Supplementary Information

Lysosomal-specific Cholesterol Reduction by Biocleavable Polyrotaxanes for Ameliorating Niemann-Pick Type C Disease

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Methods

Reagents. Pluronic P123, a triblock copolymer of poly(ethylene glycol) (PEG)-*b*-poly(propylene glycol) (PPG)-*b*-PEG, was obtained from Sigma-Aldrich (Milwaukee, WI, USA). The number of monomer repeating units in PEG and PPG segments were determined to be 25.2 × 2 ($M_{n,PEG}$: 1,100 × 2) and 71.5 ($M_{n,PPG}$: 4,150), respectively, by ^{1}H nuclear magnetic resonance (NMR) spectroscopy. β-Cyclodextrin (β-CD), acryloyl chloride, 2-aminoethanol, cystamine dihydrochloride, cysteamine hydrochloride, *N*,*N*-dimethylaminoethyl amine (DMAEA), 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM), and triethylamine (TEA) were obtained from Wako Pure Chemical Industries (Osaka, Japan). Cystamine dihydrochloride was desalted just before use according to a previously described procedure[S1]. Fluorescein isothiocyanate ethylenediamine (FITC-EDA) was synthesized according to a previously described procedure[S2]. *N*,*N'*-Carbonyldiimidazole (CDI), heptakis(2,6-di-*O*-methyl)-β-cyclodextrin (DM-β-CD), (2-hydroxypropyl)-β-cyclodextrin (HP-β-CD, averaged molecular weight of 1,460), and *N*-(triphenylmethyl)glycine (Trt-Gly-OH) were obtained from Sigma-Aldrich. 3,9-Bis(3-aminopropyl)-2,4,8,10-tetraoxaspiro[5.5]undecane was obtained from TCI (Tokyo, Japan). Other solvents and reagents were obtained from Kanto Chemicals (Tokyo, Japan). The Milli-Q water used in this study was prepared using an ultrapure water purification system (Academic A10) (Millipore, Billerica, MA).

Characterization of polymers and polyrotaxanes. Size exclusion chromatography (SEC) was carried out on an 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 from the calibration curve of PEG standard (Agilent Technologies, Wilmington, DE, USA). ^{1}H NMR spectra were recorded on a Bruker Avance III 500 MHz spectrometer (Bruker BioSpin, Rheinstetten, Germany) in $CDCl_3$ or DMSO-d_6 (Sigma-Aldrich) at room temperature.

Synthesis of α,ω-biscarbonylimidazolyl Pluronic P123 (P123-CI). Pluronic P123 (20.0 g, 3.14 mmol) and CDI (19.8 g, 62.8 mmol) were loaded into a round-bottomed flask and dissolved in 270 mL of tetrahydrofuran (THF). The solution was stirred for 24 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. Finally, the recovered polymer was dried under reduced pressure to obtain α,ω-biscarbonylimidazolyl Pluronic P123 (P123-CI) (19.75 g, 95.9% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ = 1.13 (m, 214H, C$\boldsymbol{H_3}$ of PPG), 3.40 (m, 71H, -CH_2-C***H***- of PPG), 3.54 (m, 142H, -C$\boldsymbol{H_2}$-CH- of PPG), 3.64 (m, 202H, -C$\boldsymbol{H_2}$-C$\boldsymbol{H_2}$-O- of PEG), 4.56 (t, 4H, -C$\boldsymbol{H_2}$-O-C(=O)-), 7.07 (m, 2H, -C(=O)-N-CH=C***H***-N-), 7.44 (m, 2H, -C(=O)-N-C***H***=CH-N-), 8.16 (s, 2H, -C(=O)-N-C***H***= N-).

Synthesis of α,ω-bisamino Pluronic P123 bearing disulfide linkers (P123-SS-NH_2). Desalted cystamine (2.02 g, 27.4 mmol) was loaded into a round-bottomed flask and dissolved in 72 mL of *N*,*N*-dimethylformamide (DMF). Then, P123-CI (9.0 g, 1.37 mmol) dissolved in 8 mL of DMF was added dropwise into the flask. The solution was stirred for 24 h at room temperature. After the reaction, the polymer was purified by dialysis against methanol for 3 days (Spectra/Por 6, molecular weight cut-off of 1,000) (Spectrum Laboratories). Finally, the solvent was removed under reduced pressure to obtain α,ω-bisamino Pluronic P123 bearing disulfide linkages (P123-SS-NH_2) (4.95 g, 53.3% yield). ^{1}H NMR (500 MHz, $CDCl_3$, **Figure S2B**) δ = 1.16 (m, 214H, C$\boldsymbol{H_3}$ of PPG), 2.69 (m, 4H, -O(=O)-NH-CH_2-C$\boldsymbol{H_2}$-S-S-), 2.87 (t, 4H, -S-S-C$\boldsymbol{H_2}$-CH_2-NH_2), 2.93 (t, 4H, -S-S-CH_2-C$\boldsymbol{H_2}$-NH_2), 3.20 (t, 4H, -O(=O)-NH-C$\boldsymbol{H_2}$-CH_2-S-S-), 3.40 (m, 71H, -CH_2-C***H***- of PPG), 3.54 (m, 142H, -C$\boldsymbol{H_2}$-CH- of PPG), 3.64 (m, 202H, -C$\boldsymbol{H_2}$-C$\boldsymbol{H_2}$-O- of PEG), 4.30 (t, 4H, -C$\boldsymbol{H_2}$-O(=O)-NH-).

Synthesis of α,ω-bisamino Pluronic P123 (P123-NH_2). Ethylenediamine (1.65 g, 27.5 mmol) was loaded into a round-bottomed flask and dissolved in 72 mL of *N*,*N*-dimethylformamide (DMF). Then, α,ω-biscarbonylimidazole pluronic P123 (9.0 g, 1.37 mmol) dissolved in 8 mL of DMF was added dropwise into the flask. The solution was stirred for 24 h at room temperature. After the reaction, the polymer was purified by dialysis against methanol for 3 days (Spectra/Por 6, molecular weight cut-off of 1,000). Finally, the solvent was removed under reduced pressure to obtain α,ω-bisamino Pluronic P123 (P123-NH_2) (5.85 g, 65.2% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ =

1.16 (m, 214H, CH_3 of PPG), 3.40 (m, 71H, -CH_2-C**H**- of PPG), 3.54 (m, 142H, -CH_2-CH- of PPG), 3.64 (m, 202H, -CH_2-CH_2-O- of PEG), 4.35 (t, 4H, -CH_2-O(=O)-NH-).

Synthesis of α,ω-bisamino Pluronic P123 bearing cyclic acetal linkers (P123-ace-NH_2). 3,9-Bis(3-aminopropyl)-2,4,8,10-tetraoxaspiro[5.5]undecane (6.81 g, 24.8 mmol) was loaded into a round-bottomed flask and dissolved in 100 mL of *N*,*N*-dimethylformamide (DMF). Then, α,ω-biscarbonylimidazole pluronic P123 (8.14 g, 1.24 mmol) dissolved in 10 mL of DMF was added dropwise into the flask. The solution was stirred for 24 h at room temperature. After the reaction, the polymer was purified by dialysis against methanol for 3 days (Spectra/Por 6, molecular weight cut-off of 1,000). Finally, the solvent was removed under reduced pressure to obtain α,ω-bisamino Pluronic P123 bearing cyclic acetals (P123-ace-NH_2) (7.0 g, 80.9% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ = 1.16 (m, 214H, CH_3 of PPG), 1.57 (m, 4H, -CH_2-O(=O)-NH-CH_2-CH_2-), 1.66 (m, 12H), 2.72 (t, 4H, -CH_2-NH_2), 3.20 (m, 4H, -CH_2-O(=O)-NH-CH_2-), 3.31-3.60 (m, 229H, -CH_2-C**H**- of PPG, -CH_2-CH- of PPG, -O-CH_2-C), 3.64 (m, 202H, -CH_2-CH_2-O- of PEG), 4.23 (t, 4H, -CH_2-O(=O)-NH-), 4.49 (m, 4H, -O-CH_2-C), 4.55 (m, 4H, -CH_2-C**H**-), 4.92 (m, 2H, -O-CH_2-C).

Synthesis of α,ω-bisamino Pluronic P123 bearing ester linkers (P123-COO-NH_2). The Pluronic P123 (10.0 g, 1.57 mmol) was dissolved in anhydrous tetrahydrofuran (THF, 133 mL) under a nitrogen atmosphere. Then, TEA (3.3 mL, 23.5 mmol) and acryloyl chloride (1.28 mL, 15.7 mmol) were successively added to the flask at 0 ºC, and the system was stirred for 24 h at room temperature. After the reaction, the polymer was purified by dialysis against methanol for 3 days (Spectra/Por 6, molecular weight cut-off of 1,000). Finally, the solvent was removed under reduced pressure to obtain α,ω-bisacryloyl P123 (P123-acrylate) (6.12 g, 60.2% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ = 1.13 (m, 214H, CH_3 of PPG), 3.40 (m, 71H, -CH_2-C**H**- of PPG), 3.54 (m, 142H, -CH_2-CH- of PPG), 3.64 (m, 202H, -CH_2-CH_2-O- of PEG), 4.28 (t, 4H, -CH_2-O-CO-), 5.81 (dd, 2H, -CH=CH_2), 6.13 (dd, 2H, -C**H**=CH_2), 6.41 (dd, 2H, -CH=CH_2).

The P123-acrylate (5.0 g, 0.53 mmol) and cysteamine hydrochloride (1.2 g, 10.6 mmol) were loaded into a round-bottomed flask and dissolved in anhydrous *N*,*N*-dimethylformamide (DMF, 40 mL). The system was stirred for 24 h at room temperature. After the reaction, DMF was evaporated and diluted with water, and the polymer was purified by dialysis against methanol for 3 days (Spectra/Por 6, molecular weight cut-off of 1,000). Finally, the solvent was removed under reduced pressure to obtain α,ω-bisamino Pluronic P123 bearing ester linkages (P123-COO-NH_2). ^{1}H NMR (500 MHz, $CDCl_3$) δ = 1.10 (m, 214H, CH_3 of PPG), 2.69 (t, 4H, -CO-CH_2-CH_2-S-), 2.86 (t, 4H, -CO-CH_2-CH_2-S-), 2.93 (t, 4H, -CH_2-CH_2-NH_3), 3.18 (m, 4H, -CH_2-CH_2-NH_3), 3.40 (m, 71H, -CH_2-C**H**- of PPG), 3.54 (m, 142H, -CH_2-CH- of PPG), 3.64 (m, 202H, -CH_2-CH_2-O- of PEG), 4.29 (t, 4H, -CH_2-O-CO-), 7.93 (m, 6H, -CH_2-CH_2-NH_3).

Synthesis of polyrotaxane. Typical procedure for the synthesis of PRX bearing disulfide linkages (SS-PRX) is as follows: first, a saturated solution of β-CD was prepared by dissolving β-CD (12.0 g, 10.57 mmol) in 600 mL of phosphate buffer saline (PBS) (Sigma). Then, P123-SS-NH_2 (1.0 g, 147 μmol) dissolved in a small aliquot of water was added to the β-CD saturated solution, and the system was stirred for 24 h at room temperature, during which a white precipitate of pseudopolyrotaxane was obtained. After the reaction, the precipitate was collected by centrifugation (7,000 rpm, 10 min) and freeze-dried for 1 day to obtain pseudopolyrotaxane as a white powder. Then, Trt-Gly-OH (1.63 g, 5.14 mmol) and DMT-MM (1.63 g, 5.87 mmol) were dissolved in 56 mL of methanol/DMF (1/4 v/v), and this solution was added to the pseudopolyrotaxane. 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 successively washed with DMF and water. This washing process was repeated four times to completely remove the free β-CD and unreacted reagents. The recovered PRX was freeze-dried to obtain SS-PRX (697.2 mg, 20.0% yield based on P123 mol%). The number of β-CDs threaded onto the P123 was determined from the ^{1}H NMR peak area between 4.84 ppm (H_1 proton of β-CD) and 1.06 ppm (-C$\boldsymbol{H_3}$ of the P123 axle). ^{1}H NMR (500 MHz, DMSO-d_6) δ = 1.06 (m, -C$\boldsymbol{H_3}$ of P123), 3.0-3.9 (m, -C$\boldsymbol{H_2}$C$\boldsymbol{H_2}$O- and -C$\boldsymbol{H_2}$-C$\boldsymbol{H}$- of P123 and H_2, H_3, H_4, H_5, and H_6 protons of β-CD), 4.44 ppm (m, OH_6 proton of β-CD), 4.84 ppm (m, H_1 proton of β-CD), 5.5-5.9 ppm (m, OH_2 and OH_3 protons of β-CD), 7.21 (t, Trt group), 7.32 (t, Trt group), 7.41 (d, Trt group).

Synthesis of hydroxyethyl group-modified polyrotaxane. The typical procedure for the synthesis of hydroxyethyl group-modified SS-PRX (HE-SS-PRX) is as follows: SS-PRX (250 mg, 11.2 μmol) was dissolved in 10 mL of anhydrous DMSO. CDI (235 mg, 1.45 mmol) was added to this solution and stirred for 24 h at room temperature. Then, HEA (439 μL, 7.25 mmol) was added to the reaction mixture and stirred for a further 24 h at room temperature. After the reaction, the PRX was purified by dialysis against methanol for 3 days (Spectra/Por 6, molecular weight cut-off of 3,500). The recovered solution was concentrated and diluted with water. This aqueous solution was lyophilized to obtain HE-SS-PRX as a white powder (231.5 mg, 76.8% yield). The number of modified HE groups on PRX was calculated by the ^{1}H NMR peak area between 3.04 ppm (-C$\boldsymbol{H_2}$-CH_2-OH of HE group) and 4.9-5.5 ppm (H_1 proton of β-CD). The $M_{n,NMR}$ of HE-SS-PRX was calculated based on the numbers of threaded CDs and HE groups. ^{1}H NMR (500 MHz, DMSO-d_6) δ = 1.06 (m, -C$\boldsymbol{H_3}$ of P123), 3.04 ppm (-C$\boldsymbol{H_2}$-CH_2-OH of HE group), 3.1-4.5 (m, -C$\boldsymbol{H_2}$C$\boldsymbol{H_2}$O- and -C$\boldsymbol{H_2}$-C$\boldsymbol{H}$- of P123 and H_2, H_3, H_4, H_5, and H_6 protons of β-CD), 4.63 ppm (m, OH_6 proton of β-CD), 4.84 (m, H_1 proton of β-CD), 5.5-5.9 ppm (m, OH_2 and OH_3 protons of β-CD), 7.00 (m, -O-CO-N$\boldsymbol{H}$-CH_2-CH_2-OH of HE group) 7.22 (t, Trt group), 7.32 (t, Trt group), 7.42 (d, Trt group).

Synthesis of fluorescein isothiocyanate-labeled HE-SS-PRX (FITC-HE-SS-PRX)[S3]. HE-SS-PRX (30 mg, 1.15 μmol) was dissolved in 3 mL of anhydrous DMSO. CDI (4.83 mg, 29.8 μmol) was added to this solution and stirred for 24 h at room temperature to activate the hydroxyl groups. Then, FITC-EDA (2.67 mg, 5.95 μmol) was added to the reaction mixture and stirred for a further 24 h at room temperature. After the reaction, the solution was purified by dialysis against water for 3 days (Spectra/Por 6, molecular weight cut-off of 3,500). Finally, the aqueous solution was lyophilized to obtain fluorescein-labeled HE-SS-PRX (FITC-HE-SS-PRX) (13.2 mg). The number of modified fluorescein molecules on PRX was determined by the absorbance of fluorescein at 494 nm, and approximately 0.52 fluorescein molecules were labeled on HE-SS-PRX (0.04 fluoresceins/β-CD.

Synthesis of fluorescein isothiocyanate-labeled HP-β-CD (FITC-HP-β-CD). HP-β-CD (200 mg, 136 μmol) was dissolved in 10 mL of anhydrous DMSO. CDI (66.6 mg, 411 μmol) was added to this solution and stirred for 24 h at room temperature to activate the hydroxyl groups. Then, FITC-EDA (61.6 mg, 137 μmol) was added to the reaction mixture and stirred for an additional 24 h at room temperature. After the reaction, the solution was purified by dialysis against water for 10 days to completely remove unreacted FITC-EDA (Spectra/Por 6, molecular weight cut-off of 1,000). Finally, the aqueous solution was lyophilized to obtain fluorescein-labeled HP-β-CD (FITC-HP-β-CD) (16.9 mg). The number of modified fluorescein molecules on HP-β-CD was determined to be 0.83. Then, the FITC-HP-β-CD was mixed with non-labeled HP-β-CD to adjust the number of fluorescein molecules on β-CD with FITC-HE-SS-PRX (0.04 fluorescein/HP-β-CD).

References.

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S2. Nukolova, N. V., Oberoi, H. S., Cohen, S. M., Kabanov, A. V. & Bronich, T. K. Folate-decorated nanogels for targeted therapy of ovarian cancer. *Biomaterials* **32**, 5417–5426 (2011).

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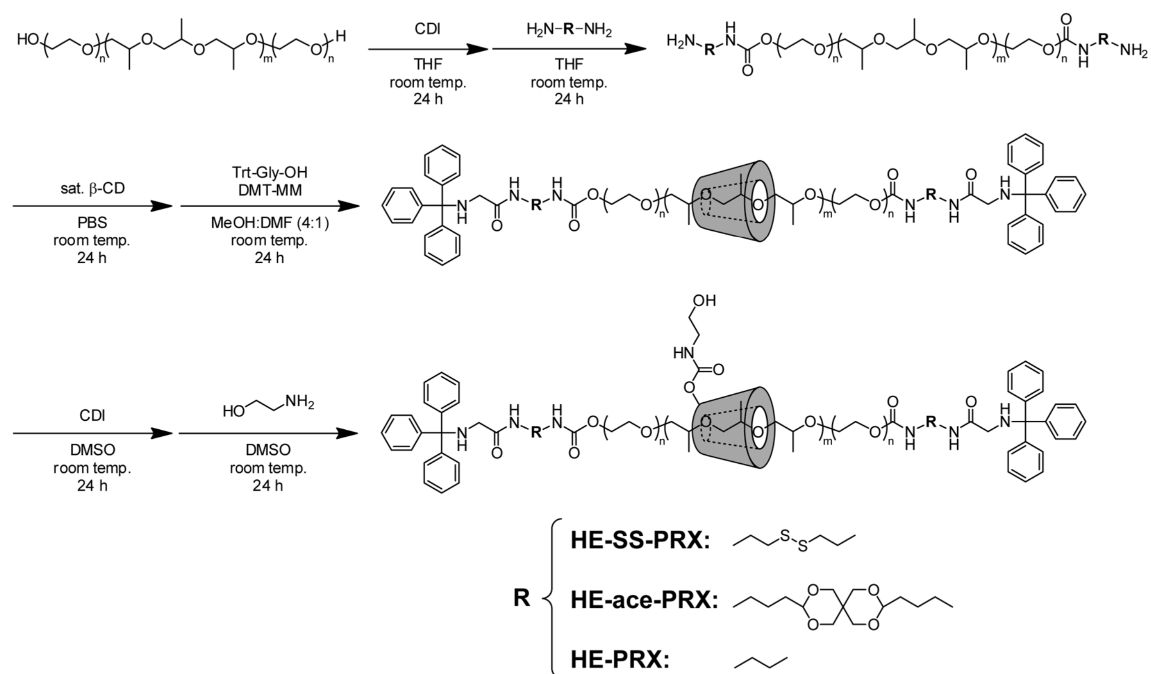
A



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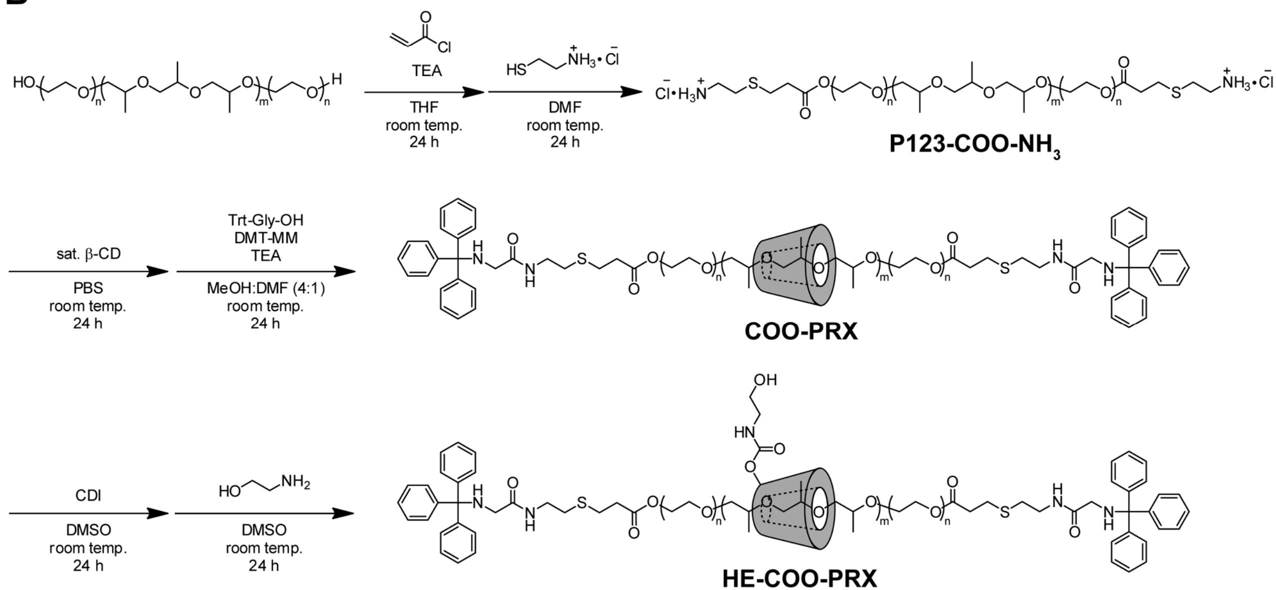

Figure S1. (A) Preparation scheme of HE-SS-PRX, HE-ace-PRX, and non-degradable HE-PRX. (B) Preparation scheme of HE-COO-PRX.

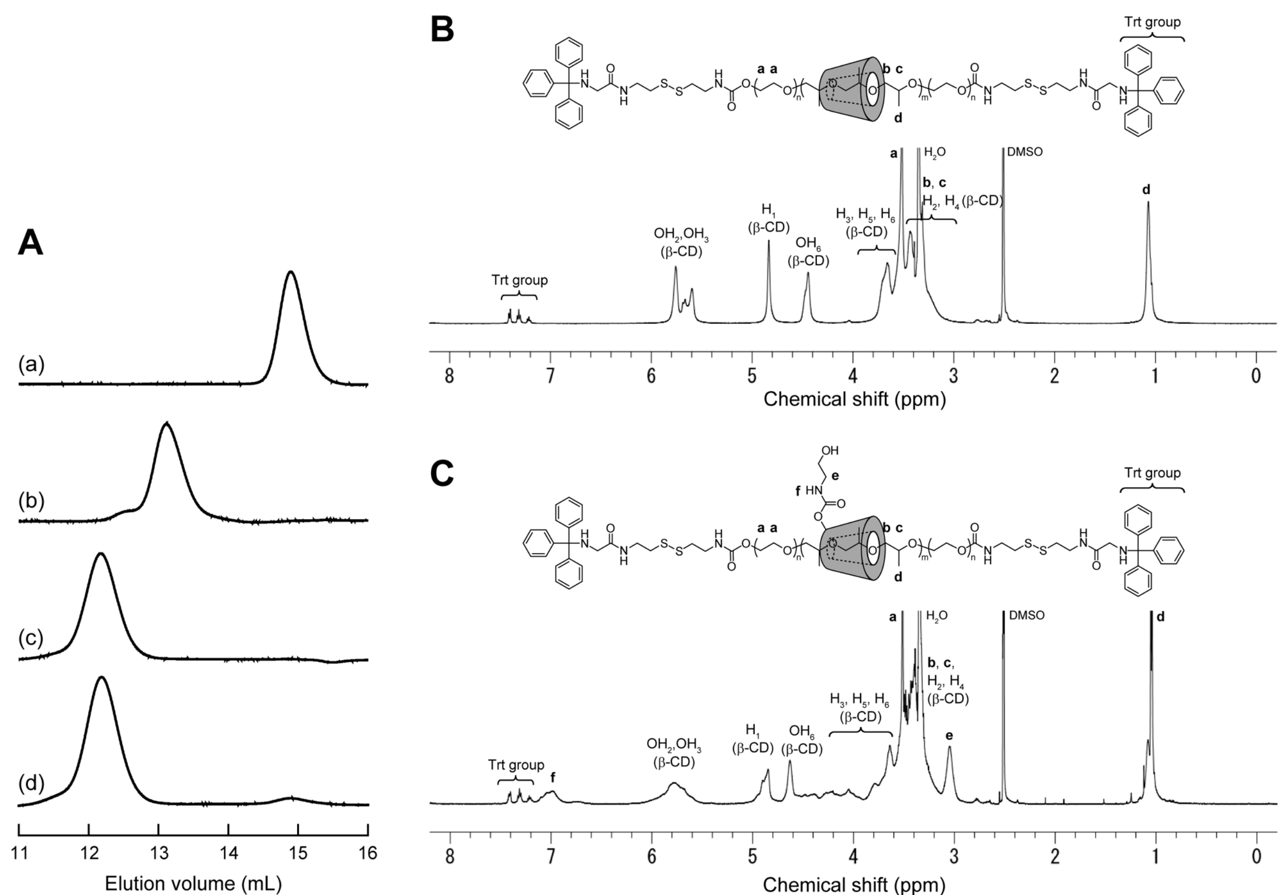

Figure S2. (A) SEC charts of β-CD (a), P123-SS-NH_2 (b), SS-PRX (c), HE-SS-PRX (d) in DMSO at 60 °C. (B, C) ^{1}H NMR spectra of SS-PRX (B) and HE-SS-PRX (C) in DMSO-d_6.

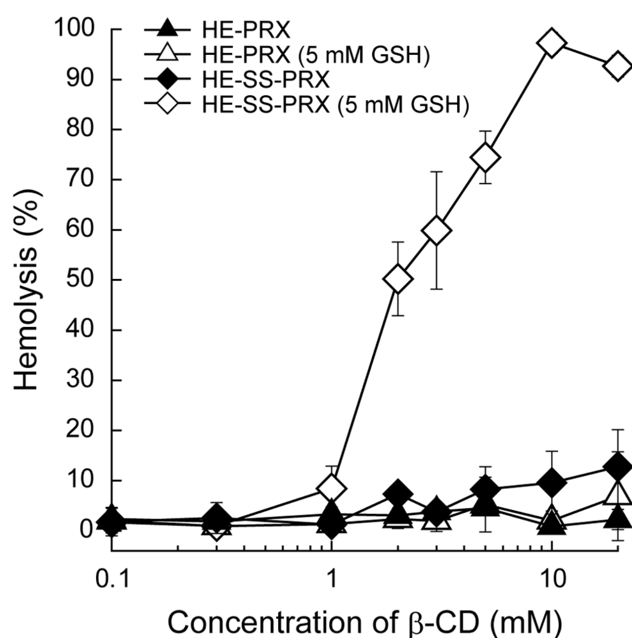

Figure S3. Hemolysis of rat erythrocytes incubated with non-degradable HE-PRX (triangles) or degradable HE-SS-PRX (diamonds) at various β-CD concentrations in the presence (open symbols) or absence (closed symbols) of 5 mM glutathione (GSH) for 3 h at 37 ºC. Data are expressed as the mean ± S.D. (n = 3).

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sample code	Number of threaded β-CDs[a]	Number of HE groups[a]	$M_{n, NMR}$[b]	EC_{50} (μM)
HE-SS-PRX	12.9	53.4	26,900	24.2 ± 2.3
HE-COO-PRX	11.7	65.9	26,500	48.6 ± 6.5
HE-ace-PRX	12.9	66.9	28,100	131.4 ± 59.9
HE-PRX	11.3	65.3	25,700	> 10,000

[a]Determined by ^1H NMR in DMSO-d_6. [b]Calculated based on the chemical composition determined by ^1H NMR.

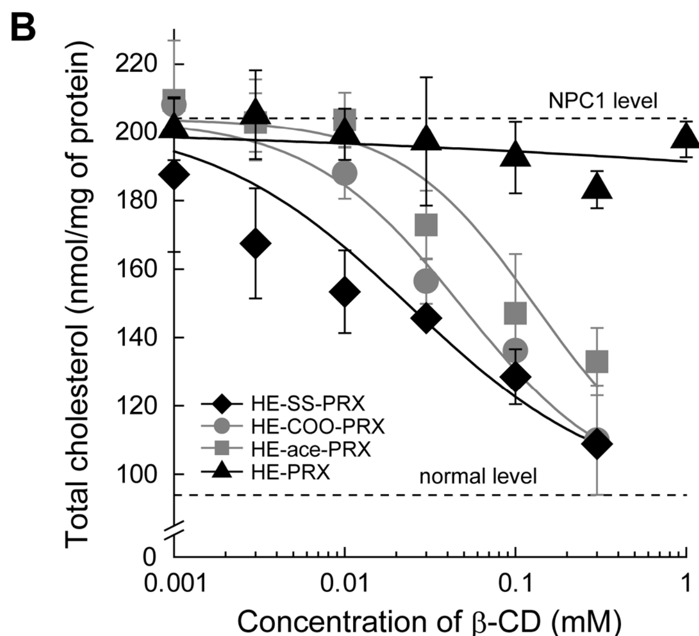

Figure S4. (A) Characterization of PRXs and their EC_{50} value in reducing accumulated cholesterols in NPC1 cells. (B) The amount of intracellular total cholesterol in NPC1 cells incubated with HE-SS-PRX, HE-COO-PRX, HE-ace-PRX, and non-degradable HE-PRX at various β-CD concentrations for 24 h at 37 ºC. The dashed lines represent the amount of intracellular total cholesterol in non-treated normal and NPC1 cells. Data are expressed as the mean ± S.D. (n = 3).

Table S1. The sequence of primer sets for real-time RT-PCR.

Target gene	Forward	Reverse
human SREBP-2 (NM_004599.3)	5'-ACA ACC CAT AAT ATC ATT GAG AAA CG-3'	5'-TTG TGC ATC TTG GCG TCT GT-3'
human HMGCR (NM_000859.2)	5'-GCC TGG CTC GAA ACA TCT GAA-3'	5'-CTG ACC TGG ACT GGA AAC GGA TA-3'
human HMGCS (NM_001098272.2)	5'-GTA TGC CCT GGT AGT TGC AGG AG-3'	5'-TGT TGC ATA TGT GTC CCA CGA A-3'
human LDLR (NM_000527.4)	5'-CAA CGG CTC AGA CGA GCA AG-3'	5'-AGT CAC AGA CGA ACT GCC GAG A-3'
human ACTB (NM_001101.3)	5'-TGG CAC CCA GCA CAA TGA A-3'	5'-CTA AGT CAT AGT CCG CCT AGA AGC A-3'