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Supporting information

Enhanced siRNA delivery of a cyclododecylated dendrimer compared to its linear derivative

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Experimental section

Materials

Generation 5 (G5) polyamidoamine (PAMAM) dendrimer with an ethylenediamine core and surface primary amine groups was purchased from Dendritech, Inc. (Midland, MI). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was purchased from Sangon Biotech Co., Ltd (Shanghai, China). Cell lysis buffer was obtained from Promega (Madison, WI). Lipofectamine 2000 (Lipo 2000) was purchased from Invitrogen (Carlsbad, CA). FITC-Annexin V apoptosis detection kit was purchased from BD biosciences (Shanghai, China). Rabbit monoclonal antibody against Bcl-2 was purchased from Cell Signaling Technology, Inc. (Danvers, MA). Mouse monoclonal antibody against β-actin was purchased from CMCTAG, Inc. (Milwaukee, WI). The siRNA targeting firefly luciferase (siLuc) (sense: 5'-CCCUAUUCUCCUUCUUCGCdTdT-3'), siRNA targeting Bcl-2 (siBcl-2) (sense: 5'-GUACAUCCAUUAUAAGCUGUCdTdT-3'), scrambled siRNA non-specific to any human gene (siNC), and fluorescently labeled siLuc (with a FAM conjugated at the 5'end of siLuc, siLuc-FAM) were synthesized by Gene Pharma Co. Ltd. (Shanghai, China). Cyclododecyl isocyanate was obtained from Sigma-Aldrich (St. Louis, MO). Dodecyl isocyanate was obtained from J&K Scientific (Shanghai, China). G5 PAMAM dendrimer was received in methanol solution and the solvent was distilled before use. All other chemicals were used as received without further purification.

Synthesis of cyclododecylated and dodecylated dendrimers

Cyclododecyl isocyanate and G5 PAMAM dendrimer were dissolved in dimethyl sulfoxide at a molar ratio of 48:1. The mixture was stirred at room temperature for 24 h and extensively dialyzed against dimethyl sulfoxide and double-distilled water (MWCO 3500 Da). This product was lyophilized and dissolved in dimethyl sulfoxide at 10 mg/mL. The stock solution was used in further characterization and gene silencing experiments. Dodecylated dendrimer was synthesized in the same way as described above. All the synthesized materials were characterized by ^{1}H-NMR in dimethyl sulfoxide-d6 (Varian 699.804 MHz).

Preparation and characterization of dendrimer/siRNA complexes

The cyclododecylated dendrimer was mixed with 0.5 μg siRNA at different weight ratios. The mixtures were incubated for 30 min at room temperature. The size and zeta potential of the

complexes were determined by dynamic light scattering (Zetasizer Nano ZS90, Malvern). Transmission electron microscopy (TEM) (HT7700, HITACHI, Japan) was further used to examine the size, as well as the morphology of the polyplexes. Nucleic acid binding capability of the cyclododecylated dendrimer was evaluated by an agarose gel (Biowest, Spain) retardation assay. The cyclododecylated dendrimer /siRNA polyplexes were run on an agarose gel at 100 V (1.5% w/v gel, 15 min). The ethidium bromide (EB) stained gel was visualized by a UV illuminator and the bands were photographed using an UVIpro Gel documentation system (Tanon-2500, China).

The interaction between modified dendrimers and siRNA was further characterized using an EB displacement assay. Generally, 1 mL of EB solution (0.5 mg L^{-1}) was mixed with 10 μL of siRNA solution (0.5 mg mL^{-1}). The cyclododecylated dendrimer was then added to the mixtures. The solutions were kept at room temperature for 10 min before measurement by a fluorescence spectrophotometer (excitation at 470 nm and emission at 500 - 700 nm).

Cell culture

HeLa (a human cervical carcinoma cell line, ATCC) and HeLa-Luc (HeLa cells stably expressing firefly luciferase) cells were cultured with DMEM containing 10% fetal bovine serum (FBS), 100 U mL^{-1} of penicillin, and 100 mg mL^{-1} of streptomycin at 37 ^{o}C and 5% CO_2. The cells were seeded in 24-well plates at a density of 10^4 cells per well and cultured for 24 h before gene silencing experiments.

Gene silencing experiments

0.5 μg siRNA (siLuc or siBcl-2) in diethylpyrocarbonate-treated water (1 O.D. siRNA dissolved in 125 μL water) was mixed with cyclododecylated dendrimer. The dendrimer/siRNA complexes were diluted with 100 μL of serum-free DMEM and equilibrated for 30 min at room temperature, further diluted with 150 μL serum-free medium. The cells were then incubated with the solutions for 6 h and replenished with 500 μL of medium containing 10% FBS. The gene silencing experiments were further continued for different periods (18 h for luciferase gene silencing and 42 h for Bcl-2 gene silencing).

The luciferase enzyme activity in the cells was analyzed according to the manufacturer's protocols (Promega, Madison, WI). Protein concentration in each well was determined using a BCA Protein Assay Kit (TIANGEN, China). The data were normalized to relative luciferase light unit per mg of protein (RLU per mg of protein), and relative to that of untreated cells. Commercial transfection

reagent Lipofectamine 2000 and dodecylated dendrimer were tested as controls. Three repeats were conducted for each material.

The efficacy of Bcl-2 knockdown was characterized at both mRNA and protein level. The mRNA expression level of Bcl-2 in the HeLa cells was analyzed by Real-time reverse transcription quantitative PCR (RT-qPCR). Total RNA isolated from the transfected HeLa cells was reverse-transcribed into cDNA using a cDNA Synthesis Kit (TOYOBO, Osaka, Japan). The cDNA was subjected to RT-qPCR analysis targeting Bcl-2 (Bcl-2-forward: 5'-AACATCGCCCTGTGGATGAC-3'; Bcl-2-reverse: 5'-AGAGTCTTCAGAGACAGCCAGGAG-3') using an SYBR Green Real time PCR Master Mix (TOYOBO, Osaka, Japan). The data were normalized to 18S as the endogenous reference (18S-forward: 5'-GGACACGGACAGGATTGACA-3'; 18S-reverse: 5'-GACATCTAAGG GCATCACAG-3'). To examine the Bcl-2 knockdown efficacy at the protein level, 60 μg total protein per lane were separated on 10% SDS-PAGE gels and transferred onto a PVDF membrane. The membrane was incubated with rabbit monoclonal antibodies against Bcl-2 overnight at 4 ^{o}C, and further incubated with IRDye 800 donkey anti-rabbit (Li-COR, USA) for 1 h. The protein bands were visualized using an Odyssey CLx infrared imaging system (LI-COR, USA). β-Actin was used as the loading control. Furthermore, cell apoptosis caused by Bcl-2 knockdown was evaluated quantitatively with flow cytometer (BD FACSCalibur, San Jose). The transfected cells were re-suspended in FITC binding buffer and stained by FITC Annexin V and propidium iodide (PI) for 15 minutes at room temperature in the dark. The fluorescence intensity of treated cells in each well was quantitatively analyzed using flow cytometry.

Uptake of dendrimer/siRNA complexes

HeLa-Luc cells were plated in a 24-well plate and incubated with complexes containing the modified dendrimers and siLuc-FAM (1 O.D. siLuc-FAM dissolved in 125 μL diethylpyrocarbonate-treated water) for 4 h. The cells were trypsinized and washed twice with PBS buffer. The mean fluorescence intensity of transfected cells was quantitatively analyzed by flow cytometry. Three repeats were conducted for each sample.

The cellular uptake of dendrimer/siLuc-FAM complexes was further investigated by confocal microscopy. After incubated with the complexes for 4 h, the cells were stained with 4, 6-diamidino-2-phenylindole (DAPI, 1 mg mL^{-1}) and observed using a confocal laser scanning microscope (Leica

SP5, Germany, excitation at 480 nm and emission at 520 nm).

Cytotoxicity analysis

Cytotoxicity of the cyclododecylated dendrimer on HeLa-Luc cells was performed by the MTT assay. Generally, HeLa-Luc cells were seeded in 96-well plates at a density of 10^4 cells per well and incubated overnight. Cyclododecylated dendrimer solution at different concentrations was incubated with the cells in 96-well plates for 24 h. Dodecylated dendrimer was treated in the same way. Five repeats were conducted for each sample.

Hemolysis assay

Hemolytic toxicity of the cyclododecylated dendrimer was test by counting haemoglobin which are released from red blood cells (RBCs). The blood was drawn from BALB/c mice obtained from SLAC Laboratory Animal Co. Ltd. (Shanghai, China). The experiment was conducted according to the regulations of Association for Assessment and Accreditation of Laboratory Animal Care in Shanghai. The red blood cells were isolated form the blood by centrifugation at 2000 rpm for 5 min and washed with PBS for three times. Each sample was incubated with red blood cells suspension (2%) diluted with PBS at 37 °C for 1 h. PBS and Triton X-100 (0.5%) were used as negative and positive controls, respectively. Finally, the solutions were centrifuged for 2000 rpm for 5 min and the supernatant of the samples was measured at 540 nm to obtain the absorbance values, respectively. The hemolysis ratio (%) was calculated as follows: (As – Anc) / (Apc – Anc) × 100, where As, Anc, and Apc represented the absorbances of samples, negative and positive controls, respectively. Dodecylated dendrimer was treated in the same way.

Statistical analysis

The data were presented as mean ± standard deviation. Student's t-test was used to analyze statistically significant differences. p-Value less than 0.05 was considered significant.

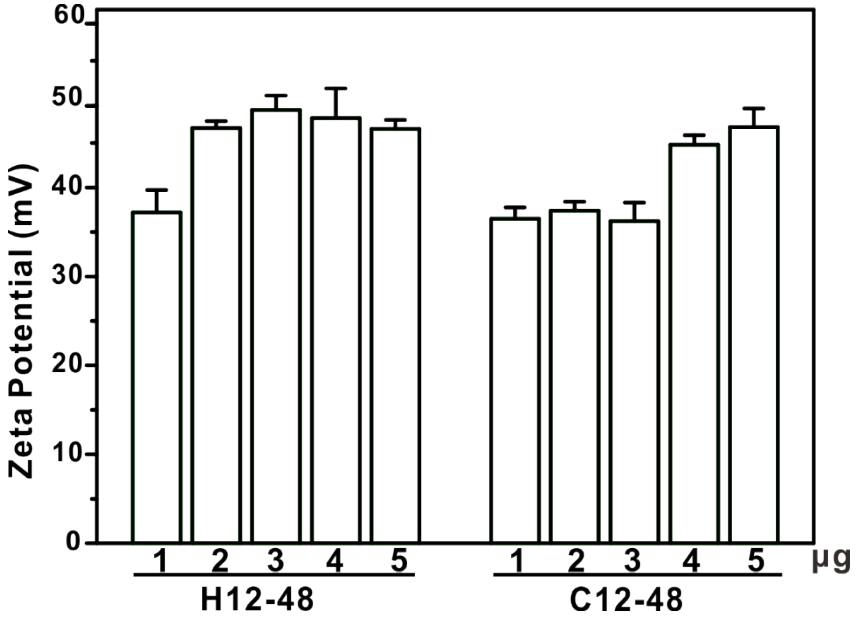
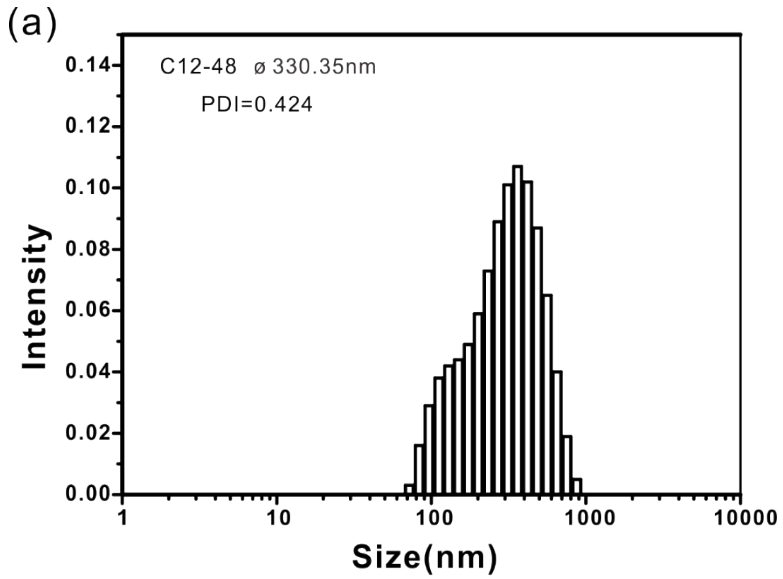


Fig. S1. Zeta potential of H12-48/siLuc and C12-48/siLuc complexes. 0.5 μg siRNA was used in each sample.



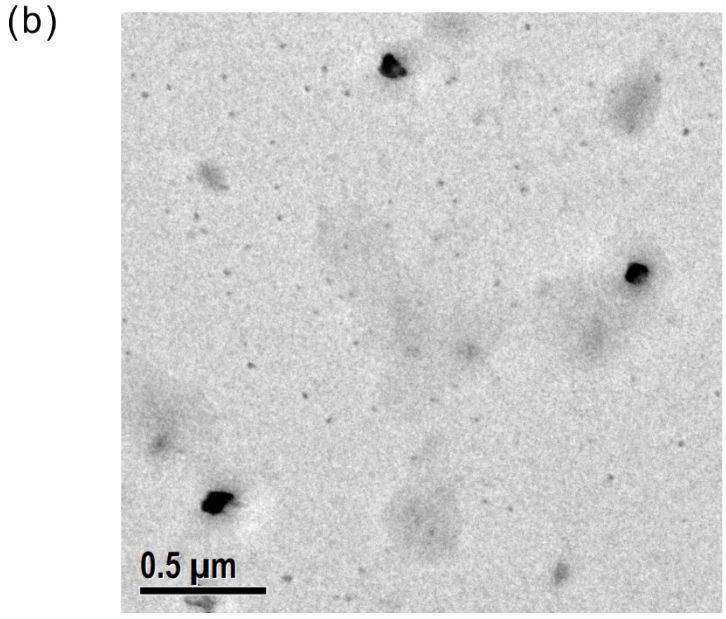


Fig. S2. Hydrodynamic size (a) and TEM images (b) of C12-48/siLuc complexes. The dose of C12-48 was 2 μg. 0.5 μg siRNA was used in each sample.

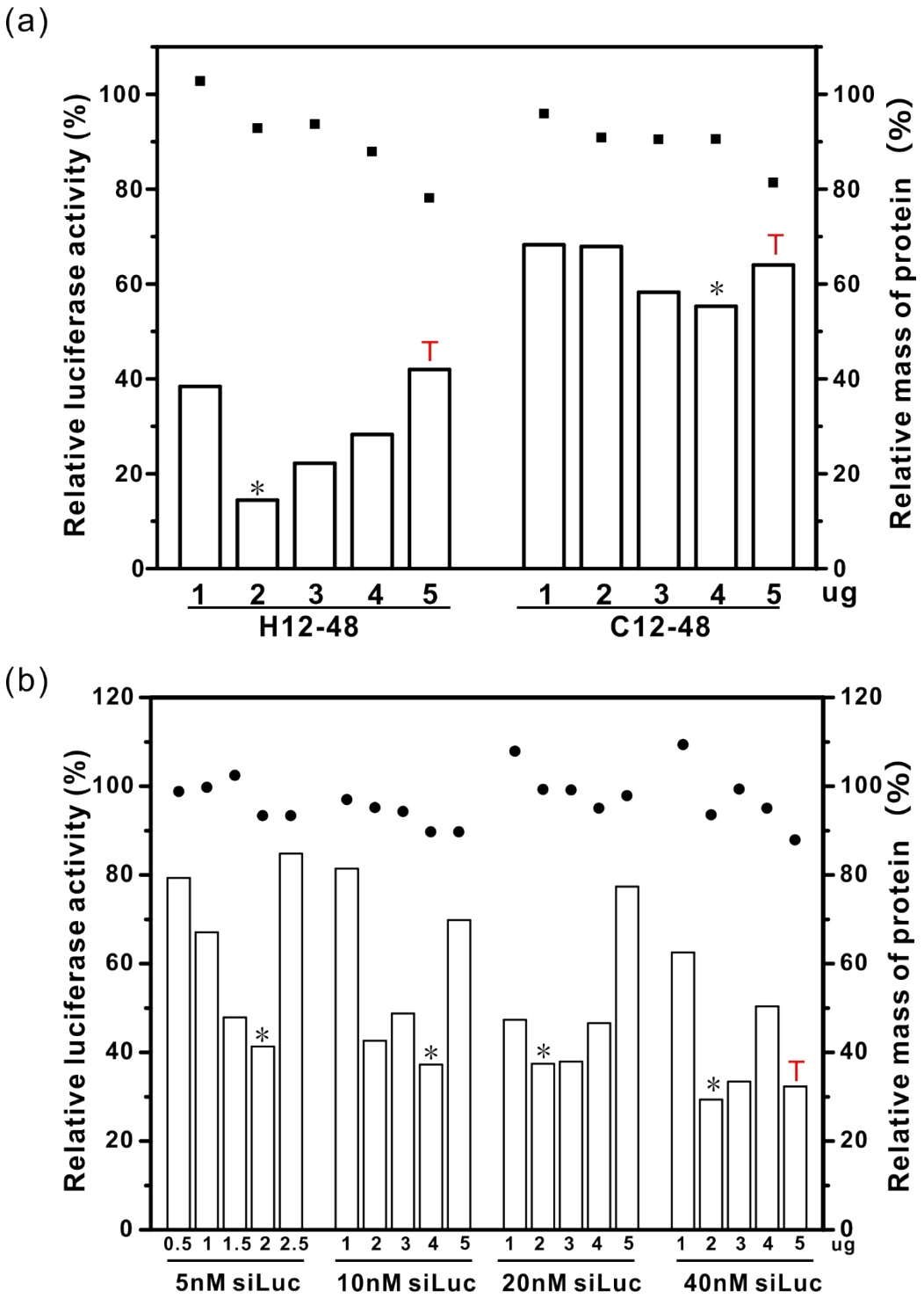


Fig. S3. (a) Gene silencing efficiencies of H12-48 and C12-48 with 50 nM siLuc in HeLa-Luc cells. (b) Gene silencing efficiencies of H12-48 with varied siRNA concentrations from 5 nM to 40 nM siLuc in HeLa-Luc cells. *Represents the optimal gene silencing weight. ^{T} Represents toxicity on the transfected cells.

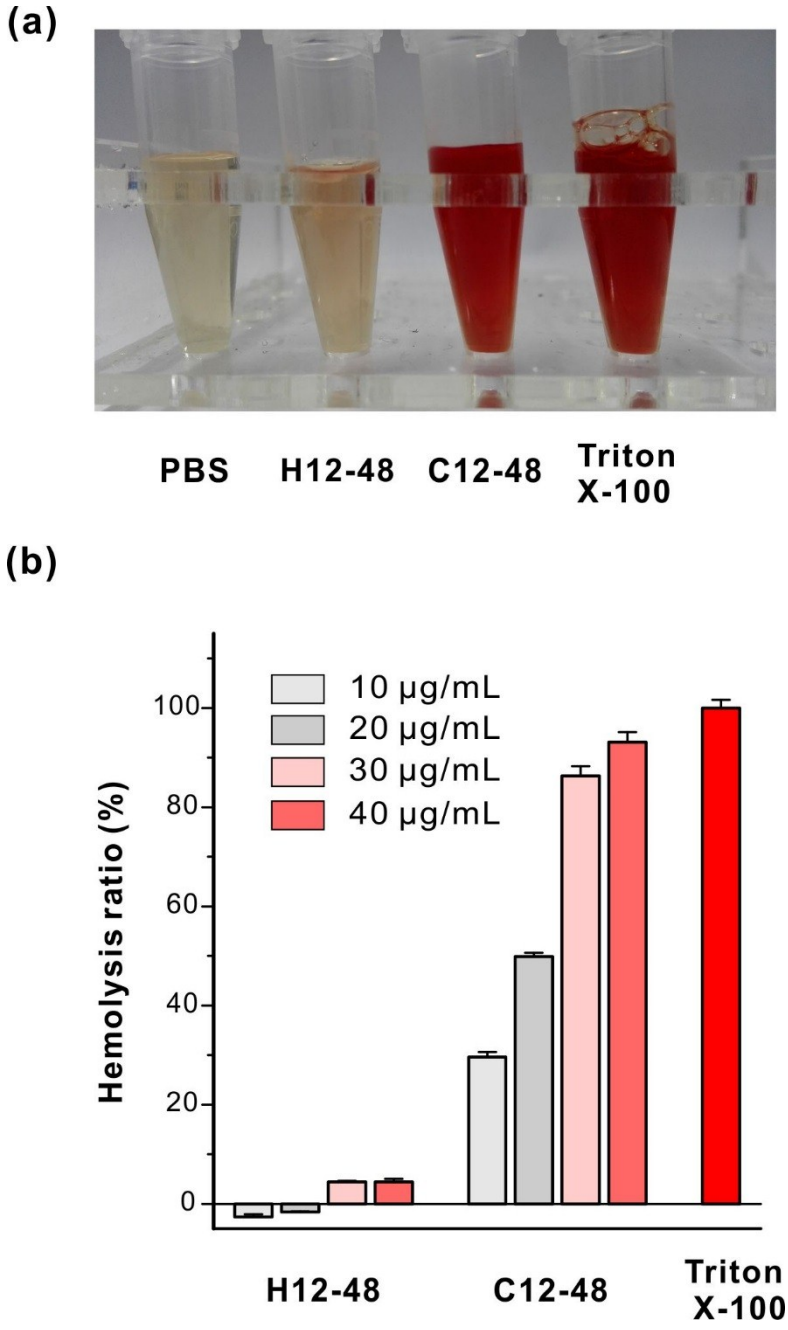


Fig. S4. (a) Photographs of supernatant of the red blood cells solution treated with H12-48 and C12-48 at 40 μg/mL. PBS and Triton X-100 (0.5%) were used as negative and positive controls, respectively. (b) Hemolysis ratio statistics of H12-48 and C12-48 at various concentrations. Triton X-100 (0.5%) were used as a positive control.