Supplementary Material for Chemical Communications

PAMAM dendrimers for efficient siRNA delivery and potent gene silencing

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Supporting Information

Materials and Methods

Samples of siRNA were purchased from Proligo LLS (Colorado, USA). For GL3Luc siRNA and GL2Luc siRNA, respectively, the sequences of antisense are 5'-UCG AAG UAC UCA GCG UAA G dTdT-3' and 5'-CGU ACG CGG AAU ACU UCG A dTdT-3', and those of sense are 3'-dTdT AGC UUC AUG AGU CGC AUU C-5' and 3'-dTdT GCA UGC GCC UUA UGA AGC U-5'. All other reagents and solvents of analytical grade were used without further purification from commercial sources. Sodium dodecyl sulfate (SDS) was supplied from Sigma. RNase A was purchased from Jingmei Biotech Co., Ltd. (Shenzhen, China). Oligonucleotides (GL3- and GL2Luc) were chemically synthesised and PAGE purified by Eurogentec (Belgium). Oligonucleotides were annealed in 1 × Annealing buffer (50 mM potassium acetate, 50 mM magnesium acetate) (Eurogentec) for 2 min at 95°C, followed by 2-4 hours incubation at room temperature. IR spectra were recorded with a PERKIN ELMER spectrophotometer. ^{1}H NMR and ^{13}C NMR spectra were measured at 300 MHz and 75 MHz relatively on a Varian Mercury-VX300 spectrometer. HPLC were performed on a Waters^{TM} 600 pump with a Waters 600 controller and the components were detected using a Waters 2996 photodiode array detector. Two HPLC C_8 columns, Agilent ZORBAX C8 (4.6 × 150 mm) and Nucleosil C8 (4.0 × 250 mm), were used for HPLC analysis.

Dendrimer synthesis and characterization

The PAMAM dendrimers were synthesized as previously described[1] and characterized by IR, NMR, MS and HPLC. FT-IR is a convenient and effective technique to characterize PAMAM dendrimers. The half generation dendrimers have the methyl ester terminating groups, which have the

characteristic IR peaks for carbonyl at 1730-1750 cm^{-1}. For the full generation dendrimers, when the methyl ester groups were converted to amide groups, the corresponding carbonyl shifted to 1660 cm^{-1}. The NMR data corroborated well with the FT-IR data to confirm the structure of the dendrimers. The characteristic methyl ester peak, which appeared in all the ^{1}H-NMR spectra of the ester terminating dendrimers, was absent in the spectra of all the amine terminating dendrimers. HPLC analysis showed a major peak for dendrimer of each generation, with purifies of 95%, 90%, 90%, 85%, 80%, 80% and 70% for dendrimers from generation 1 to 7, respectively. The molecular weights of the lower-generation dendrimers were determined by electron-spray mass spectroscopy,[1] while those of the higher-generation dendrimers could not yet be obtained. Work on determining the molecular weight of the higher-generation dendrimers are under way using MALDI TOF mass spectroscopy, GPC analysis and Diffusion-ordered NMR spectroscopy (DOSY NMR).

Potentiometric pH titration of dendrimer

The dendrimer $\mathbf{G_7}$ was diluted and the concentration of primary amine was 5 mM. The pH titrations were carried out with the above mentioned dendrimer solution (17.1 mL) and 100 mM HCl using a Mettler Toledo 320-S pH meter.

Agarose gel analysis of siRNA/dendrimer complexes

The dendrimers were diluted to an appropriate concentration in 50 mM Tris-HCl buffer (pH 7.6), with all solutions stored at 4°C. The siRNA was diluted with H_2O. Both solutions were mixed at various N/P (= [total end amines in cationic dendrimer] / [phosphates in siRNA]) and incubated at 37°C for 30 min with indicated pH and NaCl concentration. The final concentration of siRNA was adjusted to 25 ng/μL (100 ng/well). siRNA/dendrimer complexes were analyzed by electrophoretic mobility-shift assays in 1.2% agarose gel in standard TAE buffer. The siRNA bands were stained by ethidium bromide and then detected by a Kodak 290 digital camera.

Stability of siRNA/dendrimer complex against RNase A

An aliquot of 1 μg of siRNA and the indicated amounts of dendrimers was kept at 37°C for 30 min.

Then the complexes were incubated in the presence of 0.01 µg/µL RNase A for 0, 5, 15, 20, 30 and 45 min at 37°C. Aliquots (4 µL) of the corresponding solution were withdrawn, added to 1.5 µL 1% SDS solution at 4°C and then subjected to electrophoresis in 1.2% agarose gel in standard TAE buffer. The siRNA bands were stained by ethidium bromide and then detected by a Kodak 290 digital camera.

Transmission electron microscope Imaging.

Studies were performed with a Hitachi H-7000FA electron microscope instrument. 20 µL of a solution of siRNA (5 ng/µL) were mixed with 20 µL of a solution of $\mathbf{G_7}$ dendrimer in 50 mM Tris-HCl buffer (34.4 ng/µL). After equilibration (3 min), 4 µL of this mixture were dropped on a standard carbon-coated copper TEM grid, and then allowed to evaporate (1 h at 30°C, ambient pressure). The grid was then stained with uranyl acetate (2% in water, pH 4.5) for 3 min. Imaging was performed immediately after air-dried for 20 min.

Cell culture

A549 (human lung carcinoma, ATCC N° CCL-185) cells stably expressing the GL3 luciferase (*Photinus pyralis* luciferase under the control of SV40 elements) were obtained after stable transfection of pGL3Luc plasmid (Clontech). A549-GL3Luc cells were grown in RPMI (Eurobio) and supplemented with 10% fetal bovine serum, 2 mM glutamax, 100 units/mL penicillin, 100 µg/mL streptomycin and 0.8 µg/mL G418 (Promega). Cells were maintained at 37°C in a 5% CO_2 humidified atmosphere.

siRNA Transfection

One day before transfection, 2.5×10^4 cells per well were seeded in 24-well tissue culture plate in 1 mL fresh complete medium containing 10% FBS. Before transfection, complexes of siRNA/dendrimer reagent were prepared. The desired amount of siRNA and dendrimer reagent was diluted in 150 µL of serum-free medium (for a triplicate experiment). The solutions were mixed with a Vortex for 10 seconds, and left for 10 minutes at room temperature. The dendrimer reagent was added to the siRNA solution, homogenized for 10 seconds with a Vortex and left 30 minutes at room temperature. Before adding the transfection complexes, the complete medium with serum

was removed and replaced with 0.5 mL of serum-free medium. Then, 100 µL of complexes solution was added per well and the plates were incubated at 37°C. After 2 h of incubation, the complete medium was removed and replace with 1 mL of complete medium containing 10% serum. The plate was further incubated at 37°C for 24 or 48 h.

Luciferase and protein assay

Luciferase gene expression was measured using a commercial kit (Promega, France). After removing the complete medium, three washings with 1 mL of PBS solution were made. Then, 100 µL of 1 × lysis buffer were added per well, and the plate was incubated at room temperature for 30 minutes. The lysates were collected and centrifuged at 14,000 g for 5 minutes. The luciferase assay was assessed with 5 µL of lysate after injection of 100 µL of luciferin solution. The luminescence (RLU) was monitored with an integration over 10 seconds with a luminometer (Berthold, France). Results are expressed as light units integrated over 10 seconds (RLU), per mg of cell protein using the BCA assay (Pierce, France).

Cytotoxicity Assay

Cell viability was determined by MTT assay. Cells were incubated in 1 mL of PBS containing 40 µL/mL of MTT (stock solution at 5 mg/ml) at 37°C for 2 h. Then, cells were washed with 1 mL of PBS three times and 200 µL of DMSO were added per well. The absorbance was determined at 570 nm and the viability was expressed as the ratio of absorbance obtained from transfected cells to non-transfected cells (n=6).

1. J. Y. Wu, J. H. Zhou, F. Q. Qu, P. H. Bao, Y. Zhang, L. Peng, *Chem. Commun.*, **2005**, 313-315.