Supporting information of

A "self-accelerating endosomal escape" siRNA delivery nanosystem for significantly suppressing hyperplasia via blocking ERK2 pathway

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Agarose gel electrophoretic assay

To evaluate the ability of TRCs-Aco to protect siRNA against RNase degradation, agarose gel electrophoretic assay was performed. TRCs-Aco at 6/1/3 ratio was incubated with RNase for different time (0 min, 30 min, 60 min, 120 min). After digestion, sufficient heparin was added to dissociate the non-degraded siRNA from the delivery system and a UV illuminator was used to visualize the siRNA bond. Naked ERK2-siRNA was used as a control group.

Hemolysis Assay

1 mL human blood sample was freshly obtained from a healthy human donor in Hospital of the Armed Police and centrifuged at 3000 rpm for 5 min, and the supernatant was poured off. After washing until supernatant was clear, the packed cells were resuspended in 10 ml 0.01M PBS (pH =7.4). 20 μL of the diluted RBCs suspension was added to 1 mL of 0.5 mg/mL ERK2-siRNA delivery system suspension in PBS, and the mixture was incubated at 37 °C for 24 h. Meanwhile, a 20 μL amount of diluted RBC suspension incubated with 1 mL of PBS or 1 mL of ultra purified water was used as the negative or positive control, respectively. The absorbance value of the hemoglobin at 577 nm was measured with the reference wavelength of 655 nm. The percent of hemolysis was calculated as follows:

$$\text{Hemolysis}(\%) = \frac{\text{sample absorbance - negative control}}{\text{positive absorbance - negative control}} \times 100\%$$

Cell viability assay

3-(4,5-Dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) powder was dissolved in 0.01M PBS at 5 mg/mL concentration, and filtered through sterilized syringe filter (0.22 μm pore diameter). The prepared MTT stock solution was stored at 4 ℃. VSMCs (8×10^3 cells/well) were plated in 96-well plates and cultured in DMEM containing 10% FBS for 24 h to achieve 80% confluence. Then the medium was replaced by serum-free medium and cultured overnight. The cells were treated with appropriate concentration of ERK2-siRNA delivery materials and systems, and then incubated for 48 h. Then 100 μL of serum free medium with 0.5 mg/mL MTT was added, and followed by additional incubation of 4 h until purple color developed to detect the metabolically active cells. The medium was discarded and the cells were rinsed with PBS to remove remaining MTT. Finally, 100 μL of DMSO was added to each well to dissolve water insoluble formazan salt. The optical densities of each well were measured at 490 nm wavelength. Mean and standard deviation of triplicated were calculated and plotted. The relative cell viability was calculated as:

$$\text{relative cell viability}(\%) = \frac{OD_{490}(\text{sample})}{OD_{490}(\text{control})} \times 100\%$$

Cellular uptake and transmembrane mechanism study

Different Cy5-siRNA delivery systems were prepared to quantitatively evaluate their cellular uptake in VSMCs by a flow cytometry (Beckman MoFlo XDP, USA). Briefly, cells (2×10^5 cells/well) were plated in 6-well plate and transfected with these Cy5-siRNA delivery systems. After 4 h incubation, cells were washed three times with 0.01 M PBS (pH =7.4) and trypsinized with 0.25% trypsin. And then, cells were centrifuged

and resuspended in 300 μL PBS (pH = 7.4), then the suspension was analyzed with a flow cytometer. To investigate the pathways of cellular uptake, three endocytosis inhibitors were used, i.e., Chlorpromazine (CPZ, 30 μM), Amiloride hydrochloride (Amil, 50 μM) and Filipin Ⅲ (Filip, 5 μg/ml), to inhibit clathrin-mediated endocytosis, macropinocytosis and caveolae-mediated endocytosis, respectively. VSMCs were pretreated with each of the endocytosis inhibitors for 1 h and incubated with Cy5-siRNA delivery systems for 4 h at 37 ℃. The untreated cells were used as a control. Subsequently, the cells were washed three times with 0.01M PBS (pH = 7.4) and resuspended in 300 μL PBS (pH = 7.4) after trypsinization and centrifugation. The transfected cells were analyzed by a flow cytometry.

Intracellular trafficking study

The cells were seeded at 8×10^4 cells per well into a confocal dish and cultured for 24 h. Afterward, the cells were incubated with ERK2-siRNA delivery systems for 4 h in free serum medium to facilitate the cellular uptake. After that, the cells were further incubated with 10% serum-containing DMEM for another 24 h. Cells were washed with PBS twice, and added with prewarmed medium containing 0.5 mM Lyso Tracker Green to continue culture for another 1 h. Finally, the cells were washed twice with prewarmed PBS and stained with 2 μg/mL of Hoechst 33342 at room temperature for 20 min. The cells were then washed twice with PBS and observed by a confocal laser scanning microscopy (CLSM) at excitation wavelengths of 649, 504 and 350 nm for Cy5 (red), Lyso Tracker Green (green) and Hoechst 33342 (blue), respectively. The co-

localization rate (CLR) between Cy5-siRNA and lysosome was calculated by Image-Pro Plus 6.0 software according to the following equation.

$$Co-localization\ ratio = \frac{Number\ of\ yellow\ pixels}{Number\ of\ yellow,\ red\ and\ pink\ pixels} \times 100\% \quad \text{(for lysosome)}$$

Quantitative real-time PCR

Total RNA was extracted from transfected cells using TRIzol reagent and then reverse-transcribed into cDNA using the HiFiScript cDNA Synthesis Kit. The resulting cDNAs as templates were quantified via Ultra SYBR Mixture (low ROX) on 7500 Real Time PCR system according to the manufacturer's instructions. GAPDH was used as the reference gene for normalization of target gene. The forward and reverse primer sequences were 5'-GACAAGGGCTCAGAGGACTG-3' and 5'-ACGGCTCAAAGGAGTCAAGA-3' for ERK2, 5'-AGGTGAAGGTCGGAGTCAAC-3' and 5'-CGCTCCTGGAAGATGGTGAT-3' for GAPDH, respectively. All results are expressed as the means ± SD of three independent experiments.

Western blotting

Total protein was extracted from transfected cells using RIPA lysis buffer containing 1% volume of PMSF and quantified by BCA protein assay kit. Before measuring, the protein was denatured by adding 6 × SDS. All samples were separated by SDS-polyacrylamide gel electrophoresis and subjected to western blot. Membranes were

incubated with rabbit anti-ERK2 polyclonal antibody in TBST overnight and horseradish peroxidase conjugated anti-rabbit secondary antibody for 2 h. Membranes were scanned with the Tanon-5200 automatic chemiluminescence image analysis system and analyzed.

Cell migration assays

The transwell migration assay was performed to estimate the migration capability of the transfected cells. The operations of VSMCs with the treatment of different ERK2-siRNA delivery systems in 24-plate were described before. For transwell migration assay, the cells transfected 24 h were centrifuged, resuspended and then added to the upper chambers of inserts with a density of 1×10^5 cells per well. After 6 h of incubation, the migrated cells across the transwell were stained with violet crystal, photographed and counted to calculate the migration rate. By contrast, cells treated with naked ERK2-siRNA were performed as negative control.

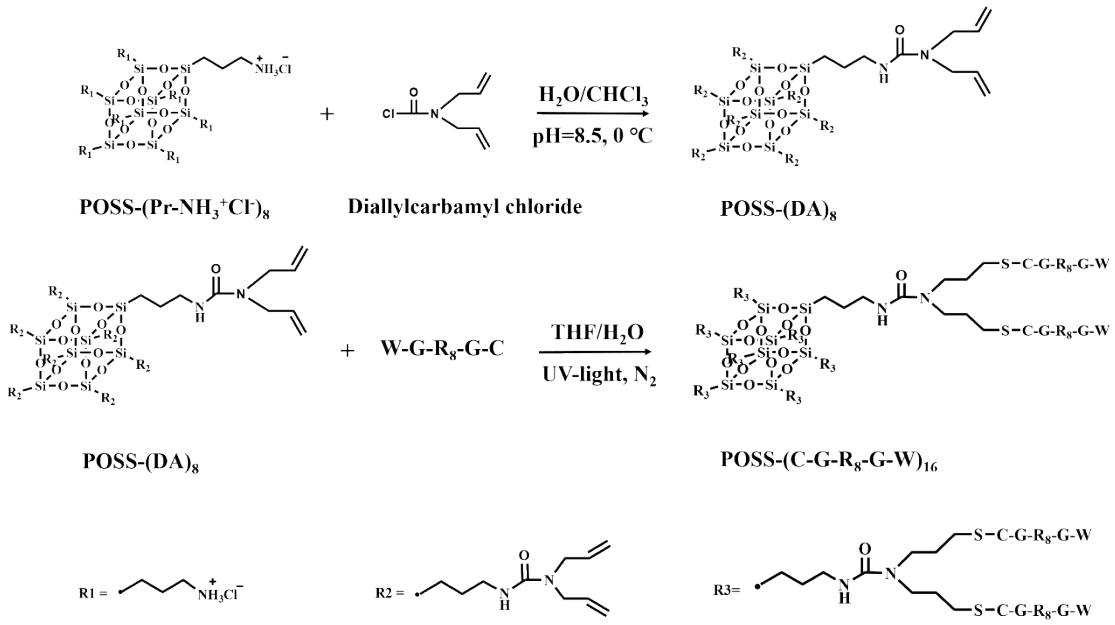

Figure S1. Synthesis pathway of POSS-(C-G-R_8-G-W)$_{16}$

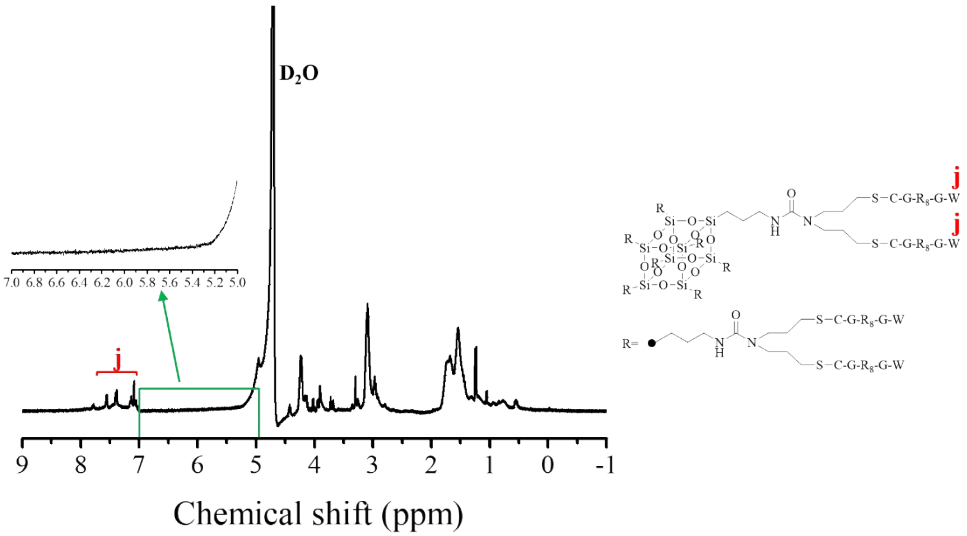

Figure S2. ^{1}H NMR spectra of POSS-(C-G-R_8-G-W)$_{16}$ in D_2O.

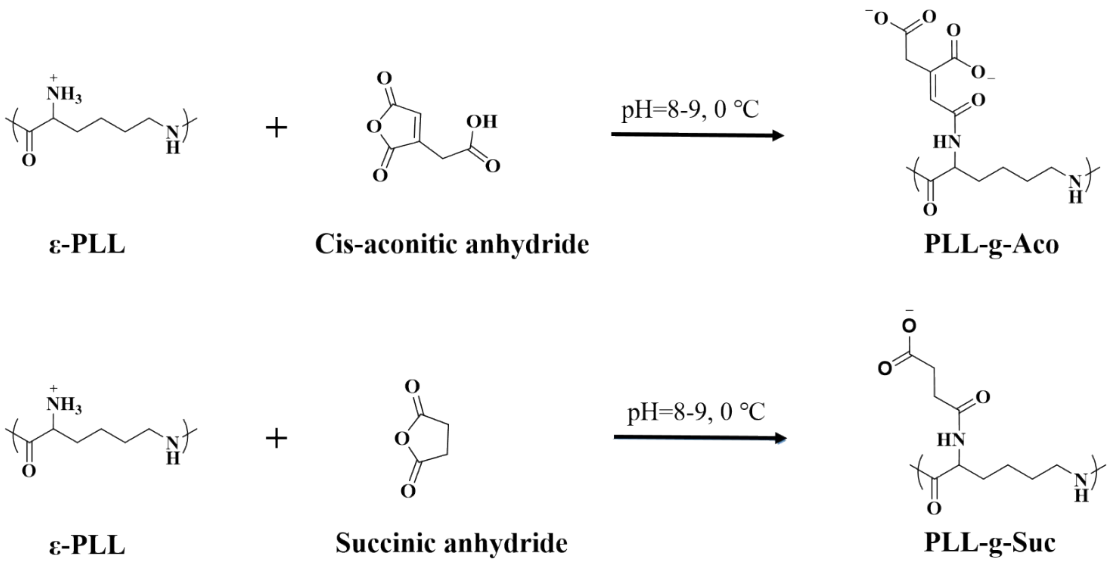

Figure S3. Synthesis pathway of PLL-g-Aco and PLL-g-Suc

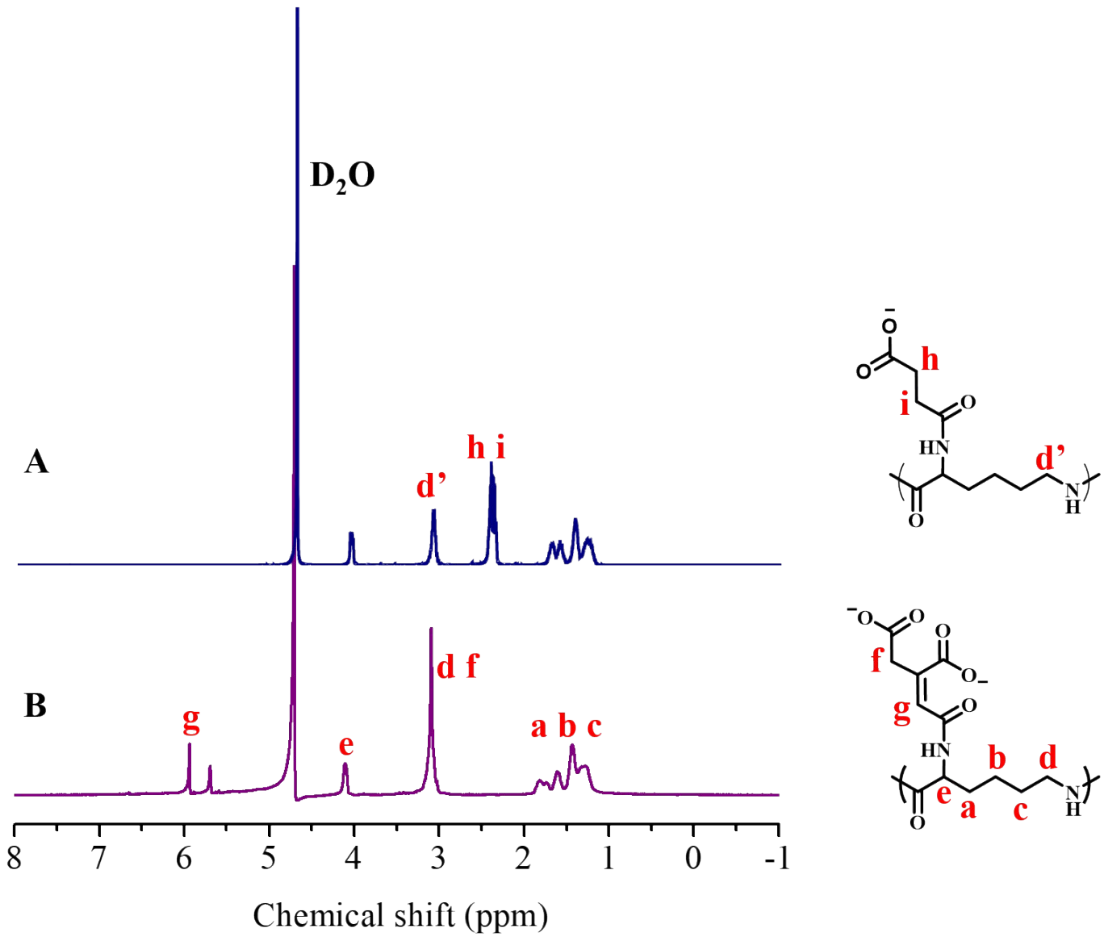

Figure S4. ^{1}H NMR spectra of (A) PLL-g-Suc and (B) PLL-g-Aco in D_2O.

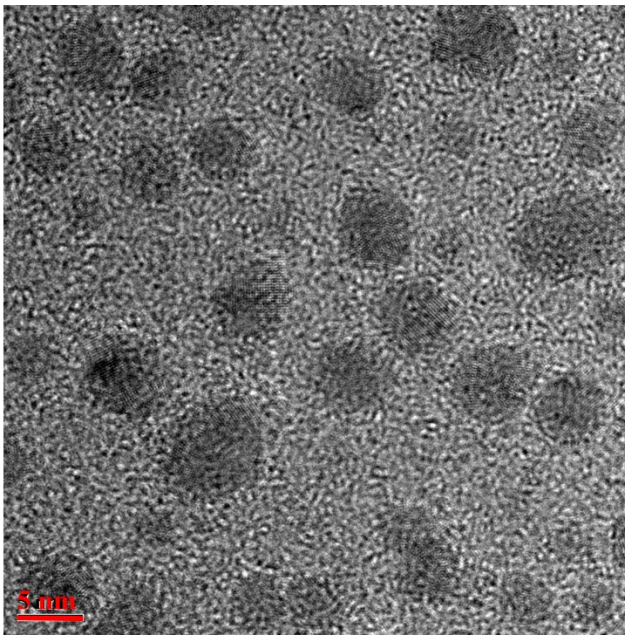

Figure S5. Morphology of POSS-(C-G-R_8-G-W)$_{16}$ in aqueous solution detected by

TEM.

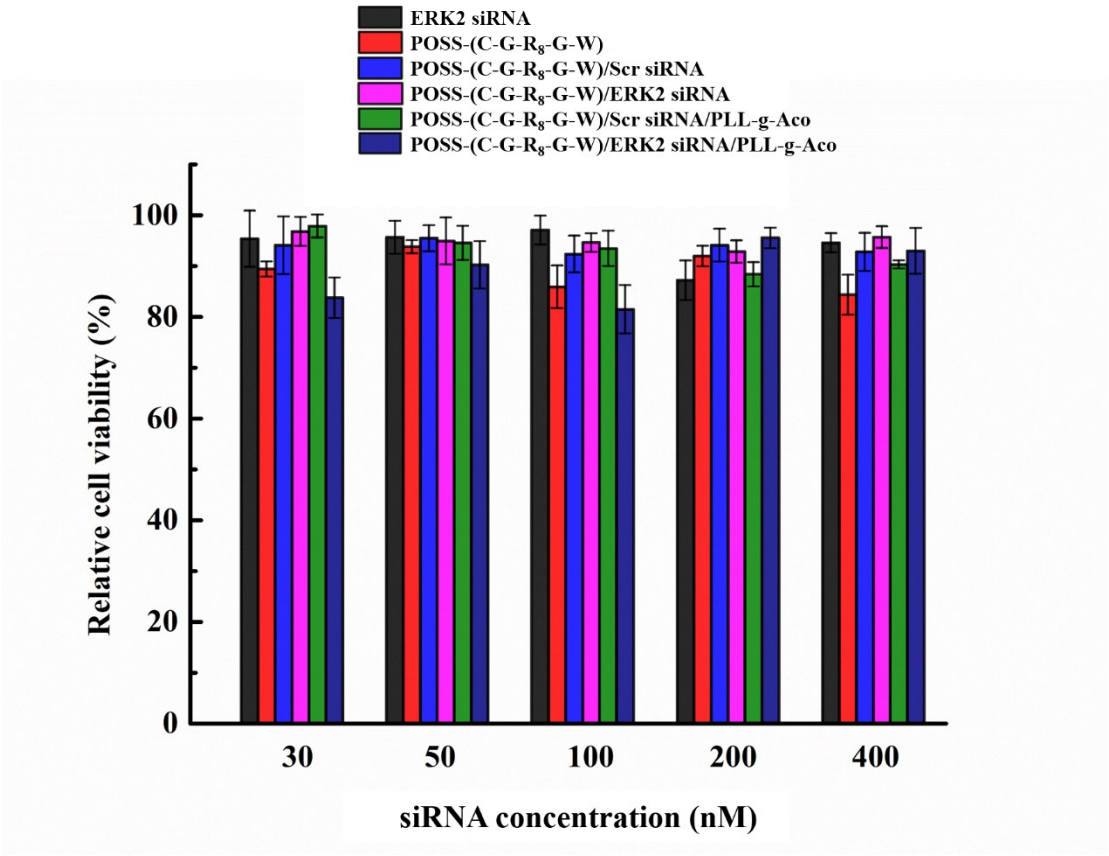

Figure S6. Relative cell viability of VSMCs treated by different concentrations of ERK2-siRNA delivery systems and incubated for another 48 h. Data were shown as mean ± SD (n = 3).

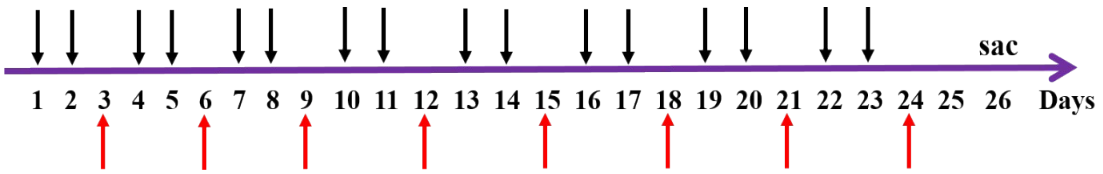

Figure S7. Injection scheme of bleomycin (black arrows) and treatments (red arrows).

Table S1. Comparison of in vitro experimental results of BRCs/siRNA complexes

In vitro experimental results	POSS-(C-G-R_8-G-W)$_{16}$/ERK2-siRNA	POSS-(C-G-R_8-G-W)$_{16}$/scrambled siRNA
ERK2 mRNA expression	0.85	1
ERK2 protein level	0.92	1
VSMCs migration per field (number)	0.80	1