Supporting Information

Lactosylated Poly(ethylene glycol)-siRNA Conjugate through Acid-Labile β-Thiopropionate Linkage to Construct pH-Sensitive Polyion Complex Micelles Achieving Enhanced Gene Silencing in Hepatoma Cells

Motoi Oishi,[†] Yukio Nagasaki,[*,†] Keiji Itaka,[‡] Nobuhiro Nishiyama,[§] and Kazunori Kataoka[*, §]

[†]*Department of Materials Science and Technology, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba 278-8510, Japan*

[‡]*Department of Orthopaedic Surgery, Faculty of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan*

[§]*Department of Materials Science and Engineering, Graduate School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan*

General

N,*N*-Dimethylformamide (DMF), triphenylphosphine, and triethylamine were purchased from Wako and used without further purification. Poly(L-lysine)-HBr (degree of polymerization (DP)=40, Mw=8300) and 2-iminothiolane (Traut's reagent) were purchased from Sigma-Aldrich and Pierce, respectively, and used without further purification. Water was purified using a Milli-Q instrument (MILLIPORE). 5'-Thiol-modified sense RNA (HS-$(CH_2)_6$-CUU ACG CUG AGU ACU UCG AdTdT–3', firefly luciferase, pGL3-control sense sequence) and unmodified antisense RNA (5'-UCG AAG UAC UCA GCG UAA GdTdT–3', firefly luciferase, pGL3-control antisense sequence) were purchased from Greiner Japan. Plasmid DNAs (pDNA) encoding firefly luciferase (pGL3-Control, Promega; 5256 bpa) and renilla luciferase (pRL-TK, Promega; 4045 bpa) were amplified using

EndoFree™ Plasmid Maxi or Mega Kits (QIAGEN). The DNA concentration was determined by reading the absorbance at 260 nm. ^{1}H NMR (400MHz) spectra were obtained in D_2O using a JEOL EX400 spectrometer. Chemical shifts are reported in ppm relative to D_2O (δ4.79, ^{1}H). Anion exchange chromatography was performed using the JASCO HPLC system equipped with an Amersham Bioscience MONOQ HR 10/10 column and 10mM Tris-HCl buffer (pH 7.4) including NaCl (gradient ranging from 0 to 0.7 M) as the eluent.

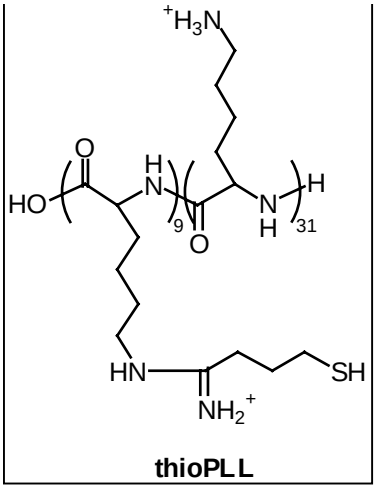
Synthesis of Lac-PEG-siRNA Conjugate.

The Lac-PEG-acrylate (M_n=4630, M_w/M_n=1.04) was prepared according to the previous report.[1] To a mixture of 5'-thiol-modified sense RNA (193 μg, 30 nmol) and excess Lac-PEG-acrylate (1.6 mg, 300 nmol, 10 eq.) in 10 mM Tris-HCl buffer pH 8.0 (300 μL), a solution of triphenylphosphine in DMF (1mM, 60μl, 60 nmol, 2eq.) was added, and Michael reaction was carried out in the dark for 48 h. After filtration, the filtrate was loaded on an anion exchange chromatograph, eluted with 10mM Tris-HCl buffer (pH 7.4) including NaCl (gradient ranging from 0 to 0.7 M). Further purification was carried out by dialyzing against distilled, deionized water (MW cutoff 3500) and then freeze-dried to obtain Lac-PEG-ssRNA conjugate (89 % yield). For the formation of Lac-PEG-siRNA conjugate, unmodified antisense RNA (50 μM) was mixed with Lac-PEG-ssRNA (50 μM) in annealing buffer (10 mM Tris-HCl pH 7.4, 50 mM NaCl, and 1mM EDTA), heated-denatured at 95 ˚C for 3 min, and annealed at room temperature overnight.

Synthesis of ThioPLL.

PLL (42 mg, 5.1 μmol) and 2-iminothiolane (7.0 mg, 50 μmol) were dissolved in distilled, deionized water (4.0 mL) in the presence of triethylamine (458 mg, 4.5 mmol), and the reaction mixture was stirred at room temperature for 48 h. The reaction mixture was directly dialyzed against distilled, deionized water (MW cutoff 3500) and then freeze-dried to obtain thioPLL (40 mg, 82 % yield). ^{1}H NMR (D_2O) δ 1.18~1.86 (m, 360H), 2.01~2.20 (brs, 18H), 2.58~2.63 (brs, 18H), 2.64~2.81 (brs, 18H),

2.84~3.07 (brs, 62H), 3.17~3.36 (brs, 18H), 4.17~4.39 (brs, 40H).



thioPLL

Preparation of PIC Micelle.

Specific amounts of the Lac-PEG-siRNA conjugate (50 μM) and PLL were separately added to 10 mM Tris-HCl buffer (pH 7.4) to prepare the 10 μM stock solutions. The solutions were filtered through a 0.1-μm filter to remove the dust. The Lac-PEG-siRNA conjugate in 10 mM Tris-HCl buffer (pH 7.4) was mixed with PLL stock solution in 10 mM Tris-HCl buffer (pH 7.4) at an equal unit molar ratio of phosphate group in the Lac-PEG-siRNA conjugate and the amino group in PLL (N/P=1), followed by the addition of 10 mM Tris-HCl buffer (pH 7.4) including 0.3 M NaCl to adjust the ionic strength of the solution to the physiological condition (0.15 M NaCl).

Preparation of Disulfide Cross-Linked PIC Micelle.[2]

Specific amount of the thioPLL was dissolved in 10 mM Tris-HCl buffer (pH 7.4) to prepare the 50 μM stock solutions. The solution was filtered through a 0.1-μm filter to remove the dust, followed by the addition of 0.1 μM DTT solution with 3 equivalent for thiol groups. After incubation for 30 min at room temperature, the Lac-PEG-siRNA conjugate stock solution (50 μM) was added to form PIC micelle at an equal unit molar ratio of phosphate group in the Lac-PEG-siRNA conjugate and the amino group in thioPLL (N/P=1). After incubation for 3 h at room temperature, the PIC micelle solution was dialyzed against 10 mM Tris-HCl buffer (pH 7.4) containing dimethyl sufoxide (DMSO) at 37 ˚C for 24 h to remove the impurities, followed by 2 days of additional dialysis for removal of DMSO. During the dialysis, the thiol groups of the thioPLL were oxidized to form the disulfide cross-links.

Polyacrylamide Gel Electrophoresis.

10 μL of each samples (sense RNA: 100 ng, siRNA: 10 ng, Lac-PEG-ssRNA: 100 ng, Lac-PEG-siRNA: 10 ng, and PIC micelle: 10 ng) was electrophoresed at 100 V for 3h on a 12 % polyacrylamide gel in 3.3 mM TBE buffer (pH 7.4). After ethidium bromide (EtBr: 0.5 μg/mL) staining for 1 h, retardation was visualized under UV irradiation.

Transmission Electron Microscopy.

The sample for the TEM study was prepared from a solution with a micelle concentration of 0.03 mg/mL. Briefly, a drop of the sample solution was allowed to settle on a carbon film grid, follwed by the air-drying. The sample was analyzed using EF-TEM (LEO 922 OMEGA, Carl Zeiss).

Cell Culture.

HuH-7 human cancer cells, derived from a hepatocarcinoma cell line, were obtained from Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University. NIH 3T3 cells, derived from a mouse fibroblast, were obtained from RIKEN Cell Line Bank. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37˚C in a humidified 5% CO_2 atmosphere.

Dual Luciferase Reporter Assay.

HuH-7 cells or NIH 3T3 cells were plated in a 24-well plate ($5X10^4$ cells/well) to reach about 50% confluence at transfection. The cells were grown for 24 h and the culture medium was changed to OPIMEM I. The cells were co-transfected with two luciferase plasmids (firefly luciferase, pGL3-control and renilla luciferase, pRL-TK) in the presence of LipofectAMINE (Invitrogen). For

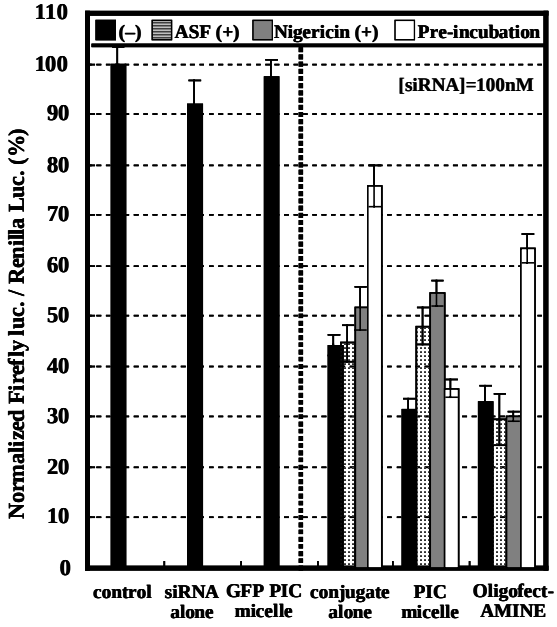


Figure S1. RNAi activities against the firefly luciferase gene generated in cultured HuH-7 cells. Normalized ratios between the firefly luciferase activity (firefly luc.) and the renilla luciferase activity (renilla luc.) are shown on the ordinate. The indicated concentrations of siRNA were the final concentrations in the total transfection volume (250 µL). The plotted data are averages of triplicate experiments ± SD

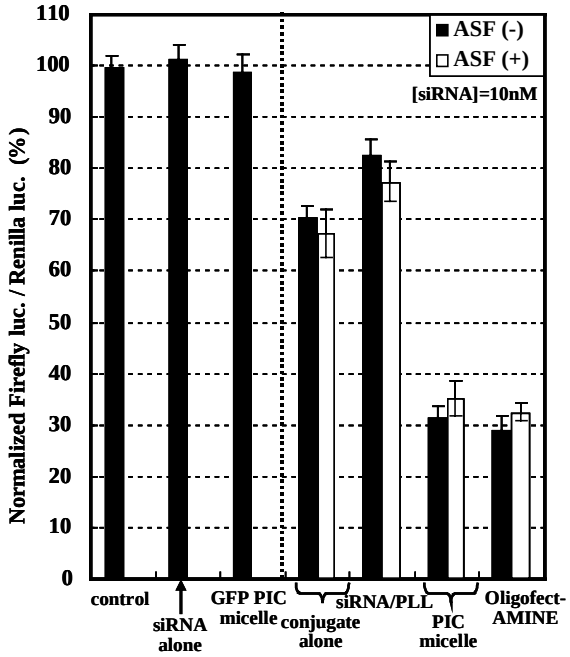


Figure S2. RNAi activities against the firefly luciferase gene generated in cultured NIH 3T3 cells. Normalized ratios between the firefly luciferase activity (firefly luc.) and the renilla luciferase activity (renilla luc.) are shown on the ordinate. The indicated concentrations of siRNA were the final concentrations in the total transfection volume (250 µL). The plotted data are averages of triplicate experiments ± SD.

each well, 0.0835 µg of pGL3 and 0.75 µg of pRL were applied; the final volume was 250 µL/well. The cells were incubated for 4 h, and then transfection medium was changed to DMEM with 10% FBS (225 µL/well). The PIC micelle (N/P=1), siRNA, or Lac-PEG-siRNA conjugate (25 µL/well) was added to the well at a prescribed concentration. After 24h incubation, transfection medium was changed to fresh DMEM with 10% FBS, and further the cells were incubated for 24 h. The luciferase expression was monitored with the dual luciferase assay kit (promega) and ARVOSX-1 (PerkinElmer).

(1) Oishi, M.; Sasaki, S.; Nagasaki, Y.; Kataoka, K. *Biomacromolecules* **2003,** *4*, 1426.

(2) (a) Kakizawa, Y.; Harada, A.; Kataoka, K. *J. Am. Chem. Soc.,* **1999,** *121*, 11247. (b) Miyata, K.; Kakizawa, Y.; Nishiyama, N.; Hrada, A.; Yamasaki, Y.; Koyama, H.; Kataoka, K. *J. Am. Chem. Soc.*, **2004,** *126*, 2355. (c) Kakizawa, Y.; Harada, A.; Kataoka, K. *Biomacromolecules* **2002,** *2*, 491.