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Mannose-Functionalized Star Polycation Mediated CRISPR/Cas9 Delivery for Lung Cancer Therapy

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Materials

Triethylamine (TEA, 99.5%, extra dry), pentaerythritol tetra (3-mercaptopropionate) (TMPER), carbon disulfide (CS_2), 3-(4,5- dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT), magnesium sulfate ($MgSO_4$, anhydrous), azodiisobutyronitrile (AIBN), 4-dimethylaminopyridine (98%), potassium chloride (KCl, 99.5%), methacrylic anhydride (95%), sodium bicarbonate ($NaHCO_3$, 99%), hydroxyethyl methacrylate (HEMA), glycidyl methacrylate (GMA, 95%) were obtained from Tokyo Chemical Industry Co. Ltd. Hydrazine hydrate, dimethyl sulfoxide (DMSO, anhydrous), isopropyl ether, dichloromethane (anhydrous, 99.8%), ethanolamine (EA, 98%), hydrochloric acid, ethyl acetate (EtOAc, 99.8%), hexane and 1,4-dioxane were purchased from Sigma-Aldrich. Boron trifluoride etherate, α-D-mannose pentaacetate (AcMan) were obtained from TCI. 4',6-diamidino-2-phenylindole (DAPI), HRP-labeled goat anti-mouse IgG were purchased from Beijing Solarbio Science & Technology. CRISPR-Cas9 mouse monoclonal antibody, Cy3-labeled goat anti-mouse IgG were purchased from beyotime. Survivin protein antibody were purchased from Abcam.

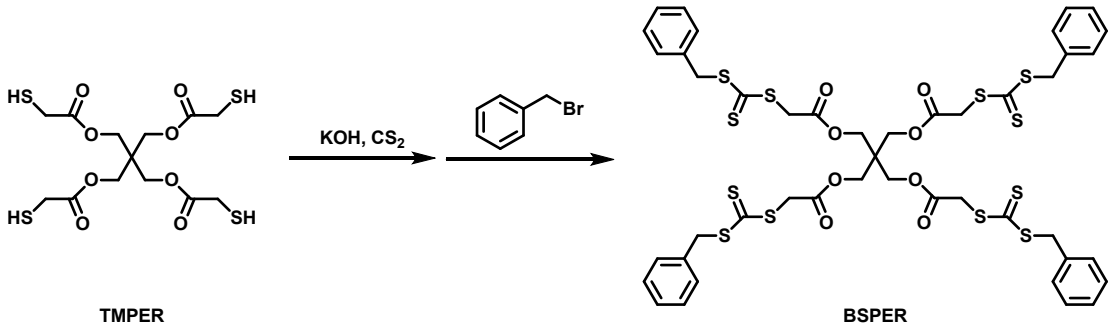
Physicochemical Characterization

^{1}H NMR spectra were measured on a Bruker ARX 400 MHz spectrometer using the solvents DMSO-d_6 (3-(benzylsulfanylthiocarbonylsulfanyl)-pentaerythritol, BSPER), $CDCl_3$ (mannose pentaacetate-functionalized hydroxyethyl methacrylate, AcManMA), DMSO-d_6 (P(GMA-*co*-AcManMA), GA), D_2O (GM). Molecular weight of GA and GM were determined by gel permeation chromatography (GPC) equipped with Waters Styagel columns and a Waters-2414 refractive index detector. Particle sizes and zeta potentials of different N/P ratio were detected with a Zetasizer Nano ZS (Malvern Instruments, Southborough, MA). The cell internalization was recorded on

the confocal laser scanning microscopy (CLSM, Leica, SP8) microplate reader (Biotek cytation 3, USA)

Synthesis of BSPER

3-(benzylsulfanylthiocarbonylsulfanyl)-pentaerythritol (BSPER) preparation followed the previous reported. Tetra (2-mercaptoacetic acid) pentaerythritol ester (TMPER) (5 mmol) and carbon sulfide (40 mmol) were dissolved in dichloromethane (15 mL). Then, triethylamine (5.5 mL in 10 mL dichloromethane) was added slowly. The mixture was stirred at room temperature for 1 h. Benzyl bromide (22 mmol) dissolved in dichloromethane (10 mL) was added into the solution, and reacted for 2 h at room temperature. The product was added into 10% HCl solution. The mixture was washed with ethyl acetate. The resultant solution was dried by using anhydrous magnesium sulfate and concentrated under reduced pressure. The product was further purified by silica gel column (ethyl acetate: hexane =1:3).

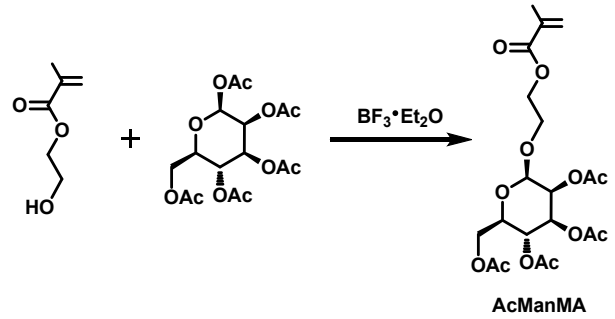


Scheme S1. Synthesis process of BSPER

Synthesis of AcManMA

Mannose pentaacetate-functionalized hydroxyethyl methacrylate (AcManMA) was obtained by reacting the hydroxyl groups of HEMA with the hemiacetal hydroxyl groups of mannose pentaacetate. Boron trifluoride etherate (3.5 mL) was added to the solution of α-D-mannose pentaacetate (1.95 g) and hydroxyethyl methacrylate (0.6

mL) in dichloromethane (10 mL) under stirring at 0 °C for 1 h, and then reacted at room temperature for 3 h. The mixture was washed with water (30 mL), sodium bicarbonate solution (30 mL) and water (30 mL). The organic layer was dried by using anhydrous magnesium sulfate and concentrated under reduced pressure. The residual product was purified by flash column chromatography with ethyl acetate and n-hexane (v/v = 1 : 3) to give a colorless powder with 45% yield.

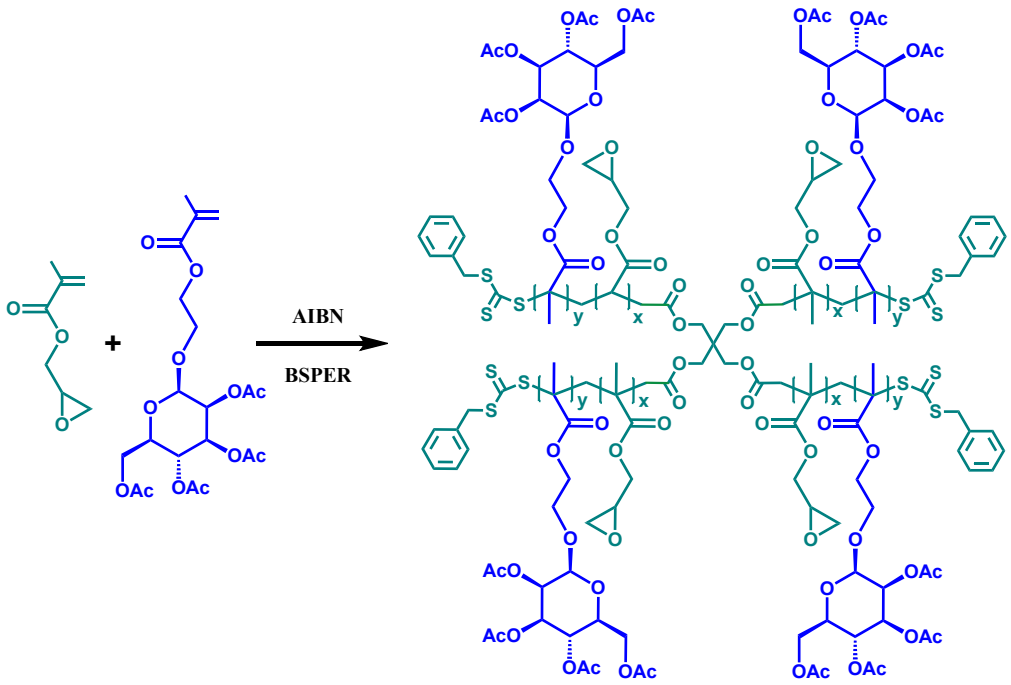


Scheme S2. Synthesis process of AcManMA.

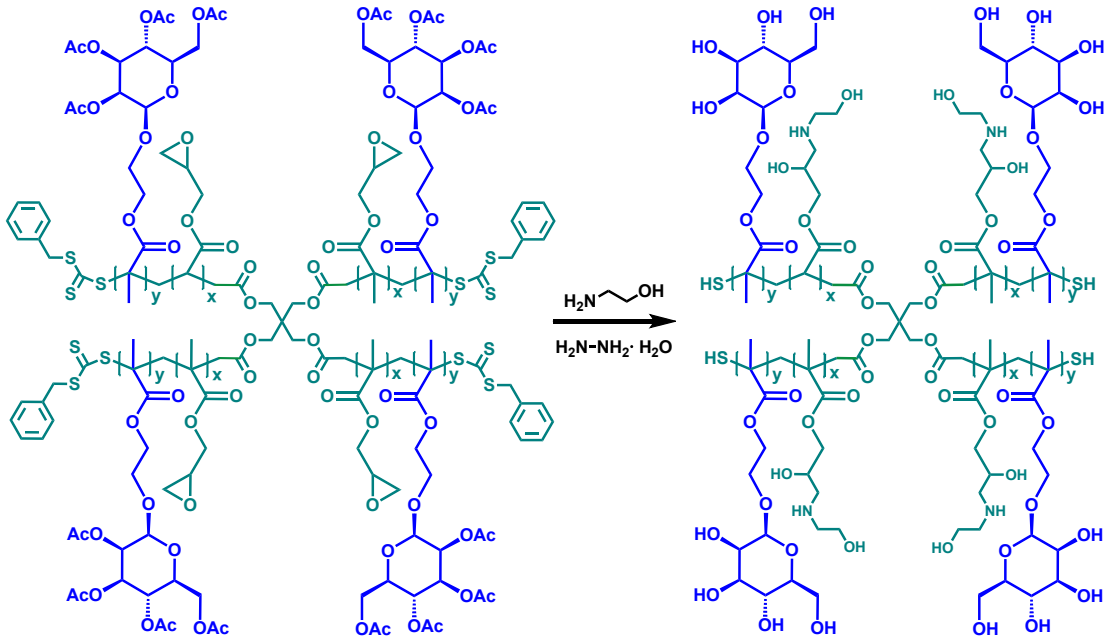
Synthesis of P(GMA-*co*-AcManMA) (GA) and P(GEA-*co*-ManMA) (GM)

For the preparation of P(GMA-*co*-AcManMA) (GA), BSPER (0.1093mmol) and AIBN (0.01093mmol) were added in 1,4-dioxane (12 mL), glycidyl methacrylate (GMA, 4 mmol) and AcManMA (40 mmol) were added into above solution. the mixture reacted at 70 °C for 12 h under Ar atmosphere. The reaction mixture was added dropwise in isopropyl ether for precipitating. The emulsion was centrifuged and the supernatant was discarded.

GA (200 mg) was dissolved in DMSO (5 mL) and ethanolamine (1 mL) was added. After reacting at 80 °C for 2 h under Ar atmosphere, hydrazine hydrate (200 μL) was added in the solution, the reaction was carried out at room temperature for another 12 h, then the reaction mixture was dialyzed (MWCO≈1000) for two days. Finally, the product was obtained by freeze-drying..



Scheme S3. Synthesis process of GA.



Scheme S4. Synthesis process of GM.

Particle Sizes and Zeta Potential Tests

pDNA was prepared at a concentration of 0.1 mg mL^{-1}. All the GM/pDNA complexes were formed by mixing equal volumes of GM with pDNA solutions at the

desired N/P ratio. The particle sizes and zeta potential were determined with Zetasizer Nano ZS (Malvern Instruments, Southborough, MA). GM were examined for their ability to bind pDNA using agarose gel electrophoresis equipped with a UV transilluminator and a BioDco-It imaging system (UVP Inc., Upland, CA).

Complex stability assay

GM/pDNA complexes were formed by mixing equal volumes of GM with pDNA solutions at the optimum N/P ratio. After 30 min, 20 μL of GM/pDNA complexes were diluted to DMEM with 10% FBS (fetal bovine serum) at 37 °C for 4 hours. The particle sizes were determined at every hour with Nano-ZS90.

DNA release ability assay

A549 cells were seeded on a glass bottom cell culture dish contains 1 mL of DMEM medium and incubated for 24 h. GM/pDNA complex (containing 2 μg of pDNA, which is pre-stained with fluorescent dye YOYO-1) was added into cell culture dish. At 3rd, 6th, and 9th hour, lysosomes were stained with LysoTracker Red for 30 min. A549 cells were fixed with 4% paraformaldehyde and nuclei were stained with DAPI. The stained A549 cells were observed by laser scanning confocal microscopy to evaluate the intracellular locations of transported pDNA.

Cell Viability Assay

For the cytotoxicity evaluation of GM, HEK 293 and lung cancer A549 cell lines were used. Both cells were cultured in DMEM with 10% FBS, 100 IU mL^{-1} of penicillin, and 100 mg mL^{-1} of streptomycin. The incubation environment requires a 5% CO_2 atmosphere with 95% relative humidity. The cytotoxicity of GM/pDNA complexes at series of N/P ratios was evaluated through the MTT assay.

Protein absorption assay.

GM solution (100 μL, 2.3 mg/mL) and PEI solution (100 μL, 0.43 mg/mL) were

respectively mixed with pDNA solution (100 μL, 0.33 mg/mL). Then all the mixtures were kept for 30 min to form complexes. 40 μL of bovine serum albumin (BSA) solution was added to the complex solution and shaken at 37 °C for 0.5-60 min. The supernatant was collected after high-speed centrifugation to remove the protein complexing with polycations. The BCA protein assay was used to determine the concentration of BSA in the supernatant.

***In Vitro* Transfection Assay with Reporter Genes**

Transfection performances of GM polycations were first investigated in HEK 293 or A549 cell lines using plasmid pRL-CMV as the reporter gene. Both cell lines were seeded in 24-well plates at 6×10^4 cells per well and incubated in 500 μL medium for 24 h with 10% FBS. Subsequently, 20 μL of GM/pDNA complexes (containing 1.0 μg of pDNA) at various N/P ratios were added to each well. 4 h later, 500 μL of new medium was added to replace original medium. After 20 h, the cultured cells were washed twice with PBS and lysed in 70 μL of cell-culture lysis buffer reagent (Promega, USA). Luciferase gene expression was quantified with a commercial kit (Promega, USA) using a luminometer (Lumat LB 9507, Berthhold Technologies, USA) as relative light units (RLUs) per milligram of cell protein lysate (RLU mg^{-1} protein). In order to prove the MR-mediated transfection ability of GM, culture medium was replaced with DMEM (containing 1mg mL^{-1} mannose) for HEK 293 or A549 cell lines and incubated for 4 h prior to the transfection with the GM/pDNA complexes. Then, the original culture medium was replaced with fresh culture medium. The GM/pDNA complex was added to both cell lines under the same procedures as mentioned above.

In addition, gene transfection was also assessed using plasmid pEGFP-N1 (0.1 mg mL^{-1}) as another reporter gene at the N/P ratios (20 for HEK 293 cells and 25 for

A549 cells) of GM/pEGFP-N1 complexes (containing 1.0 μg of pEGFP-N1) under the similar procedures as above. The transfected cells were imaged using a Leica DMIL 3000B Fluorescence Microscope. The percentage of the EGFP-positive cells was determined by a flow cytometry (FCM, Beckman Coulter, USA).

Cellular Internalization

For cellular uptake assays of the GM/pDNA complexes, pDNA was labeled with YOYO-1 staining reagent first. A total of 2×10^5 HEK 293 or A549 cells were seeded per 20 mm cell culture dish and incubated for 24 h. Then, 40 μL of the GM/pDNA complexes (containing 2.0 μg of YOYO-1 labeled pDNA) at the optimal N/P ratio were added to each culture dish and incubated for 4 h. The culture cells were washed with PBS and the nucleus were stained with DAPI (1 mg mL^{-1}) for 10 min. Finally, confocal laser scanning microscope (CLSM made in German, Leica SP8) was used to visualize the cells and flow cytometry (FCM, Beckman Coulter, USA) was used to quantify the cellular phagocytosis efficiency. In order to prove the MR-mediated targeted ability of GM, culture medium was replaced with DMEM (containing 1mg mL^{-1} mannose) for HEK 293 or A549 cell lines and incubated for 4 h prior to the transfection with the GM/pDNA complexes. Then, the original culture medium was replaced with fresh culture medium. The GM/pDNA complex was added to both cell lines under the same procedures as mentioned above. CLSM and flow cytometry were also used to analyze the cellular uptake behaviors.

pCas9-survivin expression *in vitro*

To investigate the expression of pCas9-survivin mRNA, A549 cells were seeded in 12-well plates at 1×10^5 per well. After 24 h incubation, the A549 cells were treated with 20 μL of GM2/pDNA or GM2/pCas9-survivin complexes (containing 1 μg of pCas9-survivin). After 48 h, the transfected cells were collected, and RNA was

extracted, RNA was reverse-transcribed into cDNA and then the fluorescence value was read by StepOnePlus PCR system through dye method.

human-β-actin forward：AGAAGGATTCCTATGTGGGCG

human-β-actin reverse: CATGTCGTCCCAGTTGGTGAC

Cas9 forward：GACGACAGCCTGACCTTAA

Cas9 reverse：AGGATGCCCTTCTTGATGGC

Western Blot was used to assess the Cas9 protein expressions. After 48 h transfection, both proteins from A549 cells were extracted with radioimmunoprecipitation assay (RIPA) buffer. 20 μL of protein extracts (1 mg mL^{-1}) were resolved on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. The expression levels of Cas9 protein were determined by anti-Cas9 antibody (beyotime). The β-actin was used as the loading control and its expression was measured using a monoclonal anti-β-actin (Solarbio).

To characterize the position of Cas9 protein in the A549 cells, we used cellular immunofluorescence staining. After the cells are treated with tissue fixator, DAPI and Cy3-labeled Goat Anti-Mouse IgG were used to stain the nucleus and anti-Cas9 antibody.

To investigate the editing effect for survivin, the transfected cells were collected, and genomic DNA was extracted. Genomic regions flanking the target sites were amplified by PCR with the corresponding primers (BIRC5 F and BIRC5 R). The PCR product was subjected to DNA sequencing assay. Meanwhile, 20 μL of product was reannealed by PCR, subsequently 1 μL of T7EI was added to the annealed PCR products and incubated at 37 °C for 50 min. Products were finally analyzed on 2% agarose gels and imaged by imaging system (BioRad, USA).

BIRC5 forward: TGAATCAGGATGTTTGTCCCAGGTAGC

BIRC5 reverse: CAGCCACTGTTACCAGCAGCAC

Wound Healing Assays, Apoptosis Assay, Cloning Formation Assay

For wound healing assays, A549 cells were seeded in a 12-well plate at a density of 1×10^5 cells per well in 1 mL of medium and incubated for 24 h. Then, A549 cells were treated with 40 μL of PBS, GM2/pDNA, GM2/pCas9 (containing 2 μg of pCas9). When reaching about 90% confluence, the cell layers were scratched with pipet tips. A549 cells were then continued to be cultured and wound closure was quantified at 24, 48 h.

For the apoptosis assay, 2×10^5 A549 cells per well were seeded into a 6-well plate and treated with 80 μL of PBS, GM2/pDNA, GM2/pCas9 (containing 4 μg of pCas9). After 48 h incubation, the apoptosis percentage of the above treated cells was measured by using an Annexin V-FITC/PI Apoptosis Kit and a flow cytometry (Beckman Coulter, USA).

For the cloning formation assay, a total of 2×10^5 A549 cells per well were seeded into a 6-well plate and treated with 80 μL of PBS, GM2/pDNA, GM2/pCas9 (containing 4 μg of pCas9),. 4 h later, 2 mL of new medium was added to replace original medium. After 20 h, the cultured cells were washed twice with PBS. The cells were treated with trypsin and re-cultured, a total of 2×10^3 A549 cells per well were seeded into a 6-well plate. After tenth days, the cells were washed with cold PBS twice and fixed with formaldehyde solution (3.7%). A549 cells were dyed with crystal violet solution (0.2%) and imaged.

Hemolysis Assay

Rat blood (3 mL) was suspended in 30 mL of PBS. Red blood cells (RBCs) were separated from serum by centrifuging at 2000 rpm for 15 min. The pure RBCs were

resuspended in PBS with the volume concentration of 2%. The RBCs treated with 1% Triton-X and PBS were used as the corresponding positive and negative controls. GM1, GM2 were added into the RBCs suspension with the concentrations of 0.1 and 1 mg mL^{-1}. The resultant RBC suspension was incubated at 37 °C for 3 h and then centrifuged at 2000 rpm for 15 min. The absorbance of supernatant fraction was measured at 545 nm using a microplate reader (Biotek citation 3, USA). Hemolysis ratio was calculated by using Equation

$$Hemolysis\ ratio(\%) = \frac{OD\ (test) - OD\ (negative)}{OD\ (positive) - OD\ (negative)} \times 100\%$$

where OD (test), OD (negative), and OD (positive) were the OD_{545} values of samples, negative control, and positive control, respectively. The morphologies of RBCs treated at the concentration of 1 mg mL^{-1} were also imaged by CLSM.

The GM2/pDNA complexes were injected to the mice of each group through tail vein. At different points in time after injection, blood was taken from the tail. RBCs are stained by suspending them in 1 ml of PBS containing 10^{-5} M of TO and incubating this mixture for 15 min in the dark at room temperature. The Hemolysis ratio was measured by using flow cytometry [S1].

***In Vivo* Tumor Inhibition Assay**

Animal studies were approved by Ethical Committee of Chinese Academy of Medical Sciences (CAMS). To evaluate *in vivo* cancer inhibitory activities of pCas9-survivin mediated by GM2, subcutaneous tumor-bearing mice with A549 were established. In details, 2×10^6 A549 cells were inoculated subcutaneously into the backside per nude mouse (n = 3 per group). When A549 tumors reached 150-200 mm^3, the mice were treated with 150 μL of GM2/pDNA, GM2/pCas9-survivin (containing 25 μg of pCas9-survivin) via intratumor injection every 2 days. The

GM2/pCas9-survivin complexes were prepared at the N/P ratio of 25. Mice weights and tumor volumes were measured every 2 d. After 14 d, all mice were sacrificed, and the final tumors were harvested, imaged, and weighted. The main organs including hearts, livers, spleens, lungs, and kidneys were also harvested and kept in 4% formaldehyde solution for later histological analysis. The final tumors and organs were sliced for the histological analysis. Hematoxylin and eosin were used as staining agents to investigate the damage of A549 cells and toxicity *in vivo*. For immunological analysis, Survivin rabbit monoclonal antibody and CRISPR-Cas9 mouse monoclonal antibody were respectively used to detect protein expressions in the tumor tissues.

Statistics Analysis:

Each experiment was repeated at least three times, where data are shown as means ± S.D. Statistical analysis was tested using Student's t-test. If more than two groups were compared, evaluation of significance as performed using one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test. Statistical significance was set at $*p < 0.05$

Reference

[S1] M. Makler, .L Lee and D. Recktenwald, *Cytometry,* 1987, **8**, 568-570.

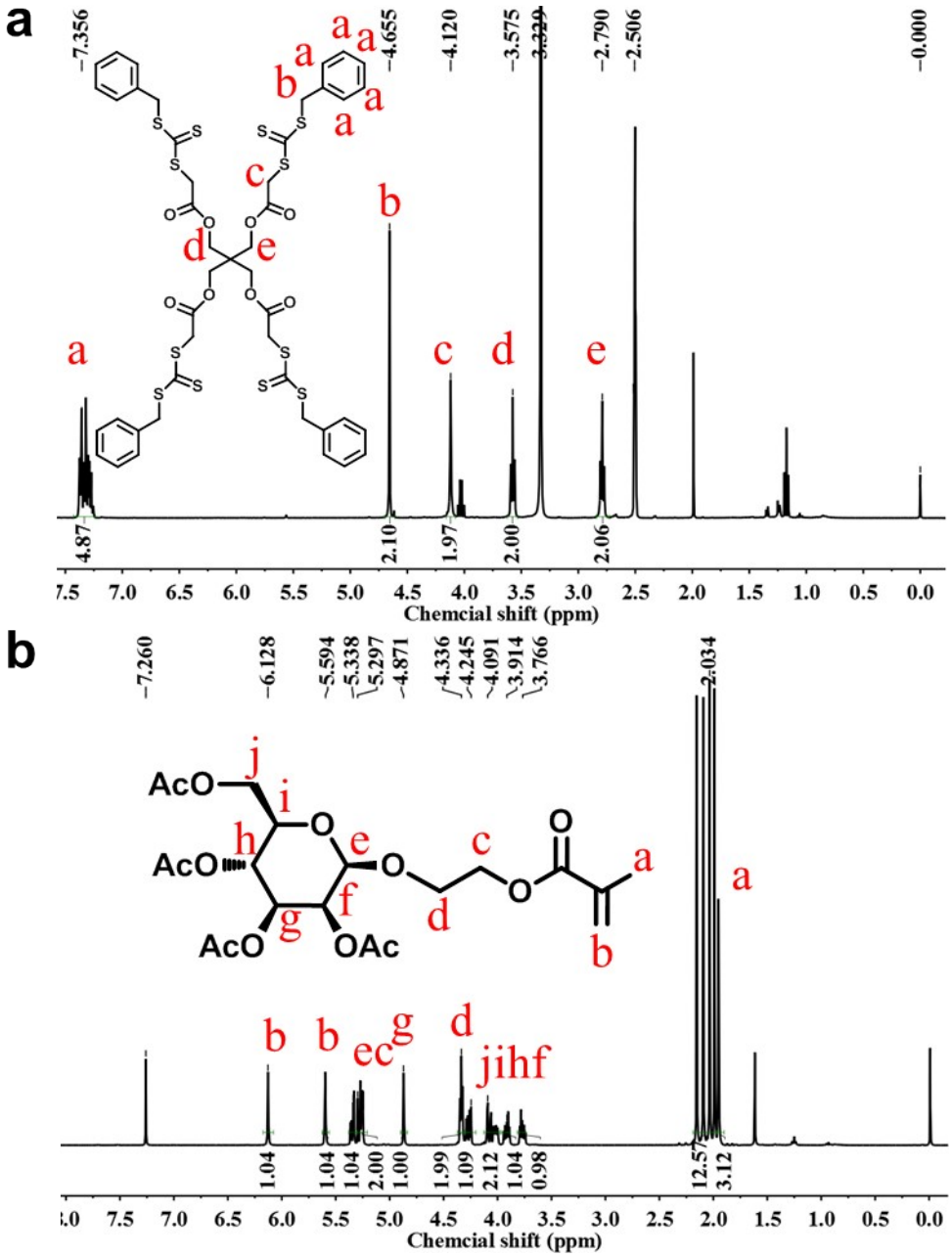


Figure S1. ^{1}H NMR spectra of BSPER in DMSO-d_6, AcManMA in $CDCl_3$.

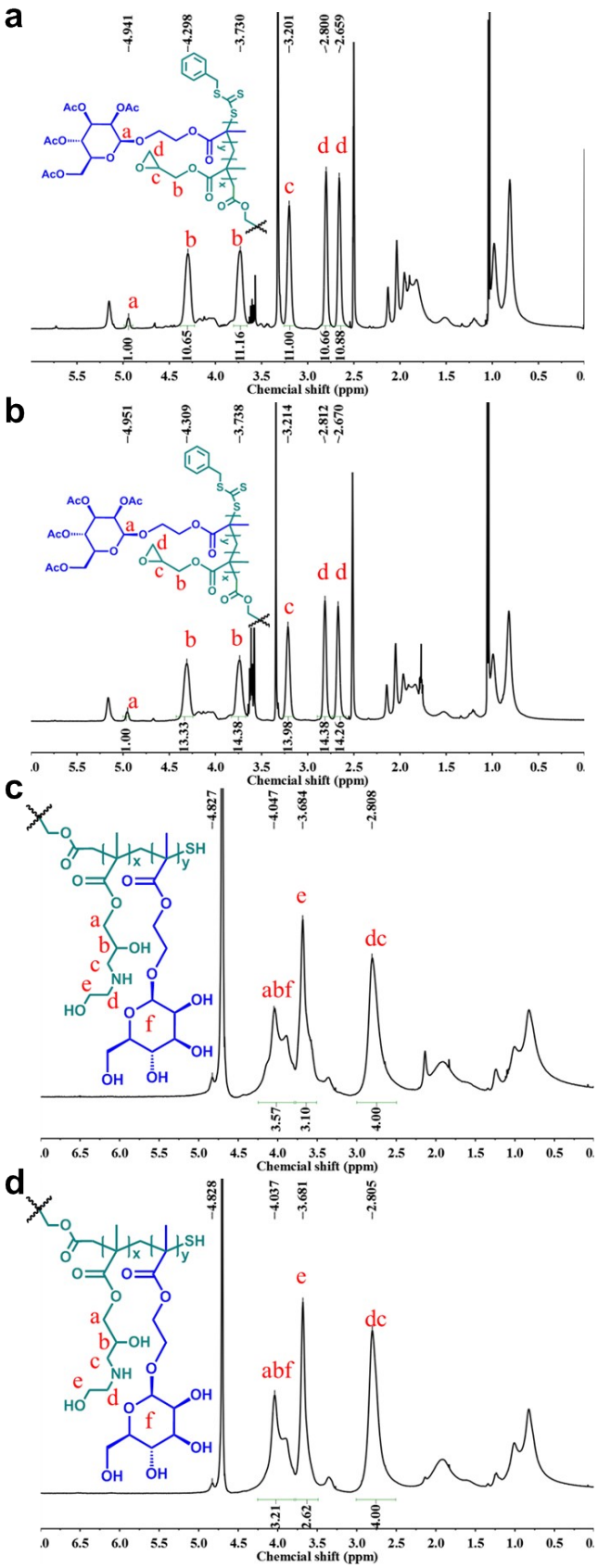


Figure S2. ^{1}H NMR spectra of a) GA1and b) GA2 in DMSO-d_6, c) GM1 and d) GM2

in D_2O.

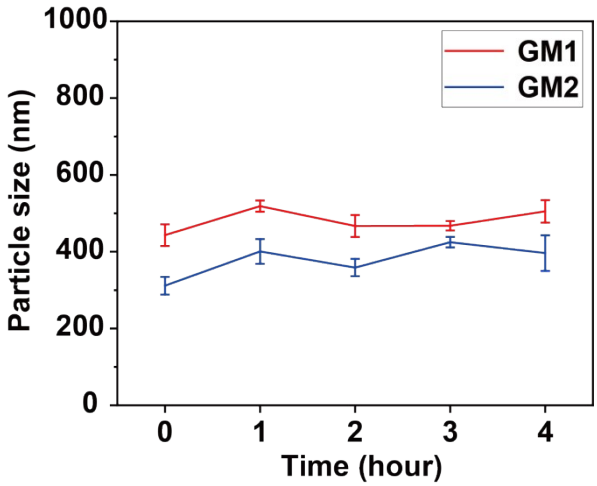


Figure S3. Particle size changes of different groups in 10% FBS for 4 h.

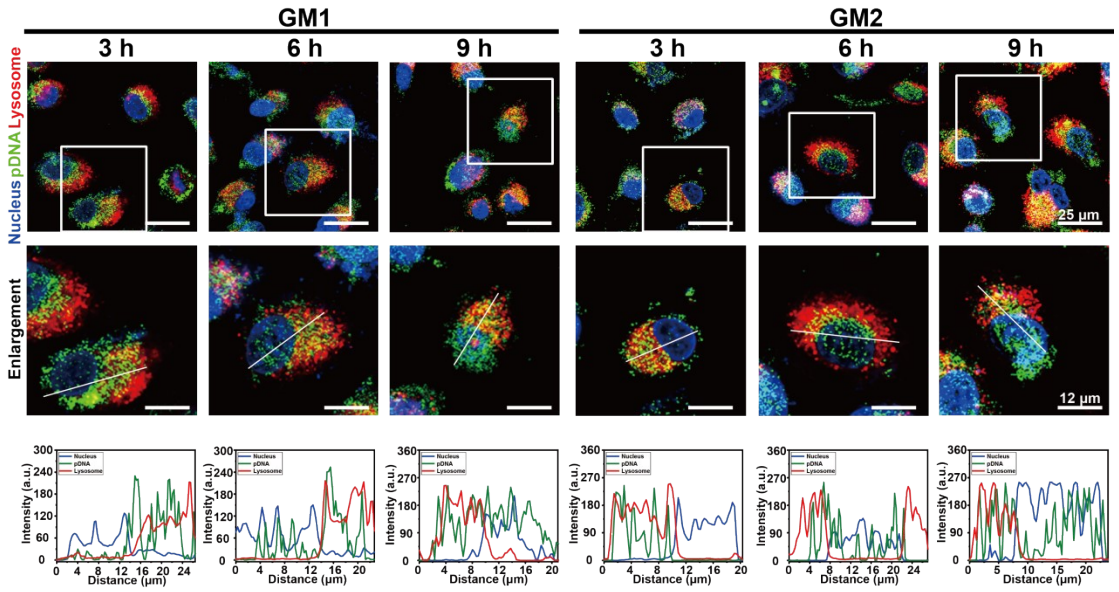


Figure S4. CLSM images of A549 cells treated with GM/pDNA under standard transfection condition at varied incubation time and fluorescence intensity of colocalization.

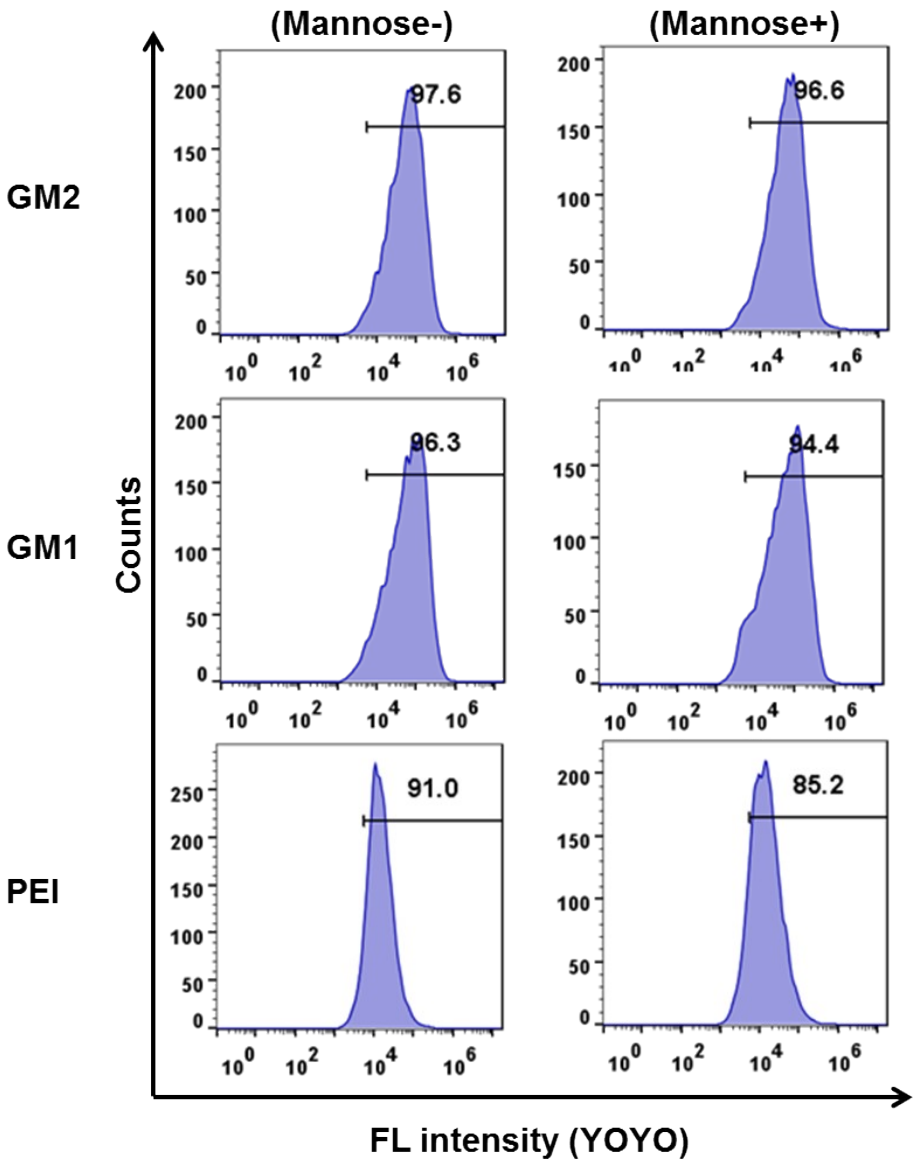


Figure S5. Flow cytometry of HEK 293 cells treated with GM2/pDNA or GM1/pDNA at the N/P ratio of 25 and PEI/pDNA at the N/P ratio of 10 in the absence (−) and presence (+) of mannose.

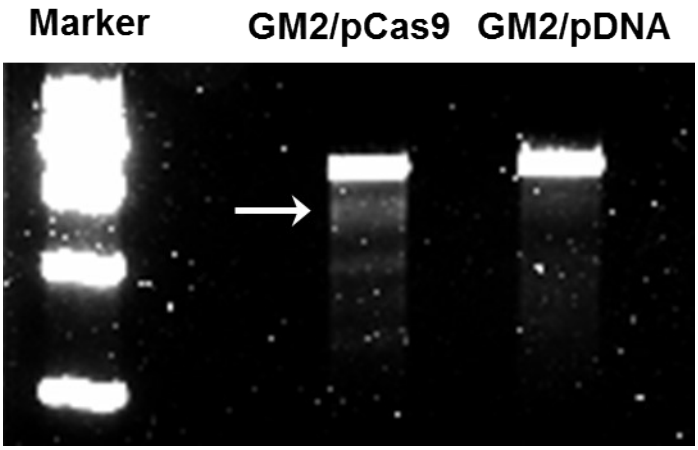


Figure S6. Cleavage detection of survivin in A549 cell lines treated with GM2/pCas9 and GM2/pDNA complexes.

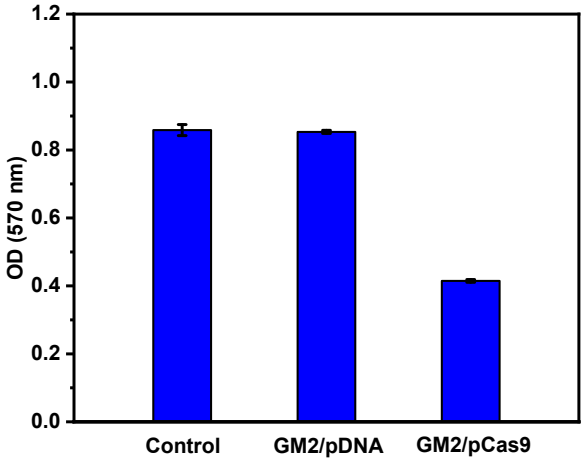


Figure S7. Statistical analysis of cloning formation assay in A549 cell lines.

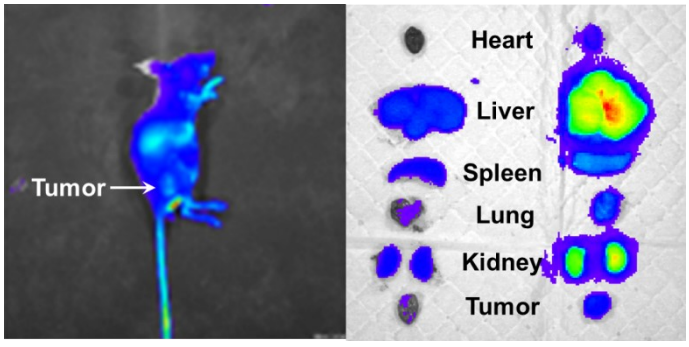


Figure S8. *In vivo* fluorescence image and *ex vivo* fluorescence image of A549 tumor-bearing nude mice after intravenous administration.

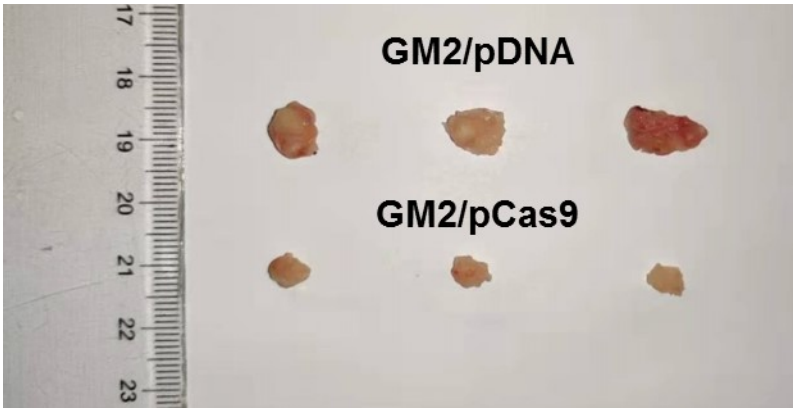


Figure S9. Tumor images after different treatment.

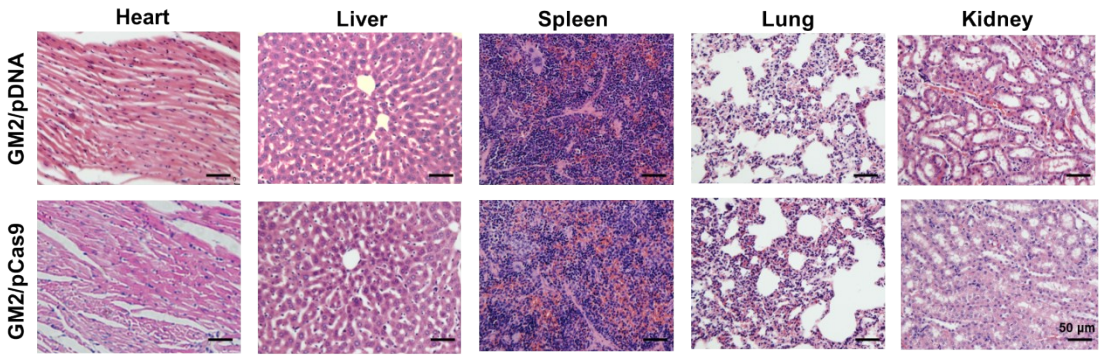


Figure S10. H&E staining images of various tissues from various treatment groups. (scale bar: 50 μm)