Supporting Information

Biocleavable Polyrotaxane – Plasmid DNA Polyplex for Enhanced Gene Delivery

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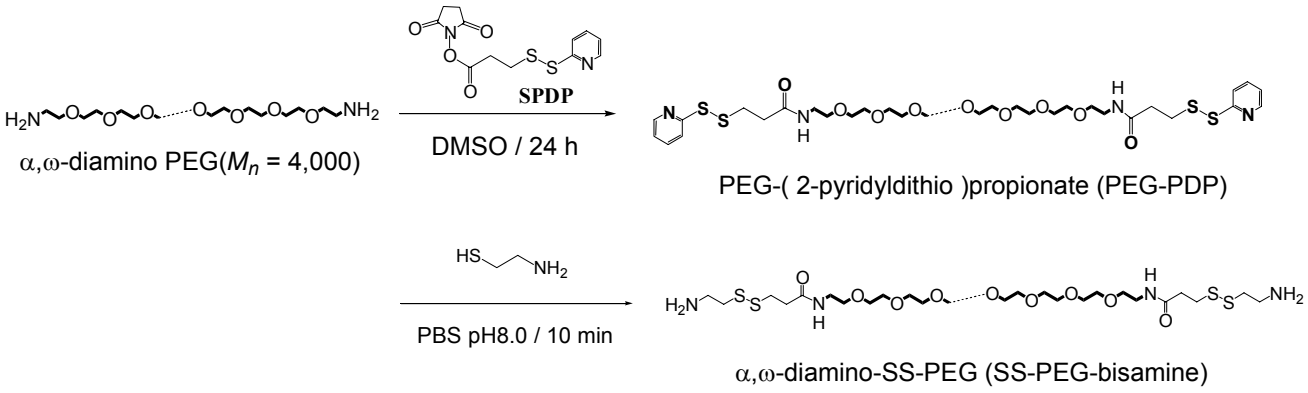
Chemicals

α-Cyclodextrin (α-CD) was purchased from Bio-Research Corporation of Yokohama (Yokohama, Japan). *N,N'*-Diisopropylethylamine (DIEA) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Wako Pure Chemical Co. Ltd (Osaka, Japan). α,ω-Diamino PEG (M_n = 4,000) was kindly supplied by Sanyo Chemical Co. (Kyoto, Japan). Benzotriazol-1-yloxytris(dimethylamino)phosohonium hexafluorophosphate (BOP reagent), DTT, *N,N'*-carbonyldiimidazole (CDI) and 1-hydroxybenzotriazole (HOBt) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). *N*-Succinimidyl-3-(2-pyridyldithio)propionate (SPDP) was purchased from Dojindo Laboratories (Kumamoto, Japan). Z-L-Tyr was purchased from Kokusan Chemical Co., Ltd (Tokyo, Japan). pDNA containing a firefly luciferase gene together with cytomegarovirus (CMV) promoter and NIH3T3 cells were kept in our laboratories and used as is. The pDNA, pCMV-luc (8454 bp) was amplified in the *Escherichia coli,* then isolated and purified using a Quiagen Plasmid Mega kit (Quiagen, Hilden, Germany). Plasmid DNA was labeled with rhodamine using a Label IT Labeling Kit (Pan Vera, Madison, WI, USA). LysoSensor DND-189 and Hoechst 33258 were purchased from Molecular Probes (Eugene, OR, USA) and Wako Chemicals (Osaka, Japan), respectively. Dual Luciferase Assay Kit was purchased from Promega (Madison, WI).

Preparation of α,ω-diamino-SS-PEG (SS-PEG-bisamine) (Scheme S1)

Scheme S1 Preparation of SS-PEG-bisamine.



The α,ω-diamino PEG (*Mn* = 4,000) (0.13 g , 3.25×10^{-5} mol) and SPDP (0.1 g, 3.2×10^{-4} mol) were dissolved in 10 mL of DMSO and stirred for 24 h at room temperature under nitrogen atmosphere. The reaction mixture was dialyzed against water to obtain PEG-(2-pyridyldithio)propionate (PEG-PDP) (0.097 g).

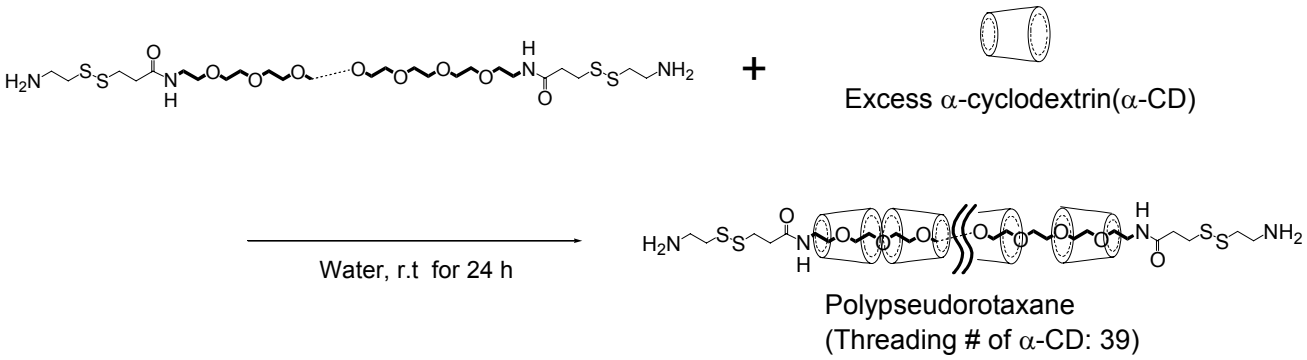
^{1}H-NMR (DMSO-d_6, 300 MHz) δ = 7.21~7.98 (m, pyridine), 3.76 (t, -O$\underline{CH_2}$$CH_2$NHCOCH$_2CH_2$SS-Py), 3.50 (s, CH_2CH_2O of PEG), 3.09 (t, -OCH$_2$CH$_2$NHCO$\underline{CH_2}$CH$_2$SS-Py), 3.01 (t, -OCH$_2$CH$_2$NHCOCH$_2\underline{CH_2}$SS-Py), 1.63 (t, --OCH$_2\underline{CH_2}$NHCOCH$_2$CH$_2$SS-Py).

The obtained PEG-PDP (0.3 g, 6.85×10^{-5} mol) was dissolved in 20 mL of 0.1 M PBS (pH 8.0), and the solution was degassed by nitrogen gas bubbling. After that, 2-aminoethanthiol (0.26 g, 3.43×10^{-3} mol) was added to the solution and stirred for 10 min under nitrogen atmosphere. The reaction mixture was then dialyzed against 3% saline and lyophilized to obtain α,ω-diamino-SS-PEG (SS-PEG-bisamine) as a white powder (0.23 g).

^{1}H-NMR (DMSO-d_6, 300 MHz) δ = 3.68 (t, -O$\underline{CH_2}$CH$_2$NHCOCH$_2$CH$_2$SSCH$_2$CH$_2$NH$_2$), 3.50 (s, CH_2CH_2O of PEG), 3.33 (m, -OCH$_2$CH$_2$NHCOCH$_2$CH$_2$SSCH$_2$CH$_2\underline{NH_2}$), 3.21 (t, -OCH$_2$CH$_2$NHCOCH$_2$CH$_2$SS$\underline{CH_2}$CH$_2$NH$_2$), 3.03~3.0 (m, -OCH$_2$CH$_2$NHCO$\underline{CH_2CH_2}$SSCH$_2$CH$_2$NH$_2$), 2.88 (brt, -OCH$_2$CH$_2$NHCOCH$_2$CH$_2$SSCH$_2\underline{CH_2}$NH$_2$), 1.57 (m, -OCH$_2\underline{CH_2}$NHCOCH$_2$CH$_2$SSCH$_2$CH$_2$NH$_2$)

Preparation of polypseudorotaxane (Scheme S2)

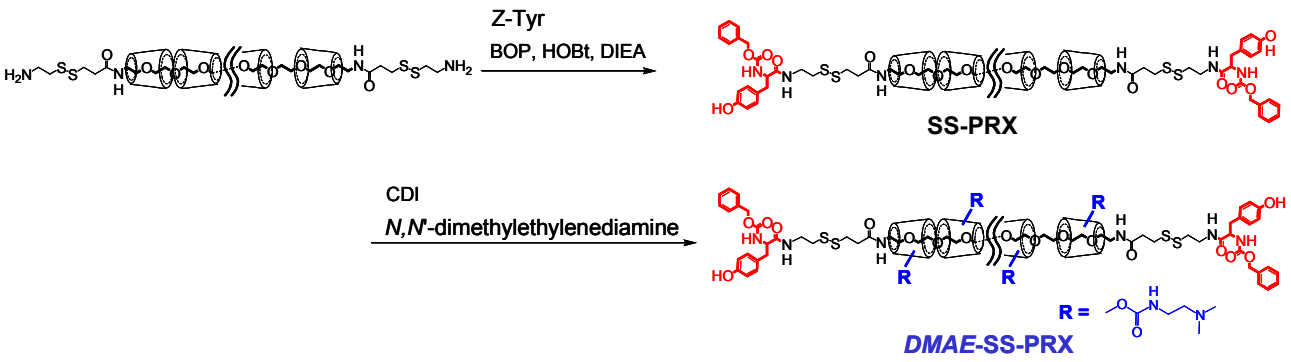
Scheme S2 Preparation of polypseudorotaxane



The SS-PEG-bisamine (0.23 g, 5.3×10^{-5} mol) dissolved in small amount of water was added to saturated solution of α-CDs (7.25g / 50 mL of distilled water). The solution was stirred for 24 h at room temperature, and white precipitate was obtained during stirring. The precipitate was collected by centrifugation and dried *in vacuo* at 60 °C to obtain a polypseudorotaxane (2.18 g). From a ^{1}H-NMR spectrum, the number of α-CDs was calculated to be *ca.* 39 by comparing peak integrations between C(1)H of α-CD and methylene protons of PEG. ^{1}H-NMR (DMSO-d_6, 300 MHz) δ = 5.4~5.6 (m, C(2)H of α-CD), 4.90 (m, C(1)H of α-CD), 4.49 (m, C(6)H of α-CD), 3.72~3.21 (m, C(3)H C(5)H, C(6)H, C(2)H, and C(4)H of α-CD) 3.50 (s, CH_2CH_2O of PEG).

Synthesis of DMAE-SS-PRX and DMAE-PRX (Scheme S3)

Scheme S3 Synthesis of DMAE-SS-PRX



Z-L-Tyr (0.82 g, 2.6×10^{-3} mol), BOP reagent (1.15 g , 2.6×10^{-3} mol), HOBt (0.35 g, 2.6×10^{-3} mol) and DIEA (0.45 mL, 2.6×10^{-3} mol) were dissolved in 3.5 mL of DMF, and then the polypseudorotaxane (2.18 g, 5.2×10^{-5} mol) was directly added to the solution. The suspension was stirred for 24 h at room temperature. During stirring, the heterogeneous condition was maintained. After the reaction, the suspension was poured into excess methanol to precipitate the crude product, and the precipitate was collected by centrifugation. Then, the precipitate was washed with acetone and water two times, respectively. After that, the obtained white precipitate was dried *in vacuo* to obtain a Z-L-Tyr-capped polyrotaxane (SS-PRX, 1.24 g). From a ^{1}H-NMR spectrum, the number of α-CDs was calculated to be *ca.* 23 by comparing peak integrations between C(1)H of α-CD and methylene protons of PEG. The SS-PRX (1.24 g, 4.56×10^{-5} mol) was dissolved in 20 mL of dry DMSO, and CDI (3.06 g, 1.89×10^{-2} mol) was added to the solution. The mixture was stirred for 3 h under nitrogen atmosphere, and then *N,N'*-dimethylethylenediamine (DMAE, 8.2 mL) was slowly added to the solution. After stirring for 13h, the reaction mixture was poured into 500 mL of ether to precipitate the crude product. The precipitate was washed with ether and acetone, and dialyzed against 2% $NaCl_{aq}$ and water. After that, the solution was lyophilized to obtain a DMAE-SS-PRX (0.79 g). DMAE-PRX was synthesized in a similar manner by using the α,ω-diamino PEG (*Mn* = 4,000) as a linear polymer instead of the SS-PEG-bisamine.

^{1}H-NMR (DMSO-d_6, 300 MHz) δ = 5.4~5.6 (m, C(2)H of α-CD), 4.90 (m, C(1)H of α-CD), 4.49 (m, C(6)H of α-CD), 3.7~3.2 (m, C(3)H, C(5)H, C(6)H, C(2)H, and C(4)H of α-CD), 3.52 (s, CH_2CH_2O of PEG), 2.4~2.25 (s, C2H, C3H of DMAE), 2.1 (s, CH_3 of DMAE).

GPC data (Figure S1 and Table S1)

Firure S1 GPC charts of DMAE-SS-PRX (black line) and DMAE-α-CD (blue line). The molecular weight was analyzed by MALS-GPC equipped with a HPLC systems 2-line degasser DG-980-51, Intelligent pump PU-980, Column oven CO-965, RI detector RI-930 (JASCO, Co. Ltd, Tokyo, Japan), a triple-angle light scattering detector (miniDAWN, Wyatt Technology Corp., CA) and an ultrahydrogel linear column (Water Corp., Milford, MA). The eluent was HPLC grade water, and flow rate was 0.5 ml/min.

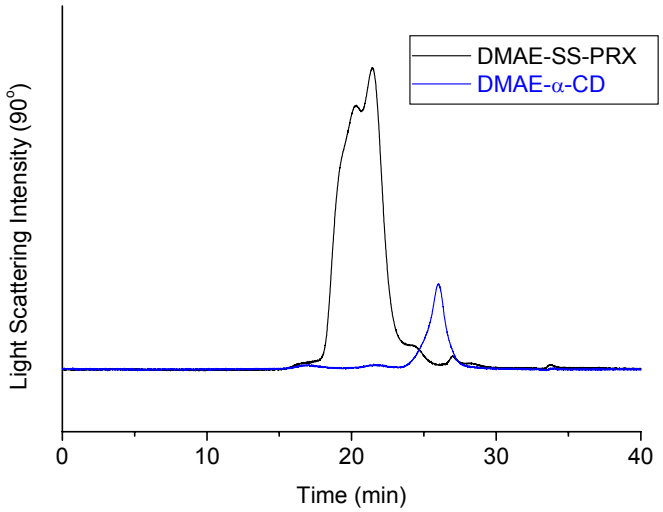


Table S1. Molecular Weight of DMAE-SS-PRX and DMAE-α-CD

Sample Code	NMR	MALS-GPC		
	M_n [a]	M_n [b]	M_w [b]	M_w/M_n
DMAE-SS-PRX	31,800	32,600	38,400	1.18
DMAE-α-CD	1,200	1,280	1,310	1.02

[a] Determined by ^{1}H-NMR spectra in d_6-DMSO.

[b] *dn/dc* of DMAE-SS-PRX and DMAE-α-CD was 0.106 and 0.105, respectively, which was determined by using a differential refractometer (DRM-3000, Otsuka Electronics Co. Ltd.)

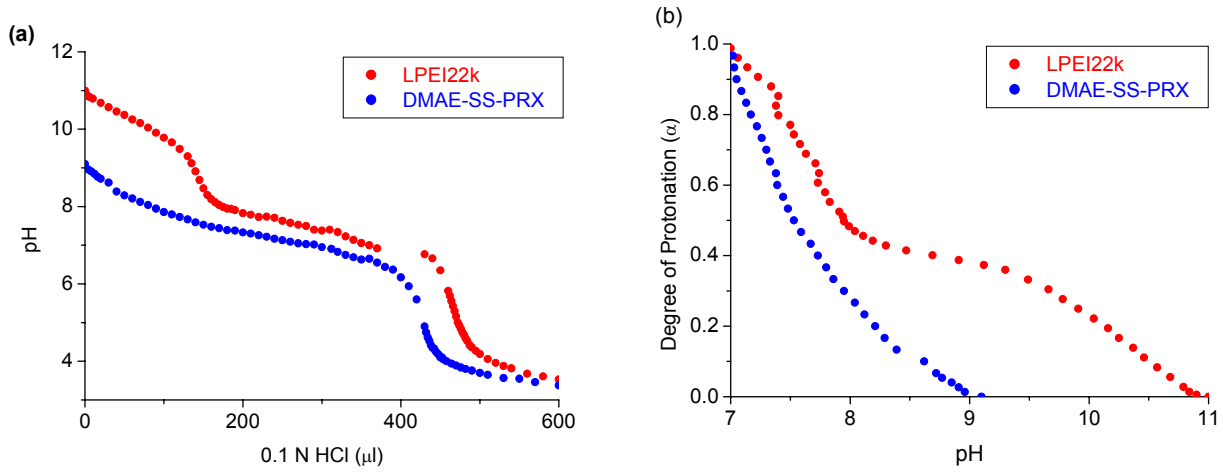
Titration of DMAE-SS-PRX and LPEI22k for determining pKa

The p*Ka* values were determined by potentiometric titration (Figure S2). A solution of DMAE-SS-PRX (24 mg, 0.03 mmol of DMAE) in 30 ml of water (pH 9) was directly titrated by using 0.1 M HCl. As for the LPEI22k, to a solution of LPEI22k (22 mg) in 3 ml of water was added 0.1M HCl to completely dissolve. After adding water to be 100 mM of amine (~ 1 ml), 250 μl of the dissolved solution was added to water (25 ml) to be 1 mM of amine. The pH of the solution was adjusted to be 11 using 1M NaOH and then titrated by using 0.1 M HCl. The p*Ka* value was calculated by the following equations:

$$pKa = pH - \log\left[\frac{(1-\alpha)}{\alpha}\right] \qquad \alpha = \frac{[protonated\ AMINEs]}{[Total\ AMINEs]}$$

where α is degree of protonation. From Figure S2 (b) and the above equations, p*Ka* of DMAE-SS-PRX and LPEI22k was calculated to be 7.5 and 8.0, respectively. The average p*Ka* value of LPEI22k was well consistent with the reported value (~7.9, see: Brissault, B; Kichler, A; Guis, C; Leborgne, C; Danos, O; Cheradame, H. *Bioconjugae Chem.* **2003**, *14*, 581-587.).

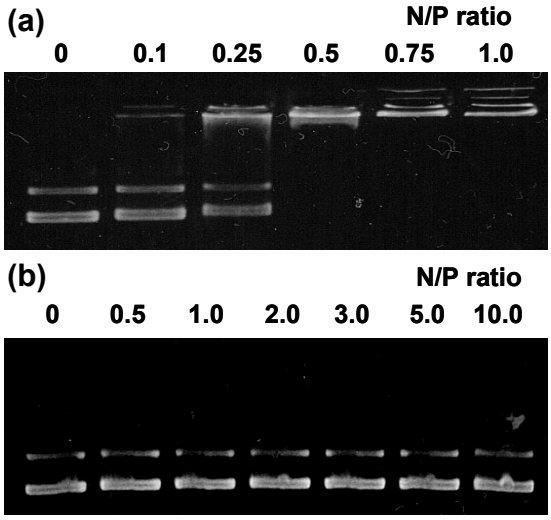
Figure S2 **(a)** Potentiometric titration curves of DMAE-SS-PRX (blue line) and LPEI22k (red line), and **(b)** the degree of protonation as a function of pH: DMAE-SS-PRX (blue line) and LPEI22k (red line).



Formation of polyplexes with DMAE-SS-PRX and pDNA

DMAE-SS-PRX/pDNA complexes were formed at a defined ratio of the highly charged amino groups to the phosphate groups of pDNA (N/P ratio) by mixing in phosphate buffer (pH 7.4). After incubation at room temperature for 20 min, the samples were electrophoresed. The polyplexes (N/P = 5) in PBS were incubated with 10 mM DTT for 0, 0.25, 0.5, 1, 2 and 3 h at 37°C to evaluate the pDNA release from the DMAE-SS-PRX polyplex. After the incubation, 1.2 μL of 73 μM dextran sulfate (*Mn* = 25,000) was added in to the solution containing the polyplexes. After incubation at room temperature for 20 min, the samples were electrophoresed using 1.0 wt % agarose gel in 40 mM Tris, 20 mM sodium acetate, and 2 mM EDTA buffer (pH 8.0) at 100 V for 20 min. Sybar-green stained bands were visualized using an UV illuminator (Bio-Rad Lab. Inc., Osaka, Japan) and photographed.

Firure S3 Agarose gel electrophoretic images of pDNA complex with DMAE-SS-PRX (a) and DMAE-α-CD (b) in TBE buffer (pH7.5).



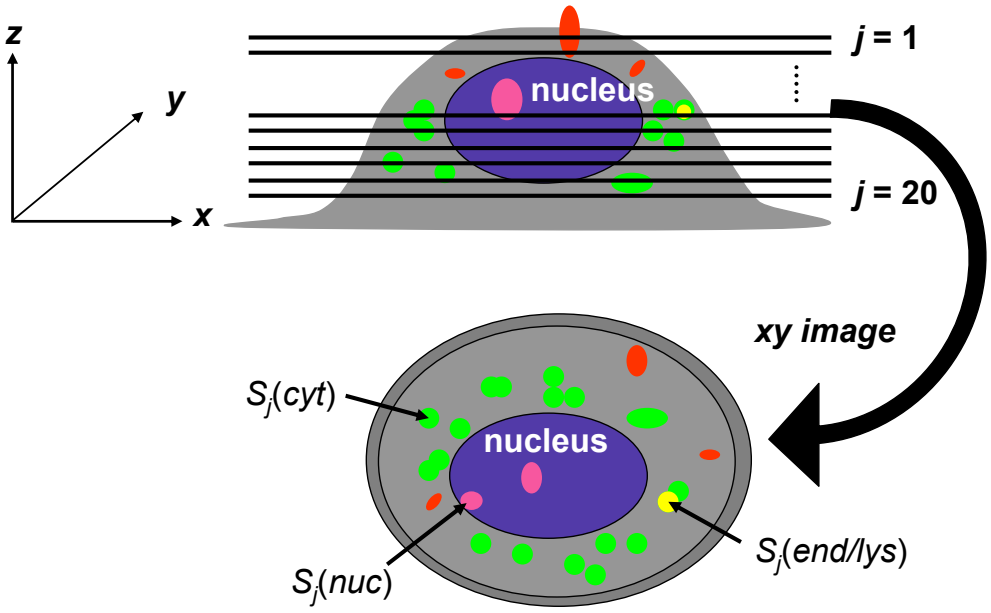
Cell culture

NIH3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (containing 1 x 10^5 mU/mL of penicillin, 0.1 mg/mL of streptmycin) supplemented with 10 % heat-inactivated fetal calf serum (FCS). Cells were grown in 10-cm dishes and incubated at 37°C under 5 % CO_2 and 95 % air atmosphere to ~ 80 % confluence. A subculture was performed every 2–3 days.

Intracellular trafficking observation

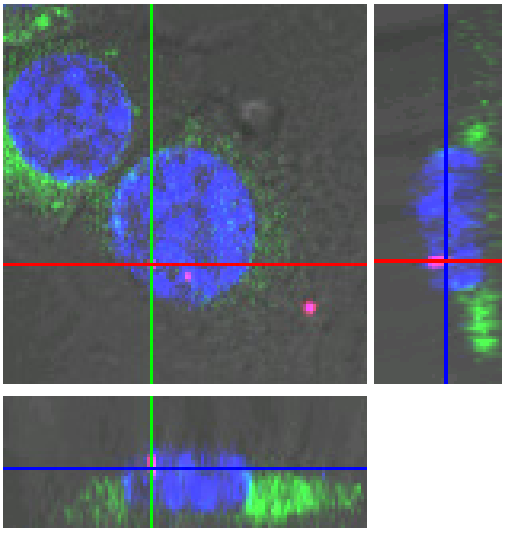
The polyplexes of rhodamine-labeled pDNA and the DMAE-SS-PRX were diluted in PBS (pH 7.4). The mixtures were allowed to stand for 20 min at room temperature before addition to NIH3T3 cells cultured at serum and antibiotics free DMEM on a poly-D-lysine coated 35-mm coverslip-bottom dish (dose of pDNA was 2.0 μg/mL while the cation-anion charge ratios were fixed at 5:1). The incubation was performed at 37°C for 1.5 h. Thirty minutes before the acquisition of the fluorescence images, LysoSensor DND-189 was applied to the transfected cells at final concentration of 1 μM to stain the acidic organelles (e.g., endosomes and lysosomes). Ten minutes before the observation, Hoechst 33258 was further applied to the transfection medium and incubated at a final concentration of 30 μM to stain the nucleus. Cells were washed three times with culture medium including 10 % FCS at the indicated times. Fluorescence and bright-field images were captured using a Zaiss Axiovert 200 inverted fluorescence microscope equipped with a 63 x NA 1.4 Planachromat objective (Carl Zaiss Co. Ltd., Jena, Germany). Twenty images obtained from the bottom of the coverslip to the top of the cells were recorded by the Zaiss LSM510 on a PC. Each 8-bit TIFF image was transferred to an AquaCosmos (Hamamatsu Photonics; Hamamatsu, Japan) to quantify the total brightness and pixel area of each region of interest as described in our previous report (Akita, H.; Ito, R.; Khalil, I. A.; Futaki, S.; Harashima, H. *Mol. Ther.* **2004**, *9*, 443-451). Schematic diagram illustrating the methodology to quantify the subcellular distribution of pDNA cluster was shown in Figure S4. Total pixel area of rhodamine-labeled pDNA in whole cell ($S_{(tot)}$) and each compartment, $S_{(k)}$ (endosome/lysosome ($S_{(end/lys)}$), cytosol ($S_{(cyt)}$), and nucleus($S_{(nuc)}$)) was calculated. The fraction of pDNA in each compartment to the whole cell is calculated as: $F_{(k)}$ (%) = $S_{(k)}$/ $S_{(tot)}$ x 100. Figure S5 shows some images of for the DMAE-SS-PRX polyplexes (N/P=5) after 90 min transfections.

Firure S4 Schematic diagram illustrating the methodology to quantify the subcellular distribution of pDNA cluster. Endosome/lysosome (green) was stained by Lysosensor and the blue fluorescence shows the Hoechst 33258-stained nuclei. The rhodamine-labeled pDNA shows a red fluorescence. If the rhodamine-labeled pDNA is located in endosome/lysosome, yellow color is observed by overlapping between green and red (i.e., endosomal digestion of pDNA).

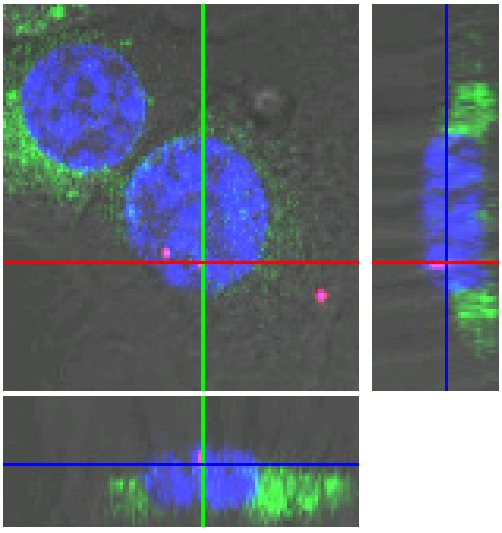


Firure S5 CLSM images of for the DMAE-SS-PRX polyplexes (N/P=5) after 90 min transfections

3D Image 1-a



3D Image 1-b



Transfection experiments

Polyplexes containing 0.4 μg pDNA suspended in 0.25 mL of DMEM without serum and antibiotics were added to 4 x 10^4 NIH3T3 cells in 24-well plates, and incubated for 3 h at 37 °C. Next, the medium containing polyplexes was removed and replaced with 1 mL of DMEM containing 10 % FCS was added to the cells,

followed by further incubation for 45 h. The cells were then washed, and solubilized with reporter lysis buffer. Luciferase activity was started by the addition of 50 μL of luciferase assay reagent into 20 μL of the cell lysate, and measured by means of a luminometer (Luminescencer-PSN, Atto Corp. Japan). The amount of protein in the cell lysate was determined using a BCA protein assay kit (PIERCE, Rockford, IL).

Cytotoxicity Assay

Cytotoxicity was evaluated by both MTT assay and total-cell count methods. Cells were grown in 96-well plates at initial density of 2,000 cells per well in 0.1 mL of growth medium for 24 h, after which the growth medium was removed and then added the 0.1 mL of serum free medium containing the polyplexes. After 3 h incubation at 37°C, the polyplexes containing medium were removed and replaced with DMEM containing 10 % FCS. The metabolic activity of the cells was measured 45 h later by using the MTT assay. Briefly, 10 μL of a 5 mg/mL MTT stock in sterile PBS was diluted for ten-folds by medium containing 10 % FCS and added to each well. After incubating the cells in the presence of MTT for 4 h, 200 μL of PBS was added to each well and removed gently by an aspirator. The obtained blue formazan crystals were dissolved in 200 μL of iso-propanol containing 4 x 10^{-2} N HCl. The absorbancies were read at 562 nm in a microplate reader (Bio-Rad Laboratories, Inc., CA, USA) and expressed as a percentage relative to control cells.

Firure S6 Cells viability of the DMAE-SS-PRX, the DMAE-PRX and LPEI22k polyplexes (n=4).