Supplementary information

Silencing-mediated enhancement of osteogenic differentiation by supramolecular ternary siRNA polyplexes comprising biocleavable cationic polyrotaxanes and anionic fusogenic peptides

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S1. Characterization of DMAE-SS-PRX. DMAE-SS-PRX composed of *N*,*N*-dimethylaminoethyl carbamate (DMAE)-modified α-CDs as a cyclic molecule, cystamine-conjugated PEG (H_2N-SS-PEG-SS-NH_2, M_n = 4,910, M_w/M_n = 1.13, degree of polymerization = 103) as an axle polymer, and *N*-carbobenzoxy-L-tyrosine as a stopper molecule was synthesized according to our previous report.[1] ^1H nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance III 400 MHz spectrometer (Bruker BioSpin, Rheinstetten, Germany). The number of threading α-CDs in the PRX was calculated from the peak area between 3.1-4.0 ppm (-O-C***H₂***-C***H₂***-O- of PEG axle and H_2, H_3, H_4, H_5, and H_6 protons of the threaded α-CD) and 4.77-4.95 ppm (H_1 proton of the threaded α-CD) in the ^1H NMR spectrum of unmodified PRX (Fig. S1A). The number of DMAE groups modified onto the DMAE-SS-PRX was calculated from the peak area between 3.2 ppm (-NH-C***H₂***-CH_2-$N(CH_3)_2$ of the DMAE carbamate) and 4.8-5.3 ppm (H_1 proton of the threaded α-CD) in the ^1H NMR spectrum of DMAE-SS-PRX (Fig. S1B). The number of threading α-CDs and the number of modified DMAE groups in DMAE-SS-PRX were determined to be 21.9 and 78.0, respectively. The $M_{n,NMR}$ of DMAE-SS-PRX was calculated to be 36,500 based on the numbers of threaded α-CDs and DMAE groups determined by ^1H NMR.

S2. Stability of the complexes against polyanion exchange reaction. The stability of the DMAE-PRX/siRNA polyplexes and GALA/DMAE-SS-PRX/siRNA ternary polyplexes against polyanion exchange reaction was evaluated by gel electrophoresis.[1] In this experiment, heparin (Sigma-Aldrich, Milwaukee, WI, USA) was used as the polyanion. The DMAE-PRX/siRNA polyplex (N/P 10) and GALA/DMAE-SS-PRX/siRNA ternary polyplex solutions (N/P 10, Glu/P 0.2) were mixed with heparin solutions at various concentrations (1 to 1,000 μg/mL) (final siRNA concentration was 2.5 μM). The resulting solution was incubated at 37 °C for 1 h. Electrophoresis was then performed on a 2% agarose gel in TAE buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA) at 100 V for 10 min.

S3. Cell viability. MC3T3-E1 cells were plated in a 96-well plate at a density of 2.5×10^4 cells/cm^2 and incubated overnight. After the medium was replaced with fresh medium (90 μL), the treatment solutions (10 μL) were added and incubated for 48 h. For determining cell viability, Cell Counting Kit-8 reagent (Dojindo Laboratories, Kumamoto, Japan) (10 μL) was added to each well. After incubation for 1 h at 37 °C, the absorbance at 450 nm was measured using a Multiskan FC plate reader (Thermo Fisher Scientific, Waltham, MA, USA). The cellular viability was calculated relative to the untreated cells.

S4. Reference

1. A. Tamura and N. Yui, *Biomaterials*, 2013, **34**, 2480–2491.

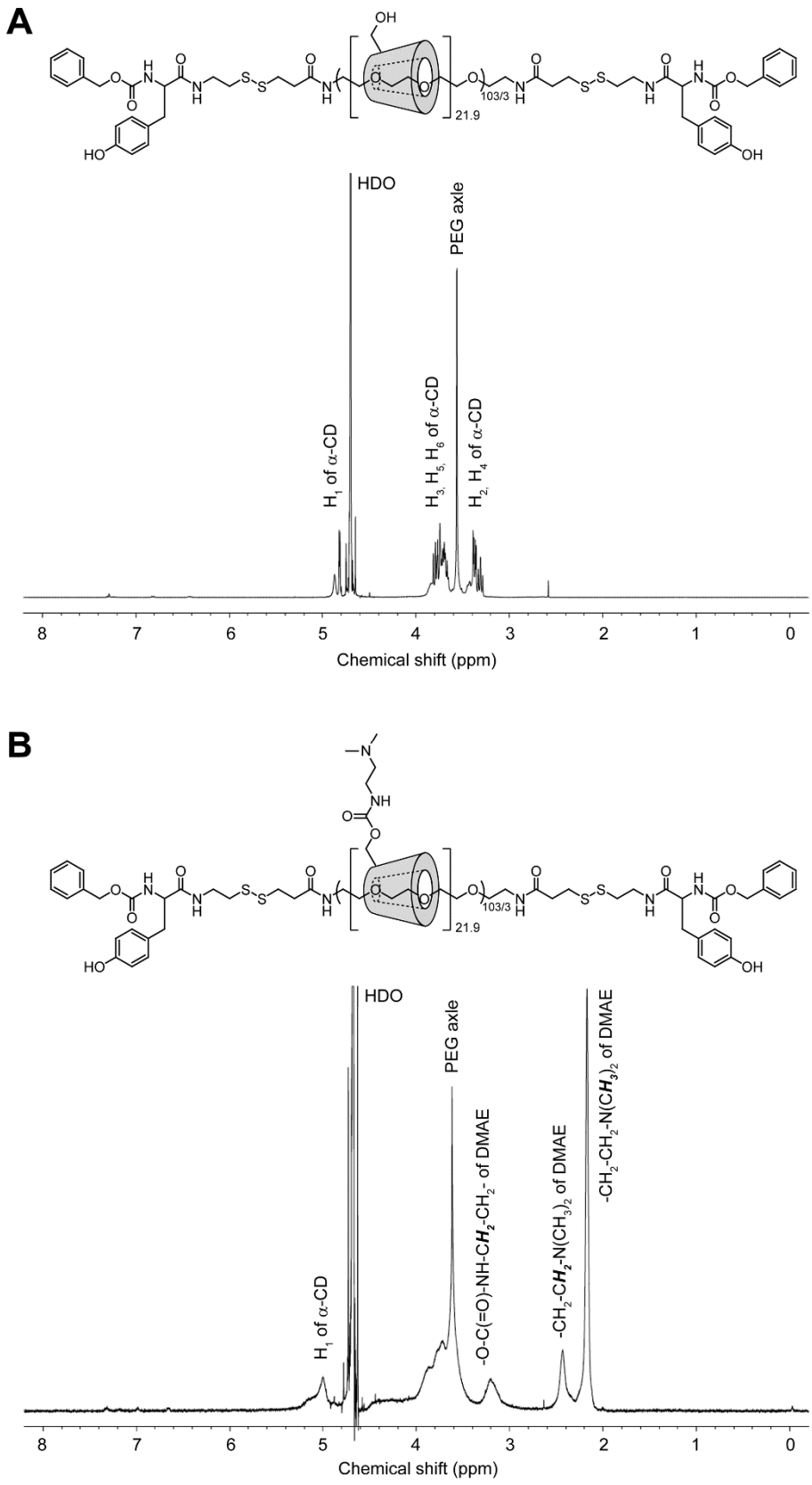

Figure S1. ^{1}H NMR spectra of unmodified PRX in NaOD/D_2O (A) and DMAE-SS-PRX in D_2O (B).

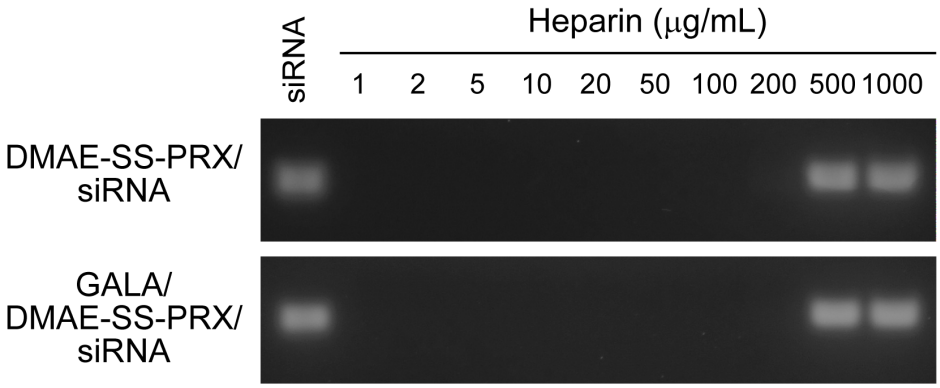

Figure S2. Release of siRNA from DMAE-SS-PRX/siRNA polyplex (N/P 10) and GALA/DMAE-SS-PRX/siRNA ternary polyplex (N/P 10, Glu/P 0.2) by the polyanion exchange with heparin.

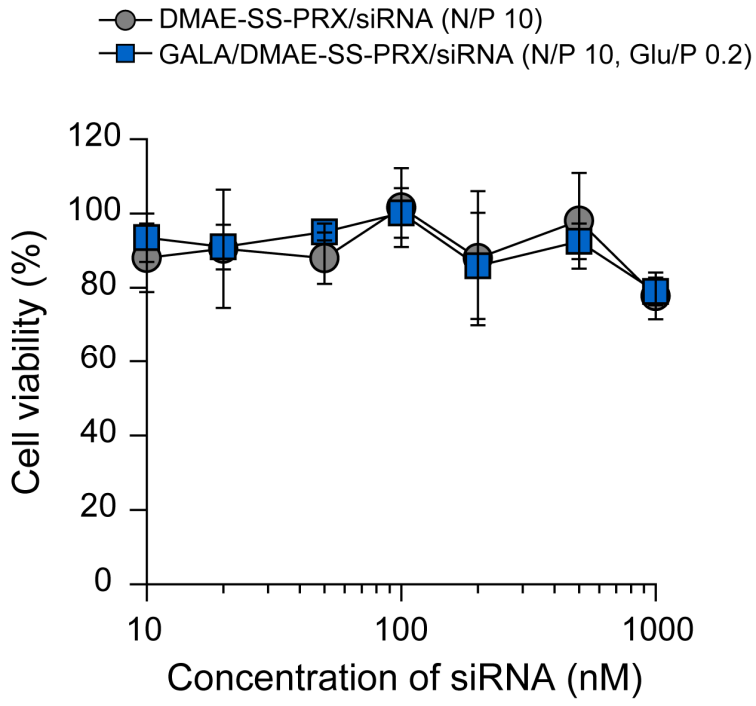

Figure S3. Viability of MC3T3-E1 cells treated with DMAE-SS-PRX/siRNA polyplex (N/P 10) and GALA/DMAE-SS-PRX/siRNA ternary polyplex (N/P 10, Glu/P 0.2) at various concentration of siRNA for 48 h. Data are expressed as the mean ± standard deviation (n = 5).