Electronic Supplementary Information

Optimized fluorodendrimer-incorporated hybrid lipid-polymer nanoparticle for efficient siRNA delivery

Qingqing Xiong,[ab] Yujing Li,[b] Kun Zhou,[c] Ping Chen,[a] Hua Guo,[d] Lu Chen,[a] Jianxun Ding,*[be] Tianqiang Song*[a] and Jinjun Shi[b]

[a] *Department of Hepatobiliary Cancer, Liver Cancer Center, Tianjin Medical University Cancer Institute & Hospital, National Clinical Research Center for Cancer; Key Laboratory of Cancer Prevention and Therapy, Tianjin; Tianjin's Clinical Research Center for Cancer; Tianjin 300060, P. R. China. E-mail: tjchi@hotmail.com*

[b] *Center for Nanomedicine and Department of Anesthesiology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA*

[c] *Vascular Biology Program, Boston Children's Hospital, Harvard Medical School, Boston, MA 02115, USA*

[d] *Department of Tumor Cell Biology, Tianjin Medical University Cancer Institute & Hospital, Tianjin 300060, P.R. China*

[e] *Key Laboratory of Polymer Ecomaterials, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun 130022, P. R. China. E-mail: jxding@ciac.ac.cn*

Experimental section

Regents and materials

Ethylenediamine-cored polyamidoamine generation 0 dendrimer (PAMAM-G0) and *N*,*N*-dimethylformamide (DMF) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 3-Perfluorobutyl-1,2-epoxypropane and 3-perfluorohexyl-1,2-epoxypropane were purchased from Tokyo Chemical Industry Co., Ltd. (Boston, MA, USA). (1,2-distearoyl-*sn*-*glycero*-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)]) with PEG molecular weight (M_w) of 3,000 Da (DSPE-PEG) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). Ester-terminated poly(D,L-lactide-*co*-glycolide) (PLGA, viscosity of 0.26−0.54 dL g^{-1}) was obtained from Durect Corporation (Cupertino, CA, USA). siRNA targeting luciferase (siLuc), siRNA targeting prohibitin 1 (siPHB1), and DY677-labelled siLuc were purchased from Dharmacon (Lafayette, CO, USA). The siRNA sequences are as follows: siLuc, 5'-CUU ACG CUG AGU ACU UCG AdTdT-3' (sense) and 5'-UCG AAG UAC UCA GCG UAA GdTdT-3' (antisense); and siPHB1, 5'-GCG ACG ACC UUA CAG AGC GUU-3' (sense) and 5'-CGC UCU GUA AGG UCG UCG CUU-3' (antisense). DY677 was labeled at the 5'-end of both the sense and antisense strand of siLuc. HyPure water (Hyclone, Molecular Biology Grade, DNase/RNase-free) was obtained from GE Healthcare Life Sciences (Logan, UT, USA).

HeLa cells stably expressing firefly luciferase (HeLa-Luc) were obtained from Alnylam Pharmaceuticals, Inc. (Cambridge, MA, USA). A549 cells were obtained from ATCC (Manassas, VA, USA). RPMI 1640 medium, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and trypsin were bought from Gibco (Grand Island, NY, USA).

Steady-Glo luciferase assay system was obtained from Promega (Madison, WI, USA). Resazurin sodium salt, Hoechst 33342, and bovine serum albumin (BSA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Cell lysis buffer (10×), phenylmethanesulfonyl fluoride (PMSF), and ECL system were obtained from Cell Signaling Technology (CST) (Danvers, MA, USA). LysoTracker Green DND-26, Lipofectamine 2000, Pierce™ protease inhibitor cocktail, and BCA protein assay kit were purchased from ThermoFisher Scientific (Waltham, MA, USA). Primary antibodies used in this study are as follows: anti-PHB1 (Abcam, ab70672, Cambridge, MA, USA), anti-caspase 3 (CST, 9665), anti-cleaved caspase 3 (CST, 9664), anti-poly(ADP ribose) polymerase (CST, 9532), anti-cleaved poly(ADP ribose) polymerase (CST, 5625), Bcl-2 (CST, 4223), and anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH) (CST, 5174). Annexin V-FITC/PI apoptosis detection kit was obtained from BD Biosciences (San Jose, CA, USA).

Synthesis and characterizations of fluorodendrimers

PAMAM-G0 was mixed with 3-perfluorobutyl-1,2-epoxypropane or 3-perfluorohexyl-1,2-epoxypropane at different molar ratios of 1:7, 1:5, and 1:3. The mixtures were then vigorous stirred at 90 °C for 48 h. The products were further purified by dialysis against methanol for 24 h and then deionized water for another 48 h. The dialysate was then recovered by lyophilization to obtain light yellow oily or powdery products. G0-C14, epoxytetradecane-modified G0 dendrimer, was synthesized by reacting 1,2-epoxytetradecane with PAMAM-G0 at a molar ratio of 7:1 as described previously.[1] The proton (^{1}H NMR) and carbon (^{13}C NMR) nuclear magnetic resonance spectra were recorded on a Varian Mercury

VX-300 spectrometer (Palo Alto, CA, USA) using DMSO-d_6 as a solvent.

Preparation and characterizations of siRNA-loaded hybrid lipid-polymer nanoparticles

The siRNA-loaded hybrid nanoparticles were prepared by a facile one-pot nanoprecipitation method. Briefly, PLGA, DSPE-PEG, and G0-C14 or G0-$(Fx)_y$ were firstly dissolved separately in DMF at concentrations of 5.0 mg mL^{-1}. PLGA (1.25 mg, 250.0 μL), and G0-C14 (0.25 mg, 50.0 μL) or G0-$(Fx)_y$ (equivalent moles with G0-C14) were mixed with 10.0 μL of siRNA (1.0 nmol) aqueous solution by pipetting. DSPE-PEG (0.5 mg, 100.0 μL) was then added into the mixture. Under vigorous stirring, the mixture was slowly added into 5.0 mL of HyPure water and then stirred for 5 min to stabilize the siRNA-loaded nanoparticles. The formed nanoparticles were then washed twice with ice-cold HyPure water using Amicon tubes (Molecular weight cut-off = 100.0 kDa; Millipore Co., Billerica, MA, USA) to remove the remaining organic solvents and free compounds, and finally concentrated into 0.5 mL of phosphate-buffered saline (PBS). The size and zeta potential of these nanoparticles were determined by dynamic light scattering technology using Brookhaven's NanoBrook 90Plus PALS instrument with a 15-mW laser incident beam of 676 nm (Long Island, NY, USA). The morphology was visualized by FEI Tecnai G2 Spirit Bio TWIN transmission electron microscope (TEM; FEI Company, Hillsboro, OR, USA) and imaged at 80 kV. The siRNA encapsulation efficiency (EE) was determined by loading DY677-labeled siLuc into the nanoparticles, and the fluorescence intensity (FI) was measured by a BioTek Synergy HT multimode microplate reader (Winooski, VT, USA) ($\lambda_{ex}/\lambda_{em}$ = 660/694 nm). Naked DY677-siLuc was used as a standard. The siRNA EE (%) was

calculated as the following equation:

$$\text{siRNA EE}\,(\%) = \frac{\text{FI}_{\text{sample}}}{\text{FI}_{\text{stadnard}}} \times 100\%$$

Cell culture

HeLa-Luc and A549 cells were respectively incubated in RPMI 1640 medium and DMEM supplemented with 10% (*V*/*V*) FBS at 37 °C in a humidified atmosphere containing 5% (*V*/*V*) carbon dioxide.

Luciferase silencing and cytotoxicity

HeLa-Luc cells were seeded into 96-well plates at a density of 3,000 cells per well and incubated at 37 °C for 24 h. The media were then replaced with fresh media containing different siLuc-loaded nanoparticles of various concentrations. After further incubation for 24 h, the cells were washed for luciferase detection or incubated in fresh media for another 24 or 48 h. The luciferase expression and cytotoxicity were respectively measured by Steady-Glo luciferase assay and resazurin (Alamar Blue) assay according to the protocols of manufacturers. The luminescence or fluorescence intensity ($\lambda_{ex}/\lambda_{em}$ = 545/590 nm) was then determined using BioTek Synergy HT multimode microplate reader (Winooski, VT, USA).

Cellular uptake

A549 cells were seeded into 12-well plates at a density of 50,000 cells per well and incubated at 37 °C for 24 h. Subsequently, the DY677-siLuc-loaded hybrid nanoparticles were added, and the cells were allowed to incubate for another 2 h. After removing the media,

the cells were washed, digested, and collected for flow cytometry analysis on a FACS Canto II flow cytometer (BD Biosciences, San Jose, CA, USA).

Endosomal escape

A549 cells were seeded into 35 mm glass-cover plates at a density of 40,000 cells per well and incubated at 37 °C for 24 h. Subsequently, NPF13-5/DY677-siLuc and NPC/DY677-siLuc were added individually, and the cells were allowed to incubate for another 2 h. After removing the media and washing with PBS twice, the cell endosomes and nuclei were stained with Lysotracker green and Hoechst 33342, respectively. The cells were then observed and imaged under a ZeissLSM710 confocal laser scanning microscope (CLSM) (Carl Zeiss, Jena, Germany).

Western blot

A549 cells were seeded into a 6-well plate at a density of 50,000 cells per well and cultured for 24 h. Subsequently, the cells were incubated with NPF13-5/siPHB1 for 24 h. After removing the media and washing with PBS twice, the cells were cultured in fresh media for another 48 h. After that, the cells were digested by trypsin, and the total proteins were extracted using cell lysis buffer supplemented with PMSF and protease inhibitor cocktail. The total protein concentration of the lysates was determined using the BCA protein assay kit. An equal amount of protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto poly(vinylidene fluoride) membrane (Millipore Co., Billerica, MA, USA), and blocked using 3% BSA at room temperature for 1 h. The

membranes with specific protein bands were separately incubated with specific primary antibodies at 4 °C overnight, followed by incubation with the corresponding second antibodies at room temperature for 1 h. The protein bands were detected using the ECL system, and the protein expression level was normalized against GAPDH expression.

Cell growth inhibition assay

A549 cells were seeded into 96-well plates at a density of 2,000 cells per well and cultured for 24 h. After that, the cells were respectively treated with NPF13-5/siPHB1 or NPF13-5/siLuc for 24 h. After removing the media, the cells were allowed to culture in fresh media for another 24 h or 48 h. Cell growth inhibition rate was determined by resazurin (Alamarblue) assay according to the manufacturer's protocol.

Cell apoptosis

A549 cells were seeded into a 6-well plate at a density of 60,000 cells per well and cultured for 24 h. After that, the cells were treated with NPF13-5/siPHB1 or NPF13-5/siLuc for 24 h at a siRNA dose of 20.0 nM. Then the media were removed, and the cells were cultured in fresh media for another 24 h. Subsequently, the cells were collected, washed with PBS, and the apoptotic cells were stained with Annexin V-FITC/PI apoptosis detection kit and then examined on FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA).

Statistical Analysis

All values in the figures are presented as means ± standard deviation (SD) of at least three independent experiments. A comparison between groups was using a two-tail Student's *t*-test. A *P* value of < 0.05 is considered statistically significant.

Notes and references

1 X. Xu, K. Xie, X. Q. Zhang, E. M. Pridgen, G. Y. Park, D. S. Cui, J. Shi, J. Wu, P. W. Kantoff, S. J. Lippard, R. Langer, G. C. Walker and O. C. Farokhzad, *Proc. Natl. Acad. Sci. U. S. A.*, 2013, **110**, 18638–18643.

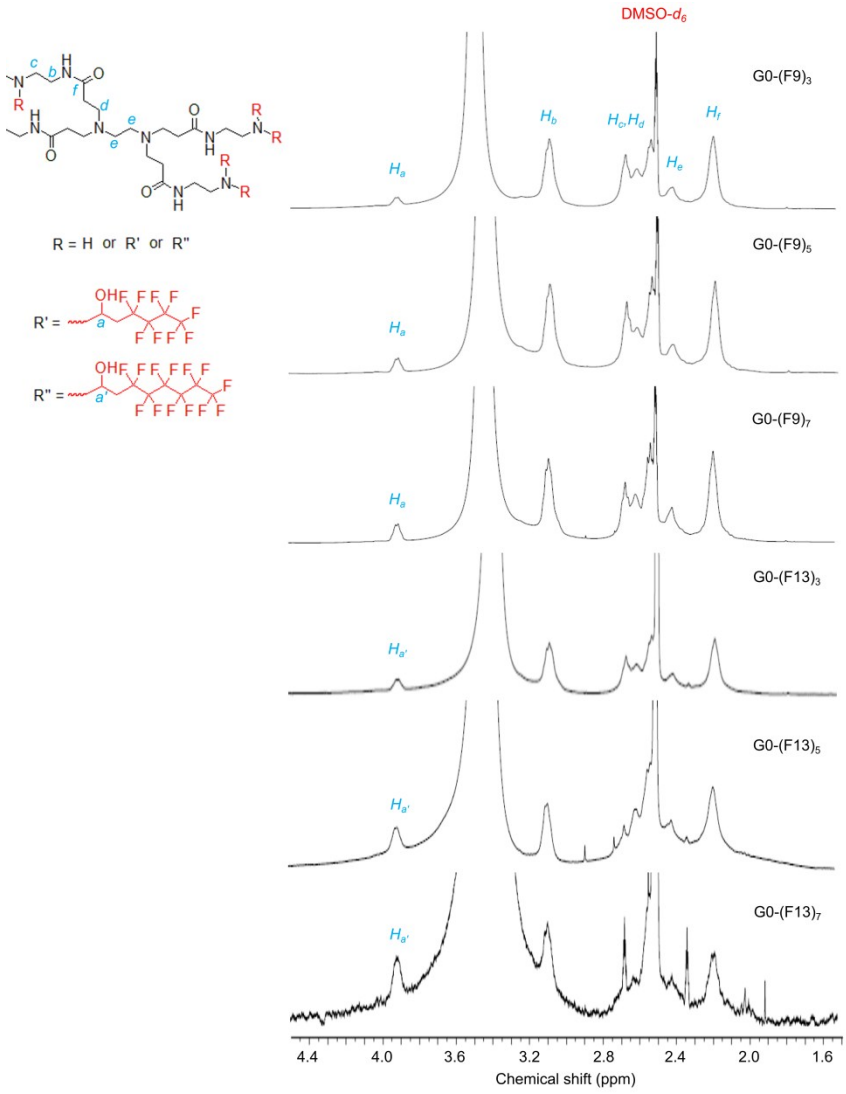

Fig. S1 ^{1}H NMR spectra of G0-(Fx)$_y$ in DMSO-d_6.

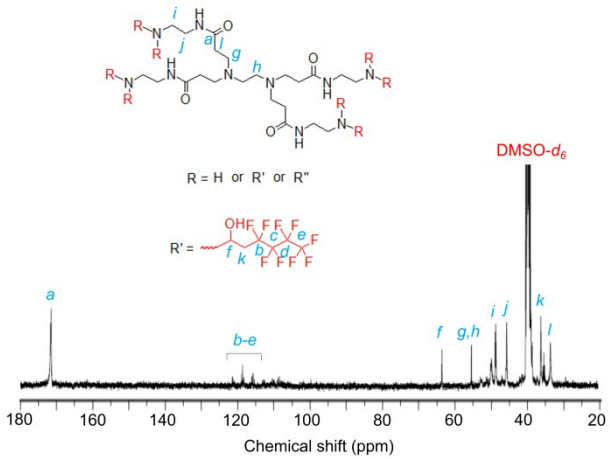

Fig. S2 ^{13}C NMR spectrum of G0-(F9)$_5$ in DMSO-d_6.

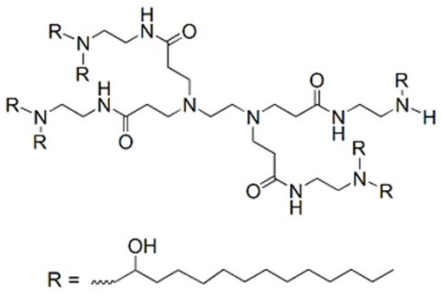

Fig. S3 Synthetic route of G0-C14.

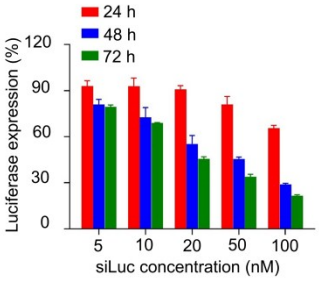

Fig. S4 Luciferase expression in HeLa-Luc cells transfected with NPF13-5/siLuc for different time (n = 5).

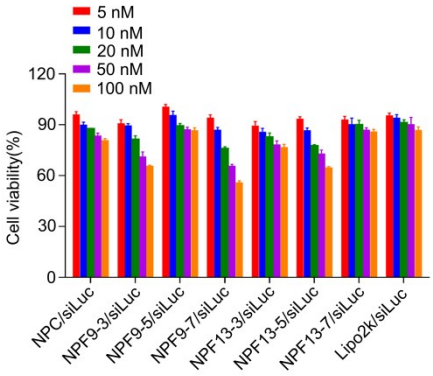

Fig. S5 Cytotoxicity of siLuc-loaded hybrid nanoparticles at various concentrations in HeLa-Luc cells (n = 5).

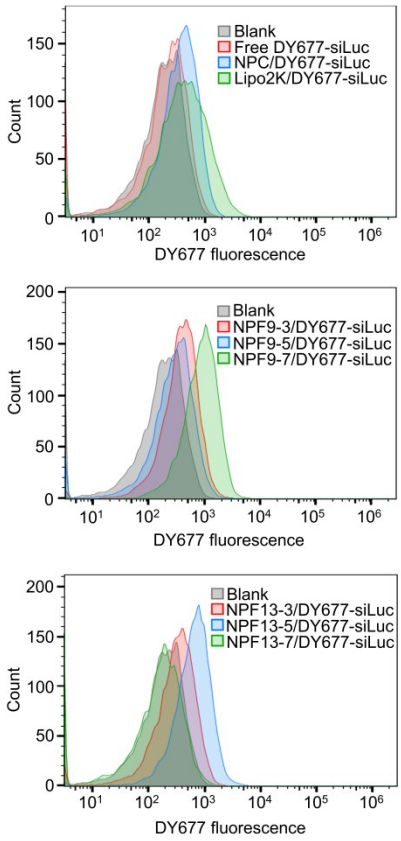

Fig. S6 Flow cytometry profiles of A549 cells incubated with different treatments for 2 h at a 20.0 nM siRNA dose.

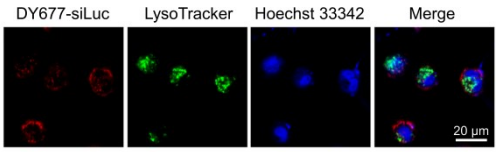

Fig. S7 CLSM images of A549 cells incubated with NPC/DY677-siLuc for 2 h at a 20.0 nM siRNA dose.

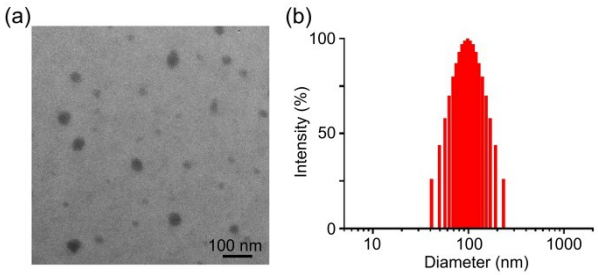

Fig. S8 Characterization of NPF13-3/siPHB1. (a) TEM image. (b) Size distribution histogram profile.

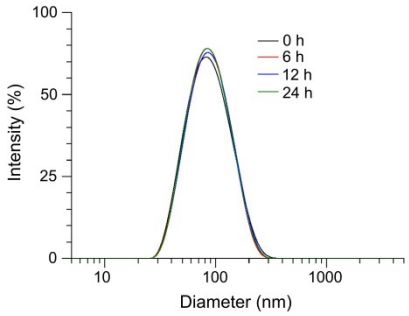

Fig. S9 Size distribution histogram profile of NPF13-5/siPHB1 in PBS after incubation for different time at 37 °C.

Table S1 Substitution degree of perfluoroalkyl groups in fluorodendrimers

Sample	Perfluoroalkane	Feeding ratio (G0 to perfluoroalkane)	Substitution degree
G0-$(F_9)_3$	3-perfluorobutyl-1,2-epoxypropane	1:3	2.33
G0-$(F_9)_5$		1:5	4.49
G0-$(F_9)_7$		1:7	6.72
G0-$(F_{13})_3$	3-perfluorohexyl-1,2-epoxypropane	1:3	2.47
G0-$(F_{13})_5$		1:5	4.63
G0-$(F_{13})_7$		1:7	6.84