Supplementary Material (ESI) for Medicinal Chemistry Communications

Supporting Information

Synthesis and efficient siRNA delivery of polyamine-conjugated cationic nucleoside lipids

Sachin Prakash Patil, Jeong Wu Yi, Eun-Kyoung Bang, Eun Mi Jeon and Byeang Hyean Kim*

Department of Chemistry, BK School of Molecular Science,

Pohang University of Science and Technology, Pohang, 790-784, Korea

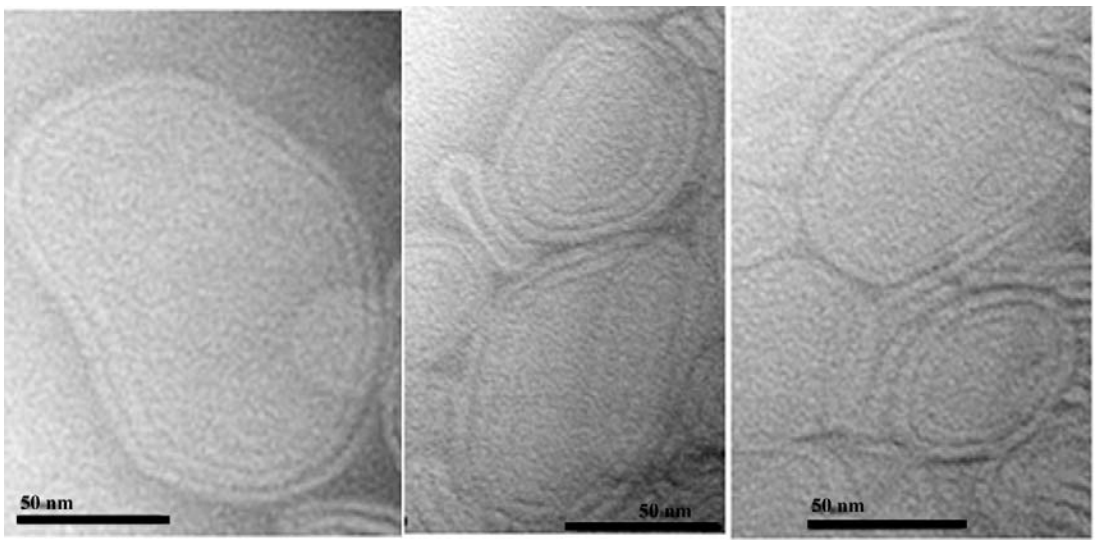

Figure S1. TEM images of siRNA complexes with 5a (left), 5b (middle), and 5c (right).

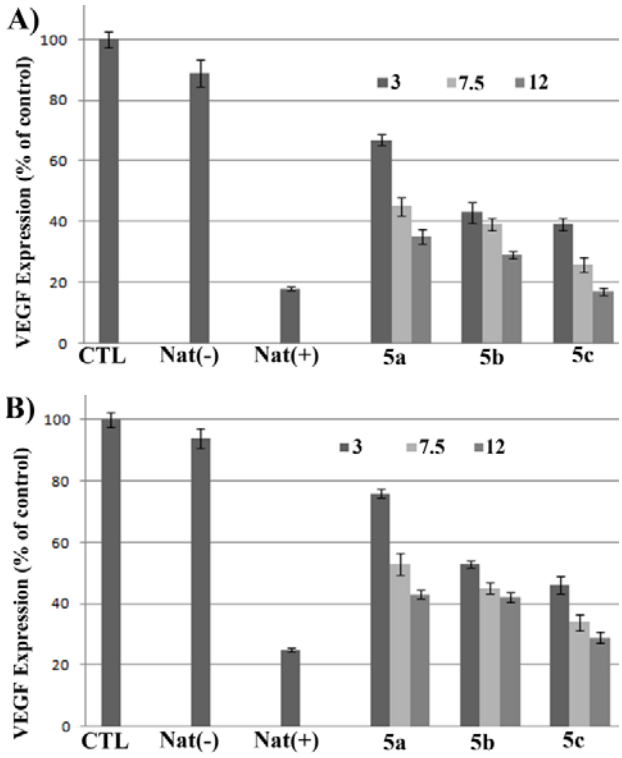

Figure S2. VEGF expression using ELISA assays of A) siRNA concentration: 10 nM (n = 3); B) siRNA concentration: 1 nM (n = 3). Control (CTL): Untreated cell; negative control [Nat (-)]: natural siRNA without lipoplexes; positive control [Nat (+)]: natural siRNA treated with lipofectamine. The N/P ratios given in Table 1.

The sequence of scrambled siRNA:

Scrambled VEGF sense strand: 5'GCC GAU CAG GAC GUA GAU UUdT 3'

Scrambled VEGF antisense strand: 3' dTU CGG CUA GUC CUG CAU CUA A 5'

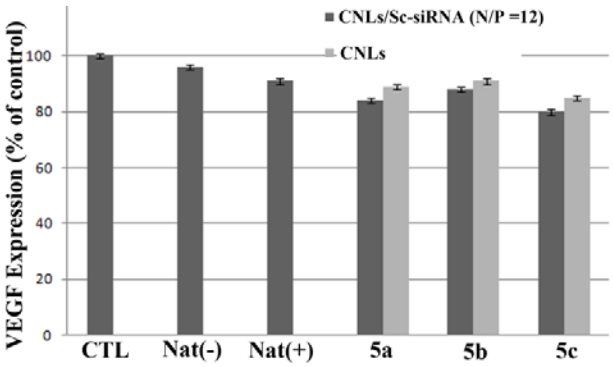

Figure S3. VEGF expression using ELISA assays. Sc-siRNA concentration: 50 nM (n = 3). Control (CTL): Untreated cell; negative control [Nat (-)]: natural Sc-siRNA without lipoplexes; positive control [Nat (+)]: natural Sc-siRNA treated with lipofectamine. The N/P ratios given in Table 1.

Experimental details

General Experiments and Analytical Conditions. All chemicals purchased from Sigma–Aldrich, Fluka, Lancaster, Proligo, or Glen Research and utilized without further purification. All reactions performed in flame-dried glassware under argon. Flash column chromatography was performed using Merck silica gel 60 (230–400 mesh). High-resolution mass spectra (FAB) were obtained using a Jeol JMS700 high-resolution mass spectrometer at the Korea Basic Science Center, Daegu, Korea. MALDI-TOF mass spectra were recorded using a Kratos Shimadzu AXIMA-CFR MALDI-TOF mass spectrometer at the Bioneer Corporation. Purification of oligonucleotides was performed using an Agilent 1100 HPLC (VyDAC C18 column; 10 × 250 mm; 5 μm; pore size: 120 Å). The RT-PCR was performed using Corbert Research RG-6600 model. The siRNAs were synthesized using an Expedite 8909 synthesizer and primer oligonucleotides were purchased from Bionics Co., Ltd. (Korea).

^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded using an FT-300 MHz Bruker Aspect 300 spectrometer. Chemical shifts of these spectra are reported in parts per million (ppm) downfield relative to the internal standard, tetramethylsilane (TMS). Coupling constants are reported in hertz (Hz). Spectral splitting patterns are designed as s, singlet; d, doublet; dd,double doublet; dt, distorted triplet; t, triplet; m, multiplet; and br, broad.

Experimental procedures of compounds **1**, **2** and **3** are as in reference 7c.

5′-*O*-(4,4′-dimethoxytrityl)uridine, 1. ^{1}H NMR (300 MHz, DMSO): δ 11.35 (s, 1H), 7.70 (d, *J* = 8.1 Hz, 1H), 7.37~6.87 (m, 13H), 5.73 (d, *J* = 2.5 Hz, 1H), 5.50 (br, 1H), 5.29 (d, *J* = 8.1 Hz, 1H), 5.15 (br, 1H), 4.06(s, 2H), 3.91 (br, 1H), 3.72 (s, 6H), 3.44~3.20 (m, 2H); ^{13}C NMR (75 MHz, DMSO): δ 163.0, 158.1, 150.5, 144.7, 140.6, 137.3, 135.4, 135.2, 129.8, 128.9, 128.2, 127.9, 127.7, 126.8, 125.3, 113.3, 101.5, 88.9, 85.9, 82.4, 73.4, 69.6, 63.0, 55.0; HRMS-FAB (m/z) : calcd for $C_{30}H_{30}N_2NaO_8^+$ $[M+Na]^+$, 569.189; found, 569.190.

2′,3′-Di-*O*-(oleylcarbamyl)-5′-*O*-(4,4′-dimethoxytrityl)uridine, 2. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.20 (br, 1H), 7.69 (d, *J* = 8.2 Hz, 1H), 7.41~6.83 (m, 13H), 6.22 (d, *J* = 6.9 Hz, 1H), 5.53~5.50 (m, 1H), 5.38~5.34 (m, 4H), 5.29 (d, *J* = 8.0 Hz, 1H), 4.81 (br, 1H), 4.22 (br, 1H), 3.79 (s, 6H), 3.47 (dd, *J* = 10.1, 38.6 Hz, 2H), 3.17~3.11 (m, 4H), 2.08~2.00 (m, 8H), 1.50~1.25 (m, 48H), 0.88 (t, *J* = 6.5 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) : δ 162.8, 159.1, 155.3, 154.8, 150.9, 144.3, 140.4, 135.4, 135.1, 130.6, 130.5, 130.4, 130.1, 129.8, 128.6, 128.5, 127.6, 115.8, 113.7, 103.2, 87.9, 85.4, 83.3, 77.8, 77.6, 77.4, 76.9, 73.8, 72.9, 72.7, 63.6, 55.6, 41.7, 41.6, 41.1, 33.0, 32.3, 32.1, 30.6,

30.3, 30.1, 30.0, 29.9, 29.7, 29.3, 29.2, 27.6, 27.3, 27.1, 23.0, 14.5; HRMS-FAB (m/z) : calcd for $C_{68}H_{100}N_4NaO_{10}^+$ $[M+Na]^+$, 1155.733; found, 1155.734.

2′,3′-Di-*O*-(oleylcarbamyl)uridine, 3. ^{1}H NMR (300 MHz, $CDCl_3$) : δ 9.39 (s, 1H), 7.89~7.86 (d, *J* = 8.1 Hz, 1H), 6.13 (d, *J* = 6.8 Hz, 1H), 5.80~5.78 (d, *J* = 8.1 Hz, 1H), 5.37~5.24 (m, 7H), 4.12 (s, 1H), 3.82 (br, 2H), 3.09~3.02 (m, 4H), 1.95~1.93 (m, 8H), 1.41~1.19 (m, 48H), 0.81 (t, *J* = 6.5 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) : δ 162.9, 154.9, 154.4, 150.4, 147.1, 140.9, 130.5, 130.3, 130.1, 128.0, 102.7, 86.5, 84.6, 84.2, 74.2, 72.7, 62.3, 62.0, 42.2, 41.6, 32.9, 32.2, 32.1, 31.8, 30.2, 30.1, 30.0, 29.8, 29.6, 29.5, 29.3, 27.5, 27.1, 23.0, 22.1, 13.6; HRMS-FAB (m/z) : calcd for $C_{47}H_{82}N_4NaO_8^+$ $[M+Na]^+$, 853.602; found, 853.730.

General procedure (4a-c): carbonyldiimidazole (58 mg, 0.36 mmol), and 4-dimethylaminopyridine (5 mg, 0.05 mmol) were added in solution of **3** (0.200 g, 0.24 mmol) in dry DMF. Solution was stirred for 1 h under Ar (g) condition. Then corresponding polyamine (3 eq.) was added and the mixture was stirred for additional 12 h. After completion of the reaction, the solvent was evaporated under reduced pressure and extracted in CH_2Cl_2 and washed with water, and brine. The organic layer was dried on Na_2SO_4 and evaporated under reduced pressure.

The crude compound was dissolved in methanol and Boc-anhydried (10 eq.) was added in the solution. Mixture was stirred for 2 h. After completion of the reaction, the solvent was evaporated under reduced pressure, extracted in CH_2Cl_2 and washed with water, and brine. The organic layer was dried on Na_2SO_4 and evaporated under reduced pressure and purified through flash column chromatography. A clear oil **4a-c** (42 ~ 70%) was obtained.

2′,3′-Di-*O*-(oleylcarbamyl)-5′-*O*-(N^4,N^9,N^{12}-tri(Boc)-1,12-diamino-4,9-diazadodecane-1-cabamyl)uridine, 4a. ^{1}H NMR (300 MHz, $CDCl_3$) : δ 8.73 (br, 1H), 7.54 (br, 1H), 6.11 (d, *J* = 6.8 Hz, 1H), 5.81 (br, 1H), 5.37~5.16 (m, 6H), 5.15 (br, 1H), 4.31 (br, 2H), 3.12~3.02 (m, 16H), 2.0~1.99 (m, 8H), 1.5 (m, 4H), 1.45 (br, 6H), 1.44(s, 27H), 1.26~1.25(m, 44H), 0.82 (t, *J* = 6.5 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) : δ 162.2, 155.6, 155.2, 154.3, 154.0, 150.0, 139.2, 130.5, 130.3, 130.1, 128.0, 103.6, 86.5, 84.6, 84.2, 79.6, 74.2, 72.7, 62.3, 62.0, 42.2, 41.6, 35.8, 32.9, 32.2, 32.1, 31.8, 30.2, 30.1, 30.0, 29.8, 29.6, 29.5, 29.3, 28.8, 27.6, 27.4, 27.0, 23.1, 22.2, 20.7, 13.6; HRMS-FAB (m/z) : calcd for $C_{73}H_{130}N_8NaO_{15}^+$ $[M+Na]^+$, 1382.955; found, 1381.960.

2′,3′-Di-*O*-(oleylcarbamyl)-5′-*O*-(N^4,N^8-di(Boc)-1,8-amino-4-azaoctane-1-carbamyl)uridine, 4b. ^{1}H NMR (300 MHz, $CDCl_3$) : δ 9.28 (s, 1H), 7.6 (br, 1H), 6.08 (d, *J* = 6.8 Hz, 1H), 5.73 (d, *J* = 8.1 Hz, 1H), 5.31~5.16 (m, 5H), 5.29 (d, *J* = 8.1 Hz, 1H), 5.15 (br, 1H), 4.31 (br, 2H), 3.12~3.02 (m, 12H), 1.95~1.93(m, 8H), 1.64~1.62(m,

4H), 1.44(br, 6H), 1.43(s, 18H), 1.42~1.19 (m, 44H), 0.81 (t, *J* = 6.5 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) : δ 162.6, 162.4, 155.7, 155.5, 155.2, 154.4, 154.0, 140.7, 130.5, 130.3, 130.1, 128.0, 103.6, 86.5, 84.6, 84.2, 79.6, 74.2, 72.7, 62.3, 62.0, 42.2, 41.6, 35.8, 32.9, 32.2, 32.1, 31.8, 30.2, 30.1, 30.0, 29.8, 29.6, 29.5, 29.3, 28.8, 27.5, 27.3, 27.1, 23.0, 22.9, 14.4, 13.6; HRMS-FAB (m/z) : calcd for $C_{65}H_{115}N_7NaO_{13}^+$ $[M+Na]^+$, 1224.845; found, 1224.844.

2′,3′-Di-*O*-(oleylcarbamyl)-5′-*O*-(N^4-(Boc)-1,4-aminobutane-1-carbamyl)uridine, 4c. ^{1}H NMR (300 MHz, $CDCl_3$) : δ 8.8 (s, 1H), 7.5 (br, 1H), 6.1 (br, *J* = 6.8 Hz, 1H), 5.73 (d, *J* = 8.1 Hz, 1H), 5.45 (br, 1H), 5.31~5.16 (m, 5H), 5.15 (br, 1H), 4.31 (br, 2H), 3.09~3.02 (m, 8H), 1.95~1.93 (m, 8H), 1.52 (s, 9H), 1.42~1.19 (m, 52H), 0.81 (t, *J* = 6.5 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) : δ 163.6, 156.5, 156.1, 155.3, 155.1, 154.6, 151.2, 147.1, 140.4, 130.5, 130.3, 130.1, 128.0, 103.6, 86.5, 84.6, 84.2, 79.6, 74.2, 72.7, 62.3, 62.0, 42.2, 41.6, 35.8, 32.9, 32.2, 32.1, 31.8, 30.2, 30.1, 30.0, 29.8, 29.6, 29.5, 29.3, 28.8, 27.5, 27.3, 27.1, 23.0, 22.9, 14.5; HRMS-FAB (m/z) : calcd for $C_{57}H_{100}N_6NaO_{11}^+$ $[M+Na]^+$, 1067.735; found, 1067.730.

General Procedure (5a-c): 4 M HCl in dioxane (2 mL) was added to a solution of **4a-c** (0.050 g, 0.04 mmol) in dry dioxane (0.5 mL) and the mixture was stirred at room temperature for 12 h and the solvent was evaporated under reduced pressure. A white solid **5a-c** (97%) was obtained.

2′,3′-Di-*O*-(oleylcarbamyl)-5′-*O*-(1,12-diamino-4,9-diazadodecane-1-carbamyl)uridine, 5a. ^{1}H NMR (300 MHz, $CDCl_3$/MeOD 4:1) : δ 8.73 (br, 1H), 7.66 (d, *J* = 8.1 Hz, 1H), 6.03 (d, *J* = 6.8 Hz, 1H), 5.81 (br, 1H), 5.37~5.16 (m, 6H), 5.15 (br, 1H), 4.31 (br, 2H), 3.12~3.02 (m, 16H), 2.0~1.99 (m, 8H), 1.5 (m, 4H), 1.45 (br, 6H), 1.26~1.25 (m, 46H), 0.82 (t, *J* = 6.5 Hz, 6H); ^{13}C NMR (75 MHz, $CHCl_3$/MeOD 4:1) : δ 162.2, 155.2, 154.3, 154.0, 150.0, 139.2, 130.5, 130.3, 130.1, 128.0, 103.6, 86.5, 84.6, 84.2, 79.6, 74.2, 72.7, 62.3, 62.0, 42.2, 41.6, 35.8, 32.9, 32.2, 32.1, 31.8, 30.2, 30.1, 30.0, 29.8, 29.6, 29.5, 29.3, 27.5, 27.3, 27.1, 23.0, 22.2, 13.6; HRMS-FAB (m/z) : calcd for $C_{58}H_{106}N_8NaO_9^+$ $[M+Na]^+$, 1081.798; found, 1081.800.

2′,3′-Di-*O*-(oleylcarbamyl)-5′-*O*-(1,8-amino-4-azaoctane-1-carbamyl)uridine, 5b. ^{1}H NMR (300 MHz, $CDCl_3$/MeOD 4:1) : δ 7.5 (br, 1H), 6.0 (br, 1H), 5.78 (br, 1H), 5.58 (br, 1H), 5.31~5.16 (m, 6H), 4.31 (br, 3H), 3.12 (br, 6H), 2.9 (m, 4H), 2.01 (m, 8H), 1.75~1.73 (m, 4H), 1.47 (br, 6H), 1.42~1.19 (m, 44H), 0.81 (t, *J* = 6.5 Hz, 6H); ^{13}C NMR (75 MHz, $CHCl_3$/MeOD 4:1) : δ 164.2, 162.6, 156.6, 155.5, 155.3, 140.2, 130.5, 130.3, 130.1, 128.0, 103.6, 86.5, 84.6, 84.2, 79.6, 74.2, 72.7, 62.3, 62.0, 42.2, 41.1, 35.9, 32.8, 32.4, 32.0, 31.5, 30.2, 30.1, 30.0, 29.9,

29.6, 29.5, 29.1, 27.9, 27.8, 27.4, 23.0, 22.8, 14.7, 13.6; HRMS-FAB (m/z) : calcd for $C_{55}H_{99}N_7NaO_9^+$ $[M+Na]^+$, 1024.740; found, 1024.740.

2′,3′-Di-*O*-(oleylcarbamyl)-5′-*O*-(1,4-aminobutane-1-carbamyl)uridine, 5c. ^1H NMR (300 MHz, $CHCl_3$/MeOD 4:1) : δ 8.8 (s, 1H), 7.65 (br, 1H), 6.0 (br, 1H), 5.78 (br, 1H), 5.45 (br, 1H), 5.31~5.16 (m, 5H), 5.15 (br, 1H), 4.31 (br, 2H), 3.12 (br, 6H), 2.96 (br, 2H), 1.95~1.93 (m, 8H), 1.42~1.19 (m, 52H), 0.81 (t, *J* = 6.5 Hz, 6H); ^{13}C NMR (75 MHz, $CHCl_3$/MeOD 4:1) : δ 163.6, 156.5, 155.3, 155.1, 151.2, 140.9, 130.5, 130.3, 130.1, 128.0, 103.6, 86.5, 84.6, 84.2, 74.2, 72.7, 62.3, 62.0, 42.2, 41.6, 35.8, 32.9, 32.2, 32.1, 31.8, 30.2, 30.1, 30.0, 29.8, 29.6, 29.5, 29.3, 27.5, 27.3, 27.1, 23.0, 22.9, 14.5; HRMS-FAB (m/z) : calcd for $C_{52}H_{92}N_6NaO_9^+$ $[M+Na]^+$, 1067.6; found, 1067.6. HRMS-FAB (m/z) : calcd for $C_{52}H_{92}N_6NaO_9^+$ $[M+Na]^+$, 967.682; found, 967.680.

Synthesis of siRNA. VEGF siRNA were synthesized using an automatic RNA synthesizer, according to standard solid phase protocols. The structures of siRNA were confirmed by MALDI-TOF mass spectrometry. MALDI-TOF MS data of RNAs: antisense strand of VEGF siRNA (5′-GAUCUCAUCAGGGUACUCCdTdT) 6603.9 (calcd. 6604.6): sense strand of VEGF siRNA (5′-GGAGUACCCUGAUGAGAUCdTdT) 6708.2 (calcd. 6709.2): Fluoroscein tagged antisense strand of VEGF siRNA (5'-AUCUCAUCAGGGUACUCCdT**Fl**).

Lipoplex preparation. Cationic lipids stock solutions were prepared by solubilizing the compounds in H_2O/EtOH (10:1, v/v). The lipid solution was vortexed for 30 s and stirred for 30 min at 45 ^oC to make it clear. After cooling to room temperature, 10 volume of nuclease free water was added and the solution was vortexed and incubated at 4 ^oC before adding the siRNA. Lipoplexes were then prepared by mixing with corresponding siRNA quantities of each formulation with N/P ratio. Finally, the formulations were mixed and incubated for 30 min at room temperature before use.

WST-1 Assay. Lipids (with N/P ratio of 3, 7.5 and 12) were complexed with VEGF siRNA (certain concentration as for lipid) and added to the cells. Cells were incubated with complexes at 37 °C and 5 % CO_2 for 24 h. After incubation, media were removed, the cells washed with 100 mL of DPBS buffer (2 times) and then a 10 μL/well of cell proliferation reagent WST-1 to the cell already cultured in 100 mL/well (1:10 final dilution, Roche) was added to each well and incubated at 37 °C for 4 h. After incubation, absorbance were measured at a wavelength of 450 nm using a micro-plate reader (Asys) and converted to percentage of cell viability (relative to control cells).

Cell culture and transfection experiment. Roswell Park Memorial Institute medium (RPMI-1640), Dulbecco's Modified Eagles medium (DMEM), penicillin-streptomycin, fetal bovine serum (FBS), and Dulbecco's phosphate-buffered saline (DPBS) were purchased from Hyclone-Themo scientific (Logan, UT). Opti-MEM was purchased from Invitrogen-Gibco (Carlsbad, CA). HeLa cells were cultured in Dulbecco's modified Eagle's medium, supplemented with 10 % FBS, 100 μg/mL of streptomycin, and 100 U/mL of penicillin at 37 °C in 5 % CO_2 incubator. Cells were split, using trypsin/EDTA medium when almost confluent. HeLa cells were seeded at a density of 2.5 x 10^5 cells per well, each well of which contained 2 mL of 10 % FBS supplemented DMEM, and incubated for 4 hours. HeLa cells were transfected in the absence of serum with VEGF siRNA using lipid (**5a**, **5b** and **5c**), Lipofectamine™ 2000 (Invitrogen), or without any transfection reagent. The cells were allowed to incubate at 37 °C for 6 hours in CO_2 incubator followed by replacement of 2 mL of DMEM containing 10 % FBS. After more 18 hrs incubation, cell medium were collected and analyzed the VEGF expression level by using VEGF ELISA kit (QIA51 VEGF ELISA Kit, Human, Calbiochem).

RT-PCR experiment. VEGF siRNA-treated cells were washed by PBS and lipholyzed in 1.0 ml of TRIzol®-reagent (Invitrogen) and total RNA was isolated. A 1 μg sample of RNA was used in reverse transcription with Improm-II™ Reverse Transcription System (Promega) and the procedures were performed by the manufacturer's protocols. The reverse transcription reaction was carried out at 25 °C (5 min), 42 °C (60 min) and 70 °C for 15 min followed by PCR: 1 cycle, 95 °C, 5 min; 30 cycles, 95 °C, 30 s; 55 °C, 30 s; 72 °C, 30 s; 1 cycle, 72 °C, 5 min. For VEGF mRNA amplification forward and reverse primers were 5'-GGGCAGAATCATCACGAAGT-3' and 5'-TGGTGATGTTGGACTCCTCA-3', respectively. The PCR products were analyzed by electrophoresis on a 1.5 % agarose gel (80 V, 50 min) stained with ethidium bromide.

Confocal Microscopy Experiment. At first, cover glasses (1.13 cm^2, Deckglaser) were put into 6-well plate, and the plate was coated with 0.2 % gelatin. HeLa cells were seeded at a density of 2.5 x 10^5 cells per well, each well of which contained 2 mL of 10 % FBS supplemented DMEM, and incubated for 12 hours. HeLa cells were transfected in the absence of serum with 100 nM of Flurorescein-tagged VEGF siRNA using Lipofectamine™ 2000 (Invitrogen), lipid **5a, 5b** and **5c** (N/P ratio: 3, 7.5 and 12) or without any reagent. The cells were allowed to incubate at 37 °C in the presence of oligonucleotides for 4 hours in CO_2 incubator followed by replacement of 2 mL of DMEM containing 10 % FBS. For preparation, 4 hours after transfection, cells were washed with DPBS, and then cover glasses were detached from bottom of plate. The cover glasses were transferred onto slide glass. Confocal images were obtained from the live cell by using Olympus FluoView™ FV1000 confocal microscope (*Olympus Optical Co., Ltd.,* Tokyo).