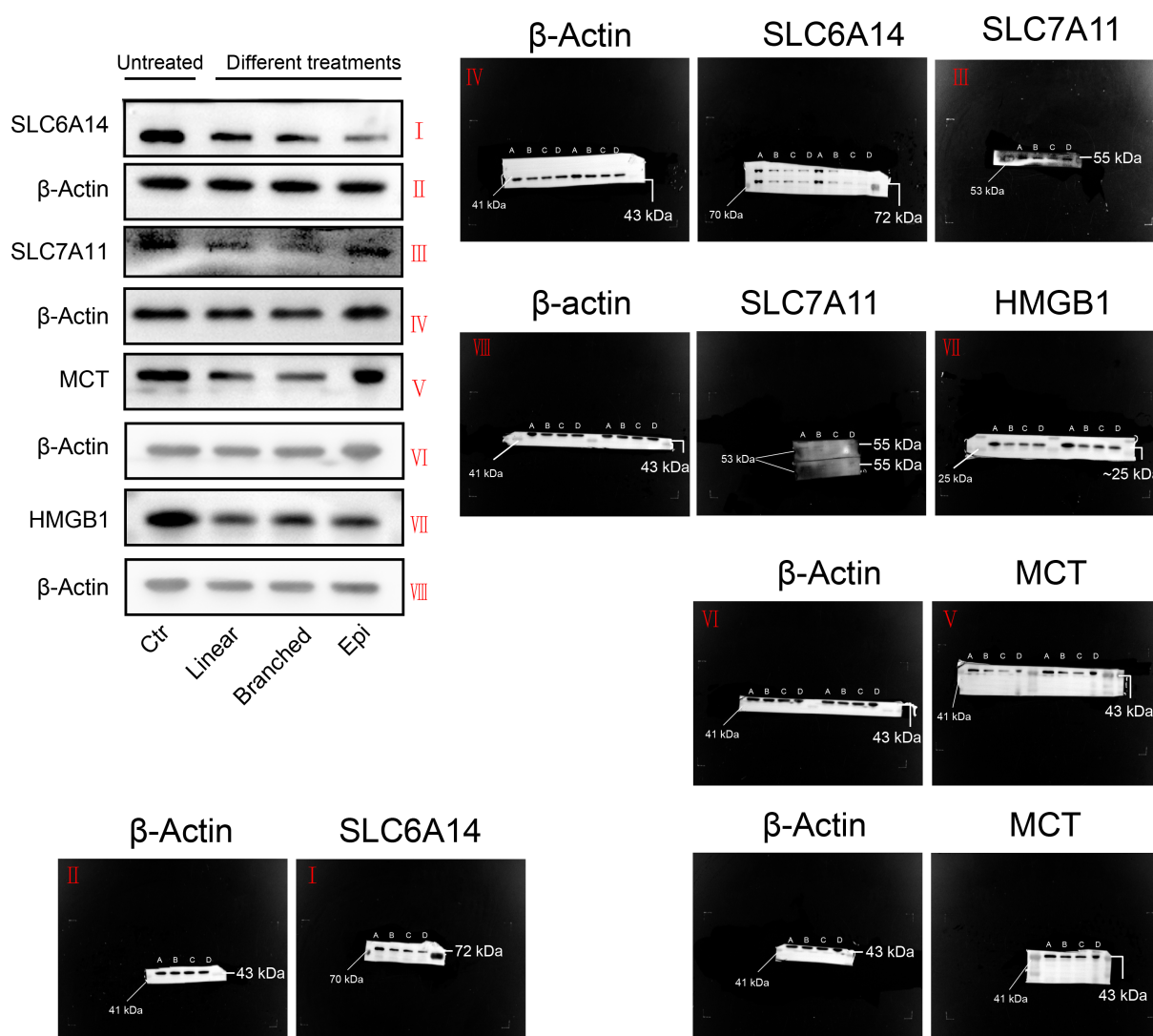


Figure S30. The expression of PARP, SLC6A14, SLC7A11 and MCT was analyzed via western blotting. Raw images of western blotting for PARP, SLC6A14, SLC7A11, HMGB1

and MCT in cells treated with the linear and branched NPs, while the protein expression in cells treated with free Epi was used as a control (A for control, B for the linear NPs, C for the branched NPs, D for free Epi).

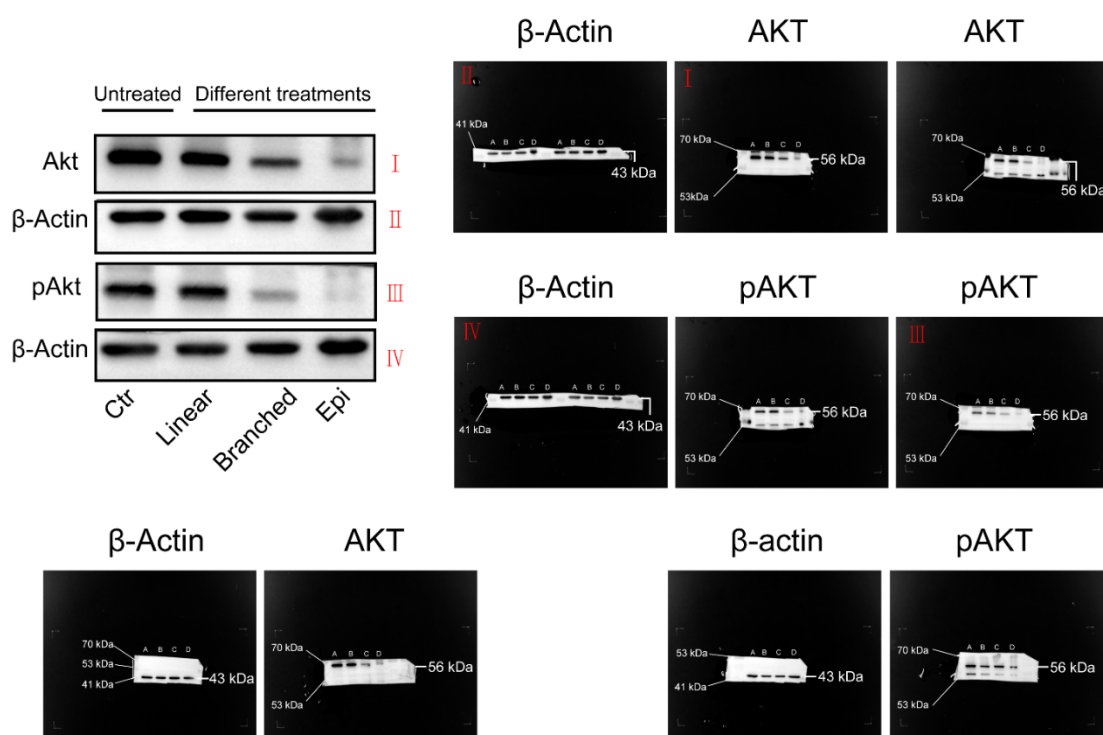


Figure S31. The expression of Akt, and pAkt was analyzed via western blotting. Raw images of western blotting for Akt and pAkt expression in cells treated with the linear and branched NPs, while the protein expression in cells treated with free Epi was used as a control (A for control, B for the linear NPs, C for the branched NPs, D for free Epi).

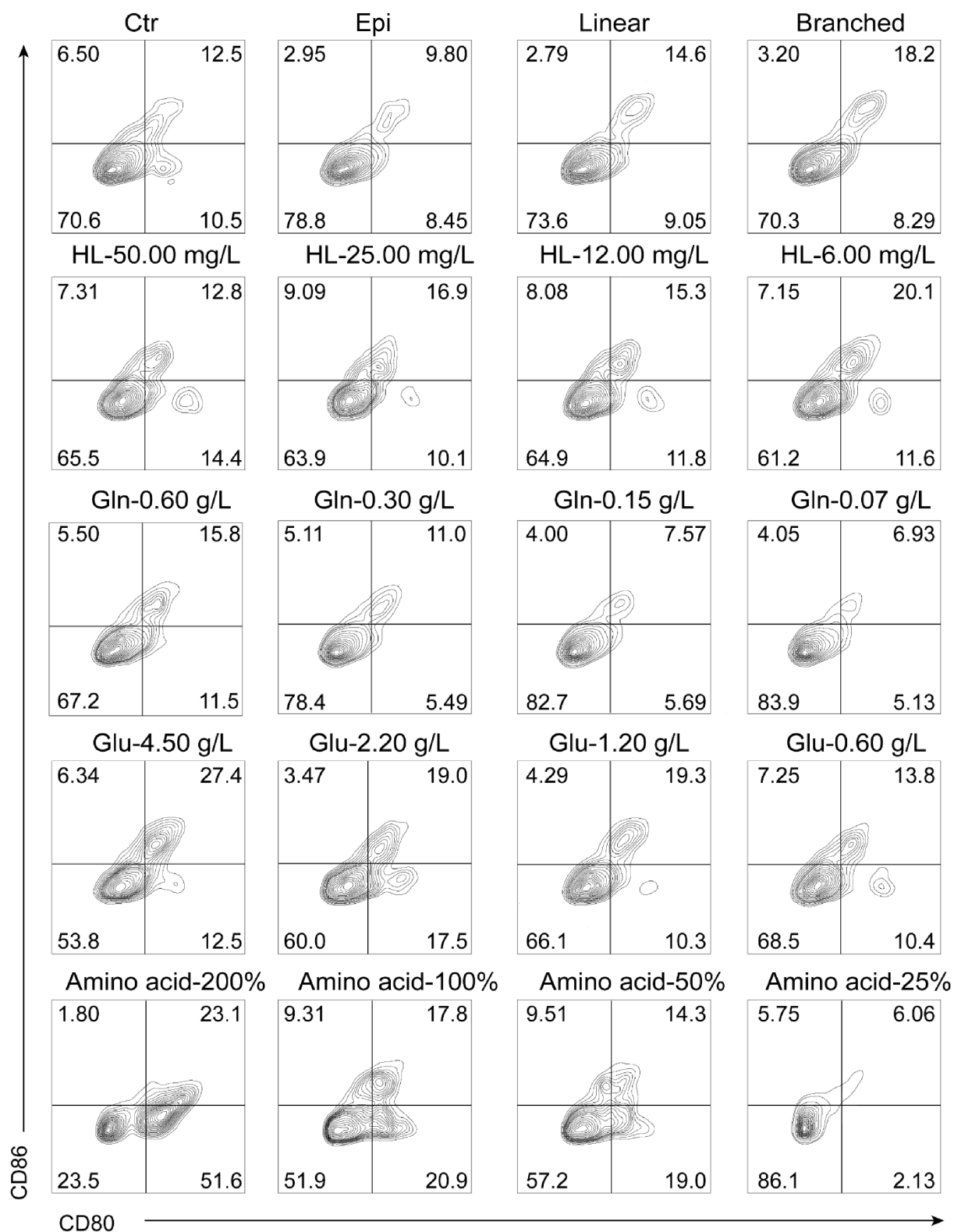


Figure S32. Mature DCs in splenocytes after incubation with nutrients (HL: lactic acid, Gln: glutamine, Glu: glucose, and amino acids) at different concentrations.

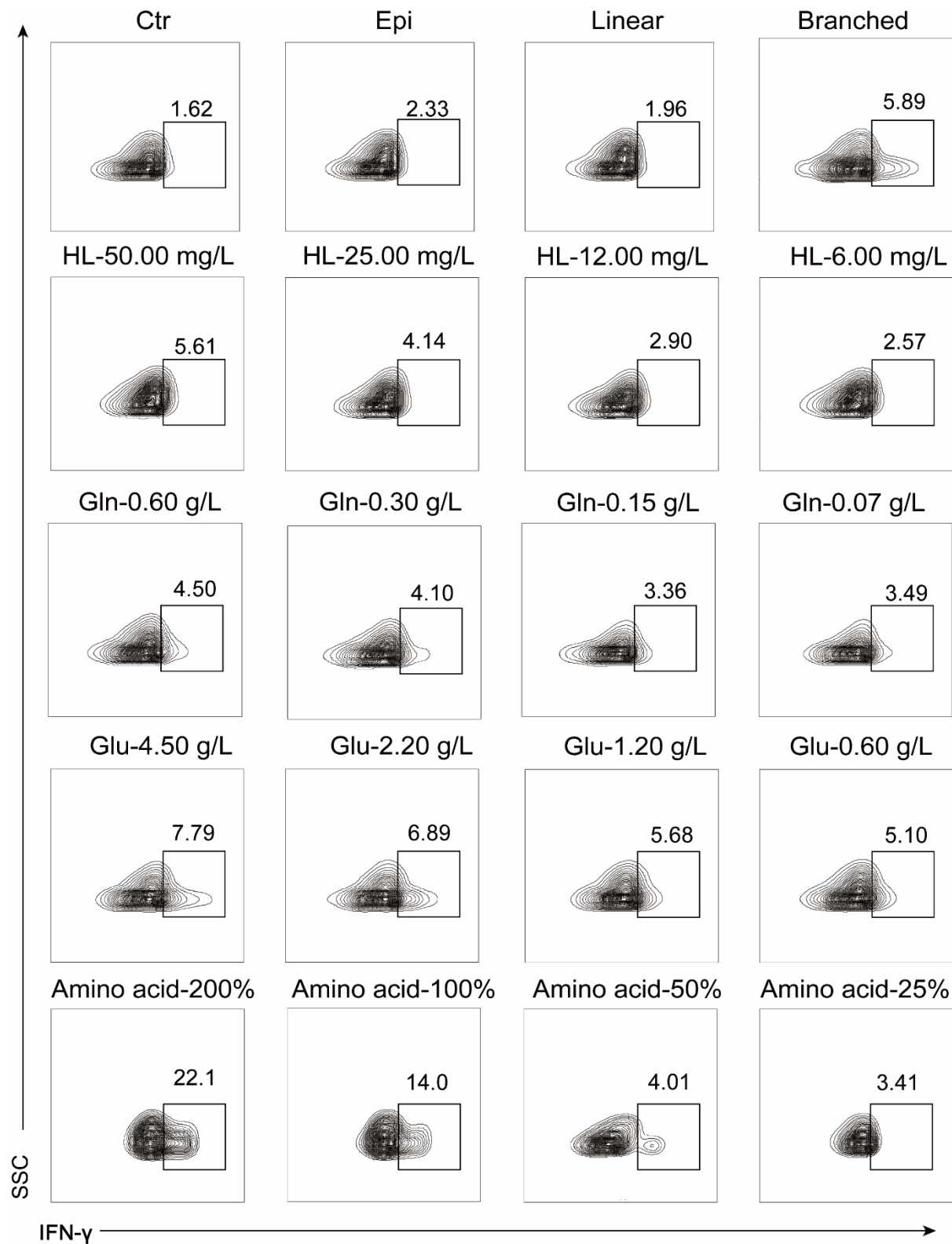


Figure S33. CD3⁺ CD8⁺ IFN-γ⁺ cells in splenocytes after incubation with nutrients (HL: lactic acid, Gln: glutamine, Glu: glucose, and amino acids) at different concentrations.

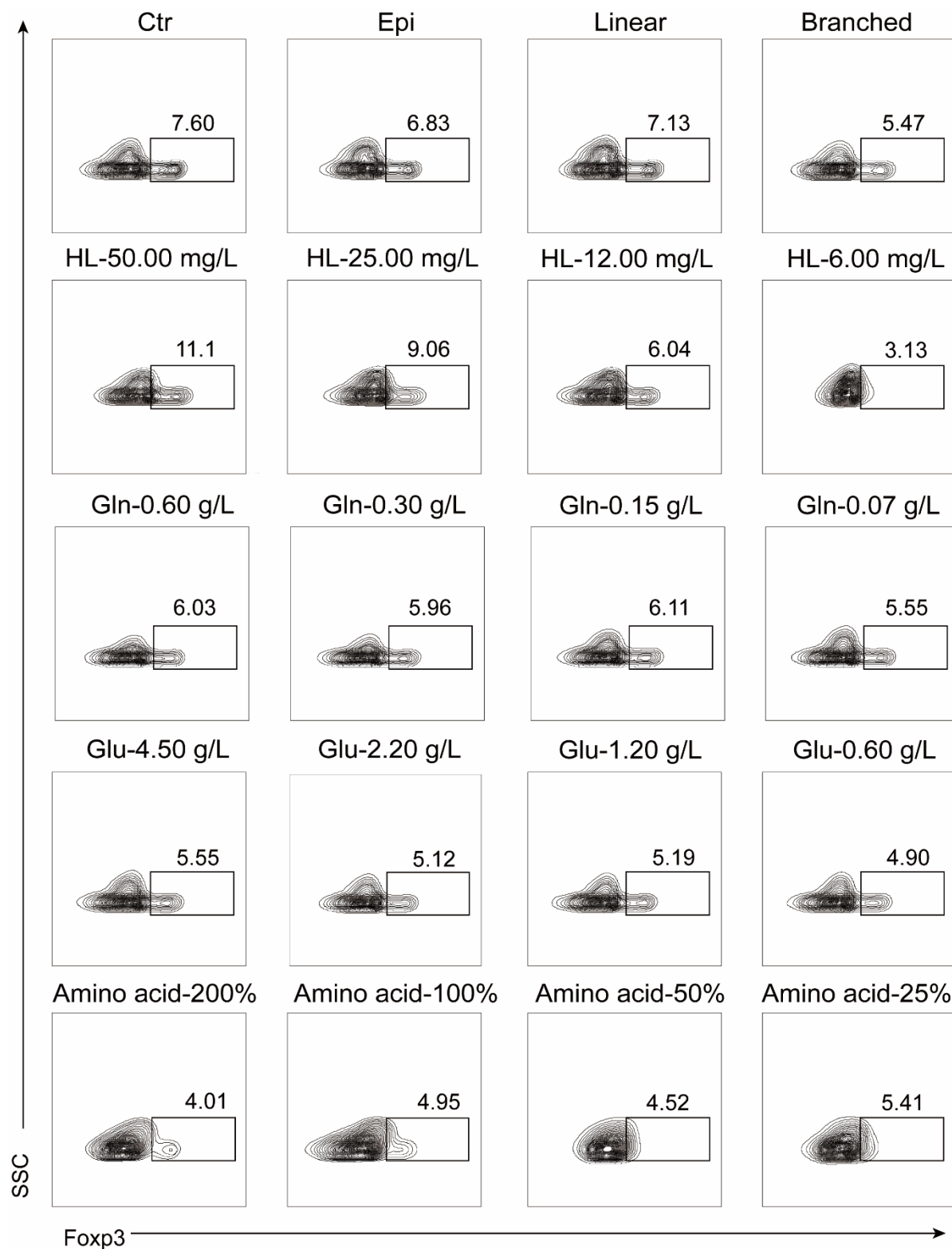


Figure S34. CD3⁺ CD4⁺ Foxp3⁺ cells in splenocytes after incubation with nutrients (HL: lactic acid, Gln: glutamine, Glu: glucose, and amino acids) at different concentrations.

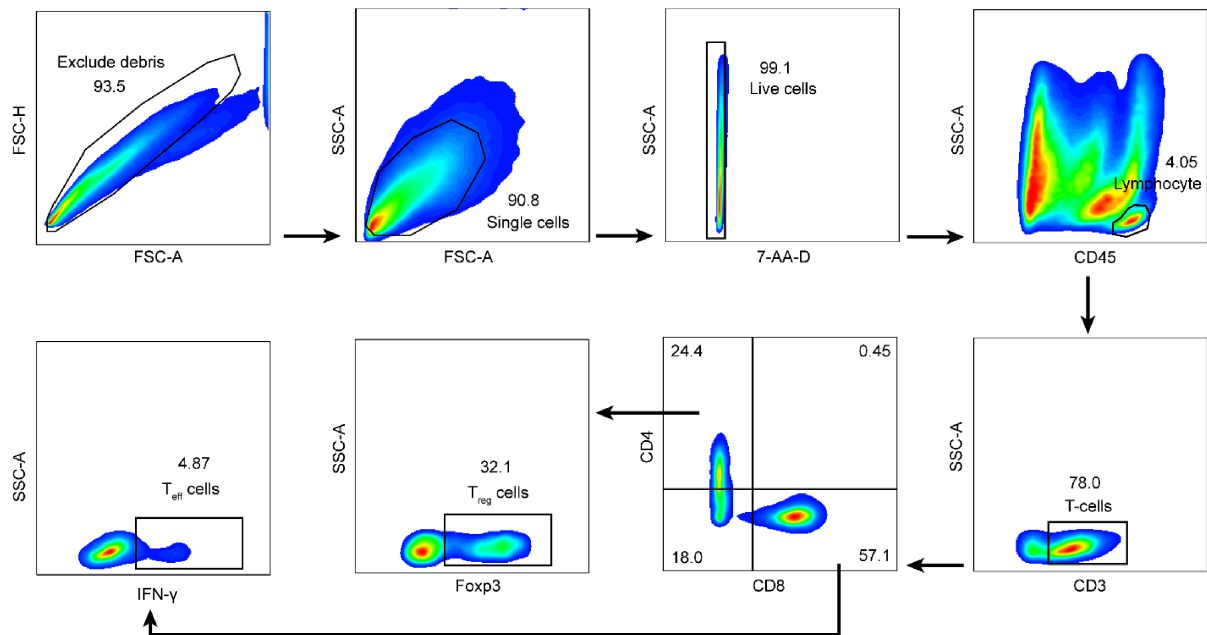


Figure S35. Gates setting for flow cytometry analysis of CD8⁺ T cells, CD4⁺ T cells, T_{reg} cells and T_{eff} cells.

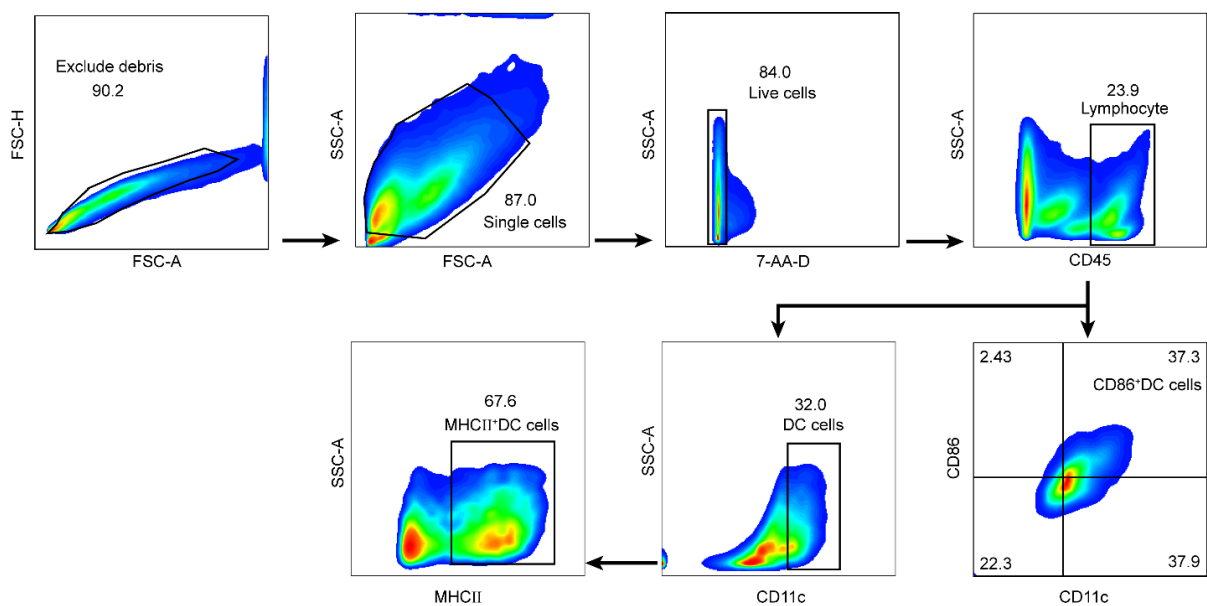


Figure S36. Gates setting for flow cytometry analysis of MHCII⁺ DCs and CD86⁺ DCs.

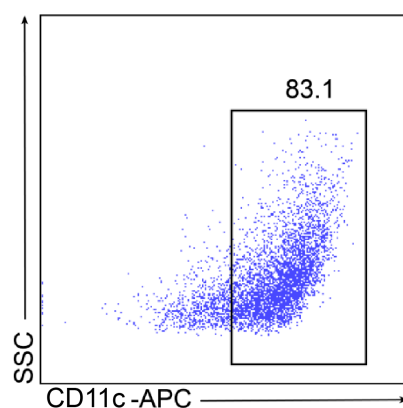


Figure S37. The proportion of CD11c⁺ cells in BMDCs after culture for 8 days *in vitro*.

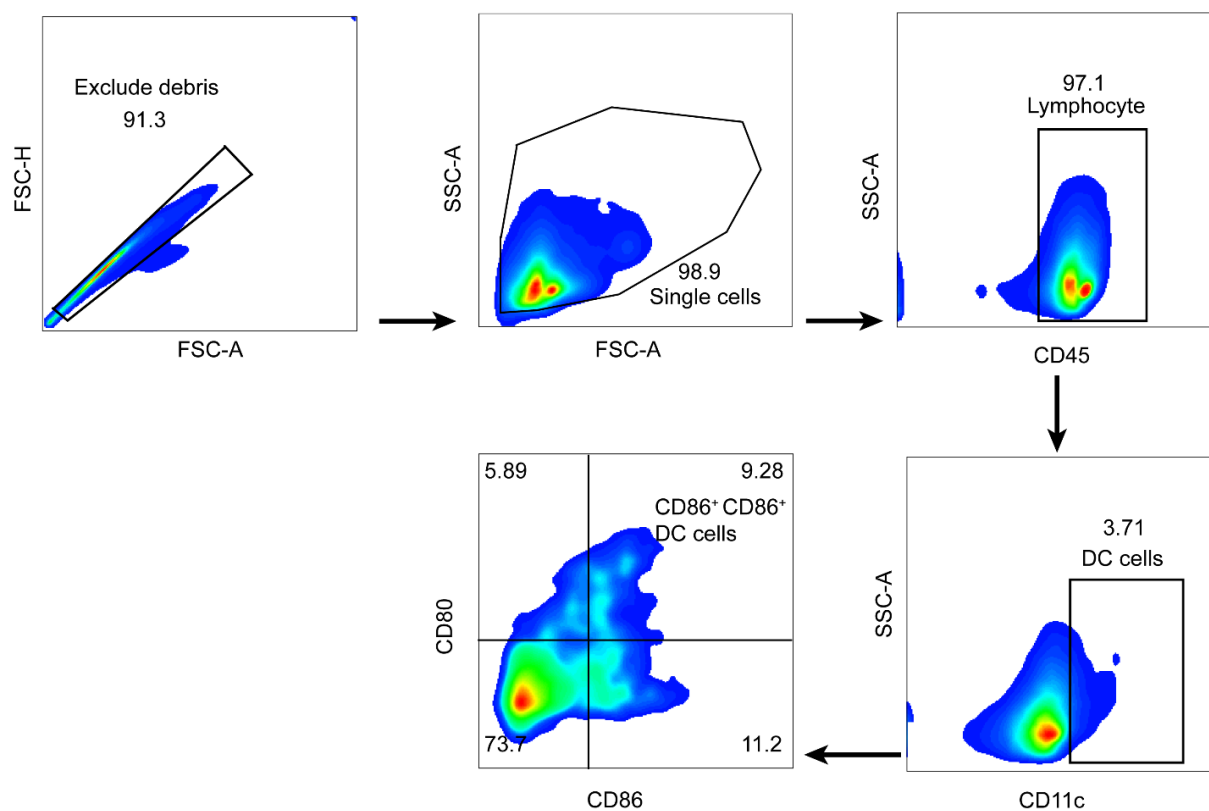


Figure S38. Gates setting for flow cytometry analysis of CD80⁺ CD86⁺ DCs.

Supplementary Tables

Table S1. Physicochemical characterizations of polymeric prodrug conjugates and their NPs.

Comp.	MW (KDa)	PDI	Zeta potential (mV)	DLS Size (nm)	Epi (w.t%)
Linear	42.6	1.28	-5.04	24.0	8.29
Branched	79.2	2.94	-2.34	59.2	8.74

Table S2. Interaction parameters between different beads for DPD simulations.

α	C	N	P	O	S	W	OH	PH
C	25.0							
N	38.6	25.0						
P	31.6	31.9	25.0					
O	84.4	142.9	56.8	25.0				
S	31.5	29.2	37.0	29.9	25.0			
W	37.6	28.3	30.6	26.9	47.3	25.0		
OH	98.5	38.6			79.0	20.0	25.0	
PH	75.0	24.0			100.0	11.0	240.0	25.0

Table S3. Pharmacokinetic parameters of the linear and branched NPs.

Group	$t_{1/2}$ (h)	AUC ($\mu\text{g/mL}\cdot\text{h}$)	C_{max} ($\mu\text{g/mL}$)	CL (L/h/kg)
Epi	2.26	1.90	1.28	427.04
Linear	7.09	9.48	4.46	62.66
Branched	10.19	18.15	5.23	24.89

Table S4. Antibodies used in this study.

Antibodies	Source	Identifier
β -actin	Cell Signaling Technology	Cat# 13E5
Bcl-2	Cell Signaling Technology	Cat# 3498S
PARP	Cell Signaling Technology	Cat# 9542S
Caspase-3	Huabio	Cat# ET1608-64
Cleaved caspase 3	Cell Signaling Technology	Cat# 9579S
Alexa Fluor 700-CD3	Thermo Fisher	Cat# 56-0037-42
eFluor 450-anti-CD4	Thermo Fisher	Cat# 48-0042-82
BV510-anti-CD8a	Biolegend	Cat# 563068
APC-Cy7-anti-CD45	BD	Cat# 557833
APC-anti-CD11C	Biolegend	Cat# 117312
FITC-anti-MHCII	Thermo Fisher	Cat# 11-5321-82
PE-anti-FoxP3	BD	Cat# 563101
eFluor 450-anti-CD86	Thermo Fisher	Cat# 48-0862-82
PE-cy5-anti-CD80	Thermo Fisher	Cat#MA5-28657
eFluor 647-anti-CRT	Abcam	Cat#ab194119
MCT	Abcepta	Cat# AP12397b
SLC6A14	Immunoway	Cat# YT5102
SLC7A11	Huabio	Cat# HA60107
Akt	Huabio	Cat# EM40507
pAkt	Huabio	Cat# HA500116
Anti-PD-1 (injection)	Bioxcell	Cat# BE0146
HMGB1 (IHC)	NOVUS	Cat# L344
HMGB1 (WB)	Huabio	Cat# ET-160-2
CD3	Abcam	Cat# ab228624
PD-1	Cell signaling	Cat# 86163T

Table S5. Critical commercial bio-reagents in this study.

Product name	Source	Identifier
Lysotracker Green DND-26	Yeasen	Cat# 40738ES50
Apoptosis assay kit	Yeasen	Cat# 40302ES20
Glucose Assay Reagent	Beyotime	Cat# S0201S-1
Lactic Acid assay kit	Jiancheng	Cat# A019-2-1
Foxp 3/TRN factor stain buffer kit	Thermo Fisher	Cat# 00-5523-00
ATP Assay kit	Beyotime	Cat# S0026B
Mouse HMGB1 ELISA Kit	Elabscience	Cat# E-EL-M0676c
CCK-8	MCE	Cat# HY-K0301
TUNEL	Roche	Cat# 11684817910
DNase I	Absin	Cat# Abs60172
Hyaluronidase	Absin	Cat# Abs47014926
Collagenase IV	Absin	Cat# Abs47014926
LPS	Sigma	Cat# L2630
Glutamine	Boster	Cat# PYG0012
Lactic acid	Sigma	Cat# L1750
Glucose	Sigma	Cat# 50-99-7
Amino acids	Procell	Cat# PB180425
Red blood cell lysate	BD	Cat# 555899

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