Electronic Supplementary Information

A Supramolecular Endosomal Escape Approach for Enhancing Gene Silencing of siRNA Using Acid-Degradable Cationic Polyrotaxanes

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1. Reagents. Homobifunctional poly(ethylene glycol) (PEG) with terminal hydroxyl groups was obtained from Sigma-Aldrich (Milwaukee, WI, USA), and its number-averaged molecular weight (M_n) and polydispersity index (M_w/M_n) were determined to be 4,550 and 1.02, respectively, using size exclusion chromatography. α-Cyclodextrin (α-CD) was obtained from Ensuiko Sugar Refining (Tokyo, Japan). *N*-Benzyloxycarbonyl-L-tyrosine (Z-Tyr-OH) and *N,N'*-carbonyldiimidazole (CDI) were obtained from Sigma-Aldrich. 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM), acryloyl chloride, triethylamine (TEA), cysteamine hydrochloride, and *N,N*-dimethylaminoethyl amine (DMAEA) were obtained from Wako Pure Chemical Industries (Osaka, Japan). Other solvents and reagents were obtained from Kanto Chemicals (Tokyo, Japan).

2. Characterization of polymers and polyrotaxanes. Size exclusion chromatography (SEC) was carried out on a HLC-8120 system (Tosoh, Tokyo, Japan) equipped with a combination of TSKgel α-4000 and α-2500 columns (Tosoh), eluted with dimethylsulfoxide (DMSO) containing 10 mM lithium bromide (LiBr) at a flow rate of 0.35 mL/min at 60 ºC. The $M_{n,SEC}$ and M_w/M_n were calculated based on the PEG standard (Agilent Technologies, Wilmington, DE, USA). Aqueous phase SEC was carried out on a Gulliver system (Jasco, Tokyo, Japan) equipped with an internal refractive index detector (RI-2031 plus, Jasco) and a combination of TSKgel α-4000 and α-2500 columns (Tosoh), eluted with 10 mM phosphate buffer containing 700 mM sodium chloride (NaCl) at pH 7.4 at a flow rate of 0.7 mL/min at 35 ºC. ^{1}H nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance III 500 MHz spectrometer (Bruker BioSpin,

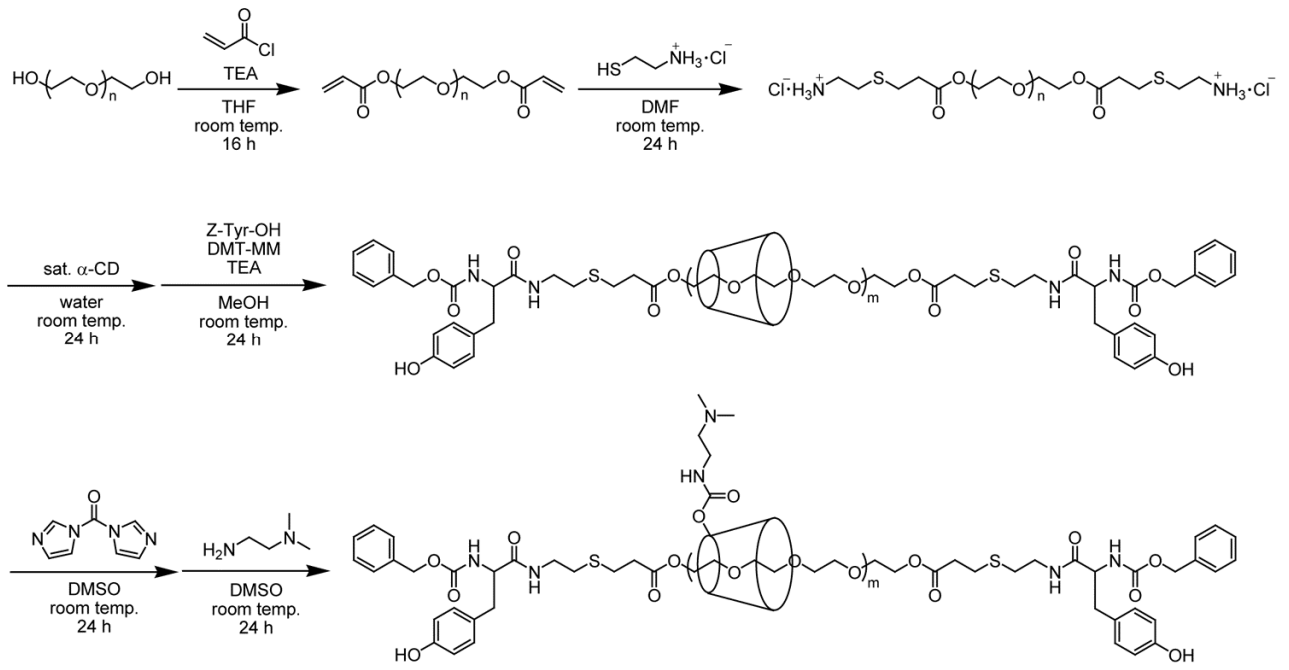

Figure S1. Preparation scheme of acid-degradable cationic polyrotaxanes.

Rheinstetten, Germany) in D_2O containing 0.1 M sodium deuteroxide (NaOD) and 0.05 wt% sodium 3-(trimethylsilyl)-propionic acid-*2,2,3,3-d*$_4$ (Sigma-Aldrich), chloroform-d_1 ($CDCl_3$) (Sigma-Aldrich), or DMSO-d_6 (Sigma-Aldrich) at room temperature.

3. Synthesis of α,ω-bisacryloyl PEG (PEG-acrylate). The PEG (10.0 g, 2.2 mmol) was dissolved in anhydrous tetrahydrofuran (THF, 133 mL) under nitrogen atmosphere. Then, TEA (4.6 mL, 33.0 mmol) and acryloyl chloride (1.8 mL, 22.0 mmol) were successively added to the flask at 0 ºC, and the system was stirred for 16 h at room temperature. After the reaction, the polymer was poured into diethyl ether to precipitate the polymer. This purification process was repeated three times to remove the unreacted reagents. The recovered polymer was dried under reduced pressure to obtain α,ω-bisacryloyl PEG (PEG-acrylate) (9.15 g, 89.4% yield). The degree of functionality of the acryloyl groups was found to be 98%, as determined by ^{1}H NMR spectroscopy. SEC (DMSO containing 10 mM LiBr) M_n = 4,710, M_w/M_n = 1.06 (calculated M_n = 4,890); ^{1}H NMR (500 MHz, $CDCl_3$, **Figure S2A**) δ = 3.61 (m, 413H, PEG backbone), 4.29 (t, J = 4.8 Hz, 4H, -C***H*****$_2$**-O-C(=O)-), 5.82 (dd, J = 1.4 and 10.4 Hz, 2H, -CH=C***H*****$_2$**), 6.13 (dd, J = 10.4 and 17.3 Hz, 2H, -C***H***=CH_2), 6.41 (dd, J = 1.4 and 17.3 Hz, 2H, -CH=C***H*****$_2$**).

4. Synthesis of α,ω-bisamino PEG bearing 3-sufanylpropionyl ester linkers (PEG-COO-NH$_2$). The PEG-acrylate (9.0 g, 1.98 mmol) and cysteamine hydrochloride (4.49 g, 39.6) were loaded into a round-bottomed flask and dissolved in anhydrous *N*,*N*-dimethylformamide (DMF, 120 mL). The

system was stirred for 24 h at room temperature. After the reaction, DMF was evaporated and diluted with water. The polymer was extracted by chloroform. The combined organic layer was dried over sodium sulfate, and poured into diethyl ether to precipitate the polymer. The recovered polymer was dried under reduced pressure to obtain acid-cleavable α,ω-bisamino-PEG (PEG-COO-NH_2) (7.53 g, 79.8% yield). The degree of functionality of the amino groups was found to be 98%, as determined by the ^{1}H NMR spectroscopy. SEC (DMSO containing 10 mM LiBr, **Figure S3B**) M_n = 3,630, M_w/M_n = 1.07 (calculated M_n = 4,890); ^{1}H NMR (500 MHz, $CDCl_3$, **Figure S2B**) δ = 2.69 (t, J = 6.6 Hz, 4H, -C(=O)-C***H*****$_2$**-CH_2-S-), 2.86 (t, J = 6.6 Hz, 4H, -C(=O)-CH_2-C***H*****$_2$**-S-), 2.93 (t, J = 7.1 Hz, 4H, -C***H*****$_2$**-CH_2-NH_3), 3.18 (m, 4H, -CH_2-C***H*****$_2$**-NH_3), 3.61 (m, 413H, PEG backbone), 4.29 (m, J = 4.4 Hz, 4H, -C***H*****$_2$**-O-C(=O)-), 7.94 (m, 6H, -CH_2-CH_2-N***H*****$_3$**).

5. Synthesis of *Z*-tyrosine-terminated acid-degradable polyrotaxane (PRX-COO-Tyr). A saturated solution of α-CD was prepared by dissolving α-CD (6.0 g, 6.17 mmol) in water (41.4 mL). The PEG-COO-NH_2 (1.0 g, 205 μmol) dissolved in small aliquot of water was added to the α-CD saturated solution, and the system was stirred for 18 h at room temperature, during which a white precipitate of polypseudorotaxane was obtained. After the reaction, the system was freeze-dried for 1 day to obtain polypseudorotaxane as a white powder. Then, Z-Tyr-OH (2.58 g, 8.18 mmol), DMT-MM (2.26 g, 8.18 mmol), and TEA (1.14 mL, 8.18 mmol) were dissolved in 56 mL of methanol, and this was added to the polypseudorotaxane. The resulting reaction mixture was stirred for 24 h at room temperature. After the reaction, the precipitate was collected by centrifugation (7,000 rpm, 10 min) and dissolved in DMSO. This solution was poured into water to precipitate the PRX, which was then collected by centrifugation (7,000 rpm, 10 min). This purification process was repeated three times to remove the unreacted reagents, α-CD, and PEG. The recovered PRX was diluted with water and freeze-dried to obtain acid-degradable PRX-COO-Tyr (2.17 g, 39.1% yield based on PEG mol%). The number of CDs threaded onto the PEG was found to be 22.2 by comparing the ^{1}H NMR peak area between 4.9-5.1 ppm (H_1 proton of α-CD) and 3.4-4.1 ppm (-C***H*****$_2$**C***H*****$_2$**O- of the PEG axis and H_2, H_3, H_4, H_5, and H_6 protons of α-CD). The CD coverage on the PEG was calculated to be 43.1%, assuming a coverage of one CD cavity to two ethylene oxide units. SEC (DMSO containing 10 mM LiBr, **Figure S3C**) M_n = 17,100, M_w/M_n = 1.08 (calculated M_n = 27,100); ^{1}H NMR (500 MHz, 0.1 M NaOD/D_2O) δ = 3.4-4.1 (m, -C***H*****$_2$**C***H*****$_2$**O- of PEG and H_2, H_3, H_4, H_5, and H_6 protons of α-CD), 4.9-5.1 ppm (m, H_1 proton of α-CD), 6.58 (d, Tyr), 6.97 (d, Tyr), 7.44 (m, Z group).

6. Synthesis of *N*,*N*-dimethylaminoethyl group-modified acid-degradable polyrotaxane (DMAE-COO-PRX). DMAE-COO-PRXs with the various number of DMAE groups were prepared by varying the feed [CDI]/[CD] molar ratio. The procedure for the synthesis of DMAE-

COO-PRX (72DMAE-COO-PRX) was as follows: PRX (150 mg, 5.53 μmol) was dissolved in anhydrous DMSO (7.5 mL). CDI (239 mg, 1.48 mmol) was added to this solution and stirred for 24 h at room temperature. Then, DMAEA (138 μL, 1.48 mmol) was added to the reaction mixture and stirred for a further 24 h at room temperature. After the reaction, DMAE-PRX was purified by dialysis against methanol for 3 days (Spectra/Por 6, molecular weight cut-off of 10,000) (Spectrum Laboratories). The recovered solution was concentrated and diluted with water. This aqueous solution was lyophilized to obtain DMAE-PRX as a white powder (171 mg, 87.1% yield). The number of modified DMAE groups on PRX was calculated by ^{1}H NMR peak area between 1.8-2.7 ppm (-N(C***H*****$_3$**$)_2$ and -C***H*****$_2$**-N$(CH_3)_2$ of DMAE group) and 4.9-5.5 ppm (H_1 proton of α-CD). The $M_{n,NMR}$ of DMAE-PRX was calculated based on the numbers of threading CDs and DMAE groups. ^{1}H NMR (500 MHz, D_2O) δ = 2.25 (m, -N(C***H*****$_3$**$)_2$ of DMAE group), 2.21 (m, -C***H*****$_2$**-N$(CH_3)_2$ of DMAE group), 3.27 (m, -C***H*****$_2$**-CH_2-N of DMAE group), 3.4-4.6 (m, -C***H*****$_2$**C***H*****$_2$**O- of PEG and H_2, H_3, H_4, H_5, and H_6 protons of α-CD), 4.9-5.4 ppm (m, H_1 proton of α-CD), 6.74 (d, Tyr), 7.08 (d, Tyr), 7.41 (m, Z group).

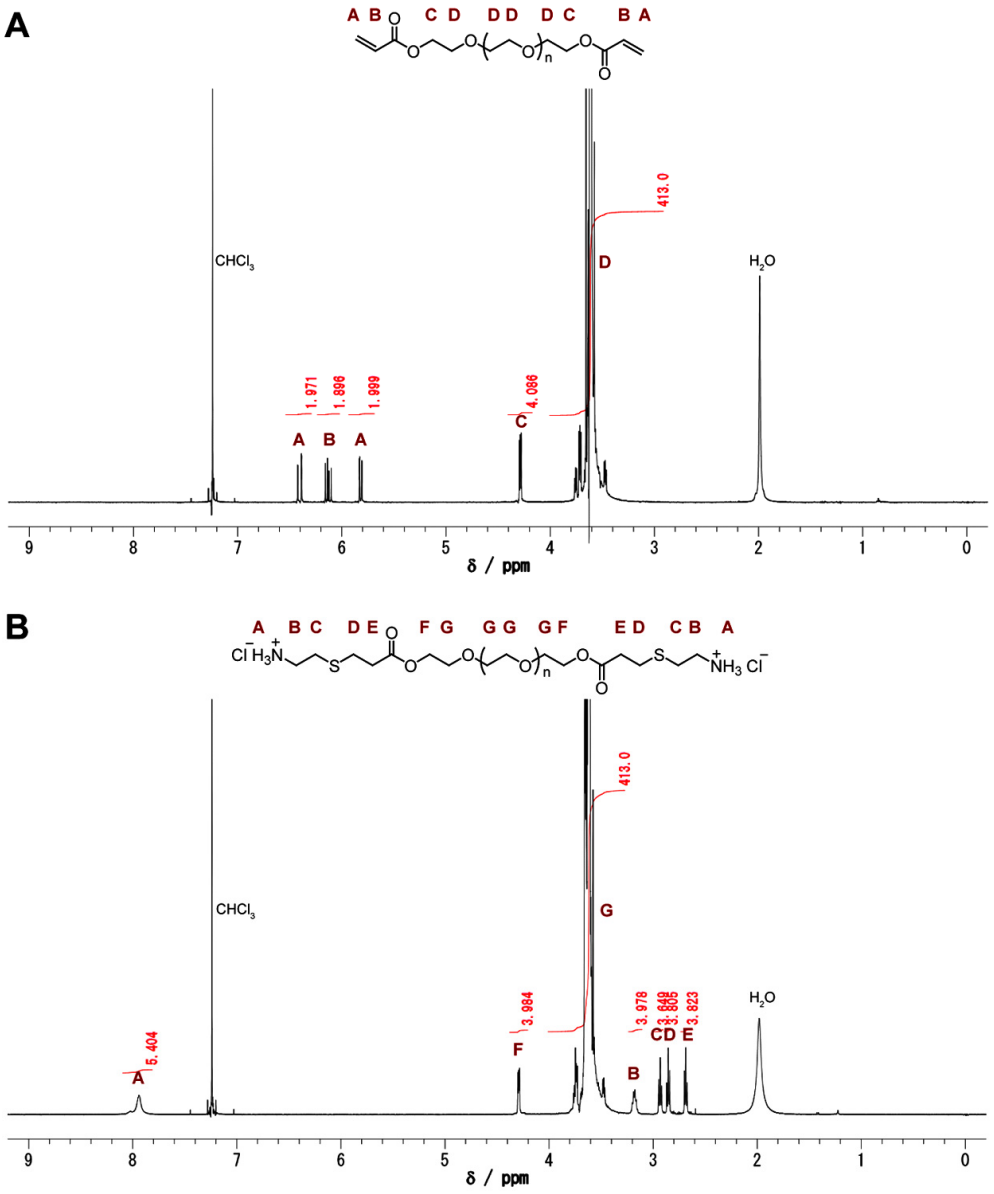

Figure S2. ^{1}H NMR spectra of PEG-acrylate (A) and PEG-COO-NH_2 (B) in $CDCl_3$.

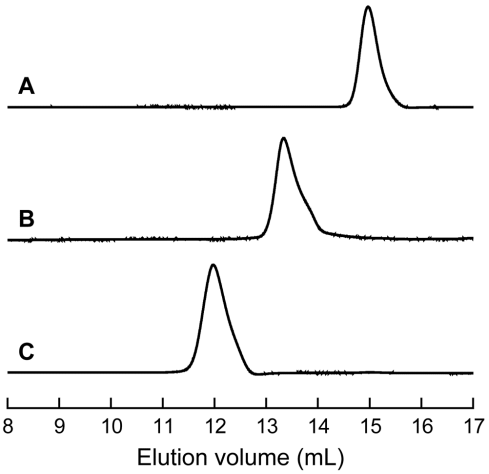

Figure S3. SEC charts of α-CD (A), PEG-COO-NH_2 (B), and PRX-COO-Tyr (C) eluted with DMSO containing 10 mM LiBr at 60 ºC.

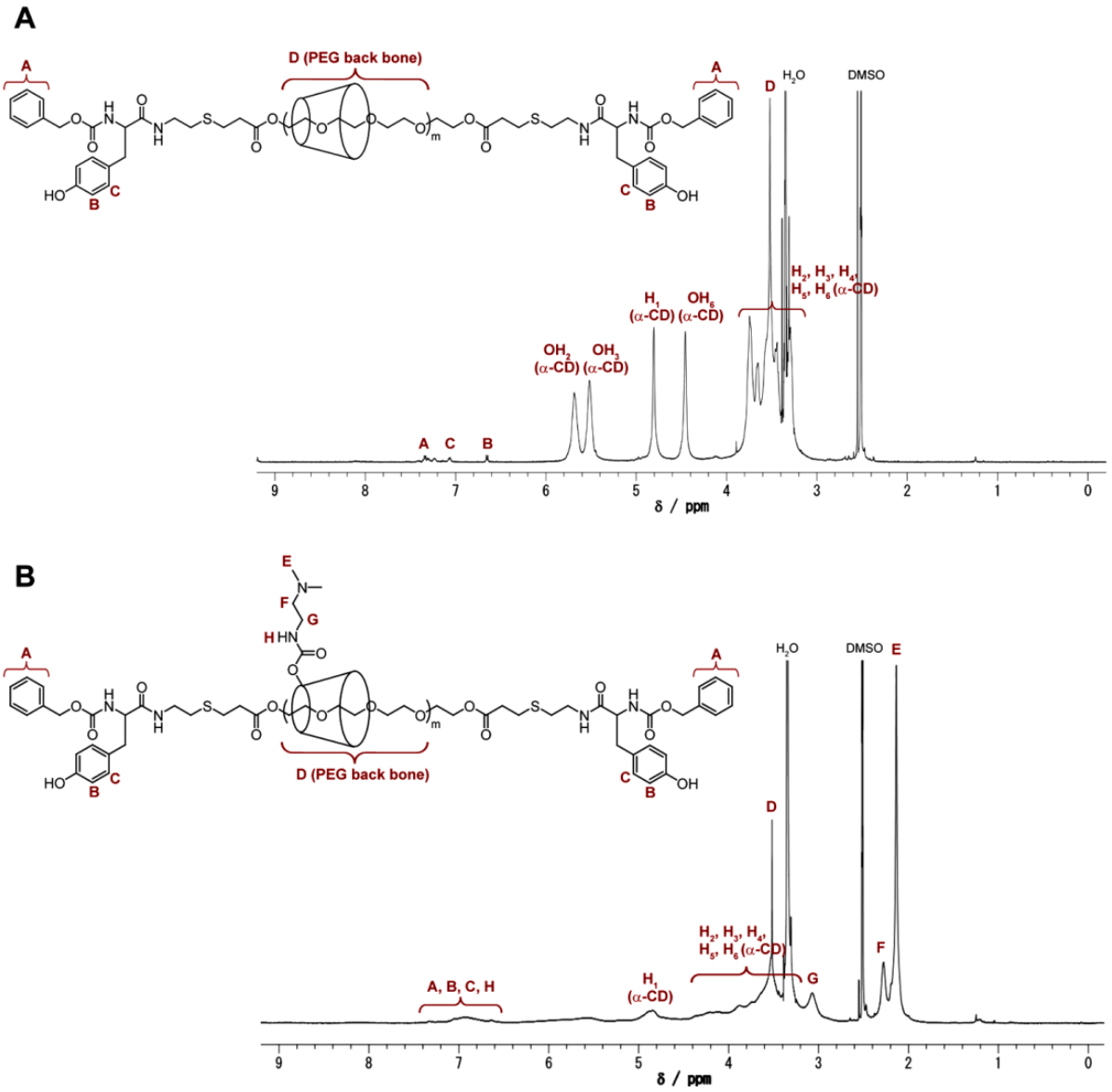

Figure S4. ^{1}H NMR spectra of PRX-COO-Tyr (A) and 72DMAE-COO-PRX (B) in DMSO-d_6.

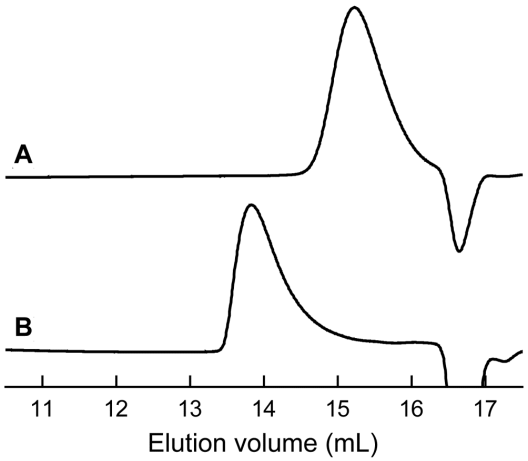

Figure S5. SEC charts PEG-COO-NH_2 (a) and 72DMAE-COO-PRX (b) eluted with 10 mM PB containing 700 mM NaCl (pH 7.4) at 35 ºC.

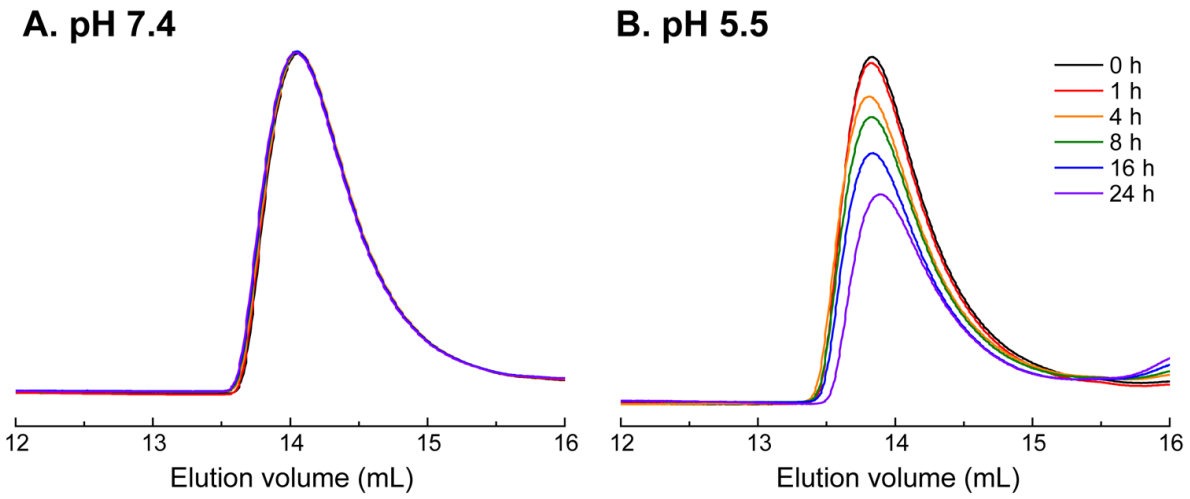

Figure S6. Time-course of SEC charts of 72DMAE-COO-PRX in pH 7.4 (A) and 5.5 (B) at 37 ºC.

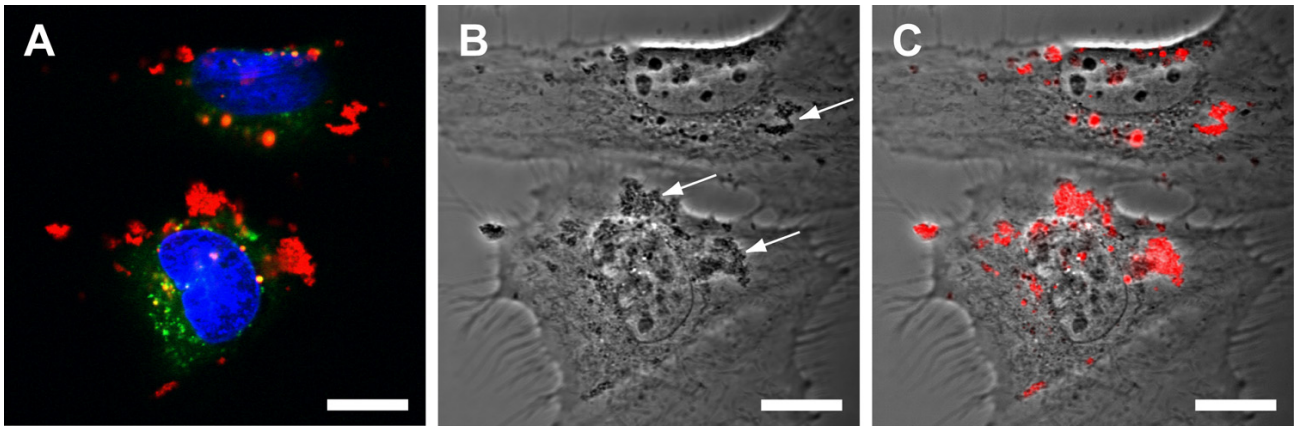

Figure S7. (A-C) Confocal laser scanning microscopic (A,C) and phase contrast microscopic (B,C) images of HeLa cells incubated with 21DMAE-COO-PRX/siRNA (N/P 10) for 6 h, followed by 42 h incubation without samples (scale bars: 20 μm). The red signals represent Alexa647-siRNA. The cell nuclei and late endosomes/lysosomes were stained with Hoechst 33342 (blue) and LysoTracker Green (green), respectively. The concentration of Alexa647-siRNA in the medium was adjusted to 100 nM. Arrows indicate the aggregation of 21DMAE-COO-PRX/siRNA adsorbed on the surface of cells.