



Supporting Information

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**Supporting Information for: Charge-conversional ternary
polyplex with endosome disruption moiety: a technique for
efficient and safe gene delivery**

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Materials and Methods

1. Materials

N,N-dimethylformamide (DMF) (Wako Pure Chemical Industries, Ltd, Japan), dichloromethane (DCM) (Wako, Japan), *n*-butyl amine, ethylenediamine (1,2-diaminoethane), diethylenetriamine (bis(2-aminoethyl)amine) (Tokyo Chemical Industry Co. Ltd, Japan) were purchased and re-distilled before use. Acetic acid and hydrochloric acid were purchased and used without further purification (Wako, Japan). 1-Methyl-2-pyrrolidinone (NMP), cis-aconitic anhydride, succinic anhydride, bovine serum albumin were purchased from Sigma (St. Louis, MO). β -benzyl-L-aspartate-*N*-Carboxy-anhydride (BLA-NCA) was obtained from Nippon Oil and Fats Co., Ltd. (Tokyo, Japan).

2. Synthesis

2-1. Synthesis of PBLA (poly(β -benzyl-L-aspartate)) (2)

PBLA was prepared by the ring-opening polymerization of BLA-NCA initiated by the terminal amino group of *n*-butylamine. The *n*-butylamine (0.0417 mmol) was dissolved in 5 mL of DMF/DCM (1:10). The BLA-NCA (4.60 mmol) solution in 8 mL of DMF/DCM (1:10) was added to the solution of *n*-butylamine and the reaction mixture was stirred for 48 hr at 35°C under argon atmosphere. The resulting polymer was precipitated into diethyl ether (150 mL). The crude precipitate was washed twice with diethyl ether to obtain the final product as a white powder. From ¹H NMR measurement, the degree of polymerization (DP) of the BLA units was calculated to be 102. All the NMR spectra were recorded using a JEOL EX 300 spectrometer at 300MHz. The chemical shifts were reported in ppm downfield from tetramethylsilane. ¹H NMR (CDCl₃): δ 0.87 (3H, CH₃CH₂CH₂CH₂NH), δ 1.27 (4H, CH₃CH₂CH₂CH₂NH), δ 2.69, 3.12 (206H, COCHCH₂COOCH₂Ph, CH₃CH₂CH₂CH₂NH), δ 4.28 (102H, COCHNH), δ 5.07 (204H, COOCH₂Ph), δ 7.27 (510H, COOCH₂Ph), δ 8.88 (102H, COCHNH).

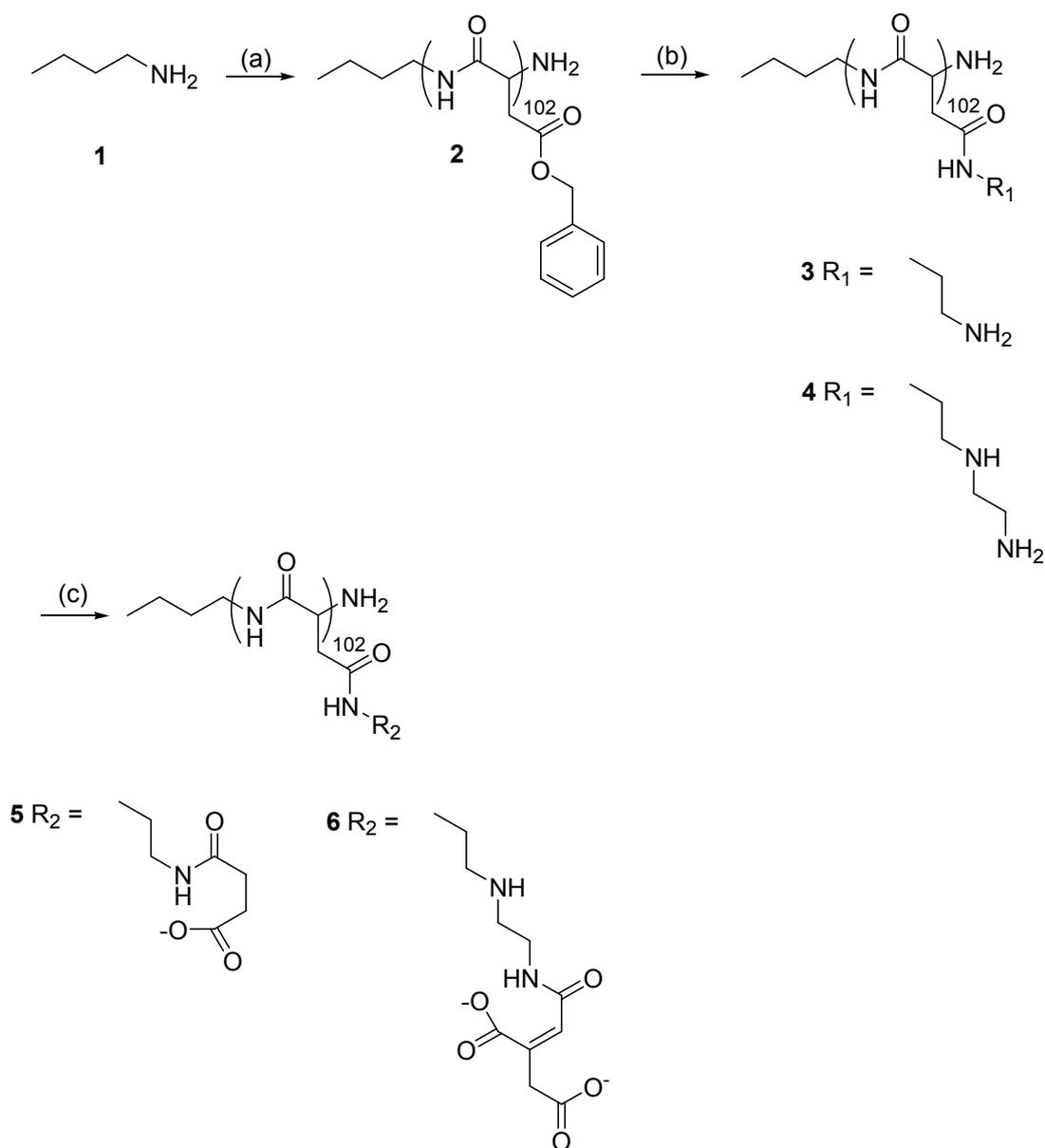
2-2. Synthesis of pAsp(EDA) (poly [(2-aminoethyl)aspartamide]) (**3**) and pAsp(DET) (poly(2-[(2-aminoethyl)amino]ethylaspartamide)) (**4**)

PBLA (0.802 mmol of benzyl ester) was dissolved in NMP (10 mL). Diethylenetriamine (DET) (40.1 mmol) was added to the solution and the reaction mixture was stirred 1 hr at 0°C. The resulting solution was added dropwise into 10% aqueous acetic acid solution (30 mL). The neutralized solution was dialyzed against 0.01M hydrochloric acid solution ($\times 3$) and distilled water ($\times 3$) at 4°C. The white powder, pAsp(DET) was obtained as hydrochloric acid salt after lyophilization. No benzyl peak was seen in ^1H NMR. The synthesis of pAsp(EDA) was similar except the use of ethylenediamine (EDA) instead of DET. ^1H NMR (D_2O): $\delta 0.87$ (3H, $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}$), $\delta 1.27$ (4H, $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}$), $\delta 2.63$ (408H, $\text{COCHCH}_2\text{CONHCH}_2\text{CH}_2\text{NHCH}_2\text{CH}_2\text{NH}_2$, $\text{COCH}_2\text{CHCONHCH}_2\text{CH}_2\text{NHCH}_2\text{CH}_2\text{NH}_2$, $\text{COCHCH}_2\text{CONHCH}_2\text{CH}_2\text{NHCH}_2\text{CH}_2\text{NH}_2$), $\delta 2.76$ (204H, $\text{COCHCH}_2\text{CONHCH}_2\text{CH}_2\text{NHCH}_2\text{CH}_2\text{NH}_2$), $\delta 2.94$ (204H, $\text{COCHCH}_2\text{CONHCH}_2\text{CH}_2\text{NHCH}_2\text{CH}_2\text{NH}_2$), $\delta 3.20$ - 3.50 (308H, $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}$, $\text{COCHCH}_2\text{CONHCH}_2\text{CH}_2\text{NHCH}_2\text{CH}_2\text{NH}_2$)

2-3. Synthesis of pAsp(EDA-Suc) (poly[(*N'*-succinyl-2-aminoethyl)aspartamide]) (**5**) and pAsp(DET-Aco) (poly(2-[(*N'*-cis-aconityl]-2-aminoethyl)amino]ethylaspartamide)) (**6**)

pAsp(DET) (0.055 mmol of primary amine) was dissolved in 0.5 M NaHCO_3 buffer (pH 9.0, 50 mL). Cis-aconitic anhydride (2.76 mmol) (Aco) was added to the solution and stirred for 3 hr at 0°C. The reaction mixture was purified with Amicon Ultra (MWCO=10,000; Millipore (Billerica, MA)) ($\times 3$ with distilled water). The final product was obtained as a white powder after lyophilization. The conversion yield was over 99%, which was calculated from ^1H NMR. The synthesis of pAsp(EDA-Suc) was similar except the use of succinic anhydride (Suc) and pAsp(EDA) instead of Aco and pAsp(DET). ^1H NMR of PEG-pAsp(DET-cisAco) (D_2O): $\delta 0.87$ (3H, $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}$), $\delta 1.27$ (4H, $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}$)

δ 1.78 (204H, COHC(COONa)CH₂COONa), δ 2.67, δ 2.76, δ 2.94, δ 3.20 (920H, CH₃CH₂CH₂CH₂NH, COCHCH₂CONHCH₂CH₂NHCH₂CH₂NH₂), δ 5.43-5.71 (102H, COHC(COONa)CH₂COONa).



Scheme S1. Synthetic scheme of pAsp(DET) (**4**), pAsp(EDA-Suc) (**5**) and pAsp(DET-Aco) (**6**).

(a) BLA-NCA, DMF/DCM; (b) DET (or EDA), NMP; (c) cis-aconitic anhydride (or succinic anhydride), pH 9.0 buffer

3. Plasmid DNA preparation

A plasmid coding for luciferase with a CAG promoter was provided by RIKEN Bioresource Center (Japan) (H. Niwa et al. *Gene*, **1991**, *108*, 193-199). Also, a fragment of SEYFP-F46L (Venus), which is a variant of yellow fluorescent protein with the mutation F46L (T. Nagai et al. *Nat. Biotechnol.*, 2002, *20*, 87-90), was provided by RIKEN and inserted into the pCacc vector (pCacc+Venus). The plasmids were amplified in competent DH5 *E. coli* and purified using HiSpeed Plasmid MaxiKit (QIAGEN Science Co., Inc., Germany). The plasmid concentration was determined by the absorption at 260 nm.

4. Ternary polyplex formation

The positive polyplex was obtained by simple mixing of pAsp(DET) (1 mg/mL) and plasmid DNA (50 µg/mL) at various N/P ratios (4~8). Here, the N/P ratio was defined as the residual molar ratio of amine units in the pAsp(DET) to phosphate units in the pDNA. After 15-min incubation at RT, the positive polyplex was added by pAsp(DET-Aco) (or pAsp(EDA-Suc)) solution (1 mg/mL). The molar ratios between pAsp(DET) and pAsp(DET-Aco) (or pAsp(EDA-Suc)) were varied from 1 to 4. The ternary polyplex was obtained after 15 min more incubation at RT.

5. DLS measurement of the polyplexes

Each polyplex was diluted in aqueous buffers. (pH 5.5 acetate buffer (10 mM) or pH 7.4 Tris-HCl buffer (10 mM)). The final concentration of the plasmid DNA was 33 µg/mL. After each sample was incubated at 37°C, dynamic light scattering (DLS) measurements were performed using Zetasizer Nano-ZS (green badge, ZEN3500, Malvern, Ltd. Malvern, U. K.) with a He-Ne ion laser of 633 nm.

The DLS distribution of DNA/pAsp(DET) positive polyplex (N/P ratio = 6) and DNA/pAsp(DET)/pAsp(DET-Aco) ternary polyplex (N/P ratio = 6) at pH 7.4 are shown below:

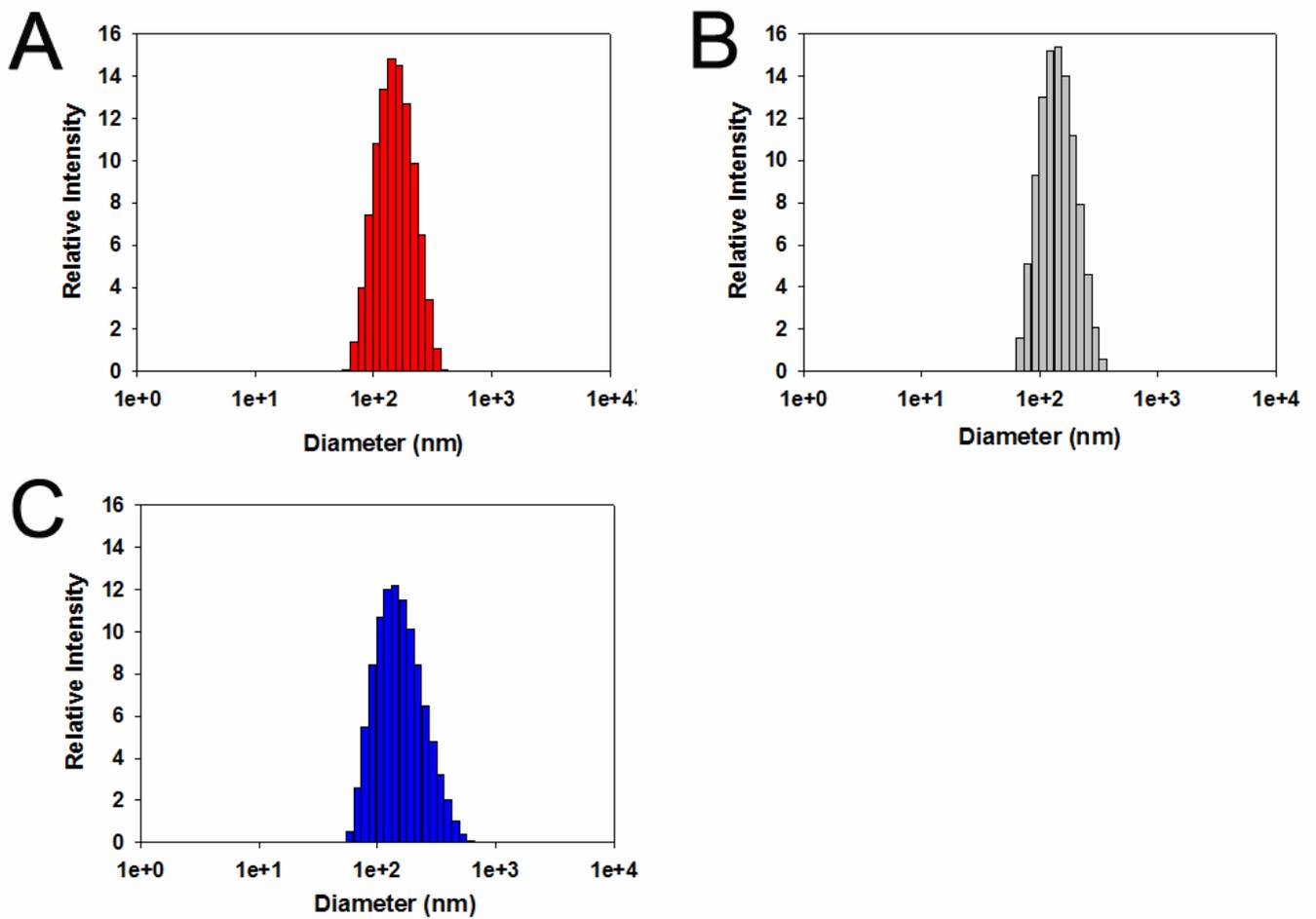


Figure S1. Size distribution of the DNA/pAsp(DET) polyplex (N/P ratio = 4) (A), the pAsp(DET)/pAsp(DET-Aco) complex (pAsp(DET-Aco)/pAsp(DET) = 2) (B), and the (DNA/pAsp(DET)/pAsp(DET-Aco) ternary polyplex (N/P ratio = 4; pAsp(DET-Aco)/pAsp(DET) = 2)



Figure S2. Gel retardation assay of polyplex system. (lane 1- pDNA only; lane 2- pDNA/pAsp(DET) polyplex of N/P ratio of 4; lane 3- pDNA/pAsp(DET)/pAsp(DET-Aco) (N/P = 4, pAsp(DET-Aco)/pAsp(DET) = 2); lane 4- pDNA/pAsp(DET)/pAsp(DET-Aco) (N/P = 6, pAsp(DET-Aco)/pAsp(DET) = 2); lane 5- pDNA/pAsp(DET)/pAsp(DET-Aco) (N/P = 8, pAsp(DET-Aco)/pAsp(DET) = 2)

6. Zeta potential measurement

The zeta-potential of the positive polyplex and the ternary polyplex were determined from the laser-Doppler electrophoresis using Zetasizer Nano-ZS (green badge, ZEN3500, Malvern, Ltd. Malvern, U. K.) with a He-Ne ion laser of 633 nm at a detection angle of 173° and a temperature of 37°C. Each complex was diluted in aqueous buffers. (pH 5.5 acetate buffer (10 mM) or pH 7.4 Tris-HCl buffer (10 mM)). The final concentration of the plasmid DNA was 33 µg/mL. Each sample was incubated at 37°C. From the obtained electrophoretic mobility, the zeta-potentials of each complex were calculated by the Smoluchowski equation: $\zeta = 4\pi\eta v/e$ in which η is the viscosity of the solvent, v is the electrophoretic mobility, and e is the dielectric constant of the solvent.

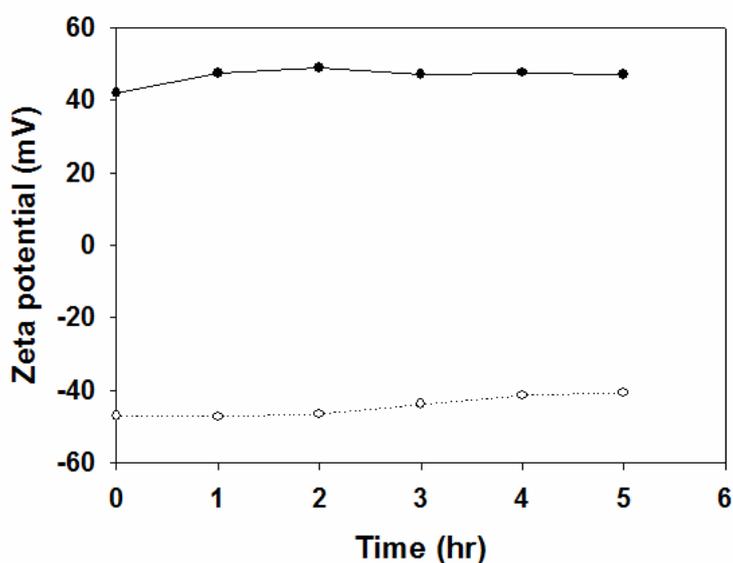


Figure S3. Zeta potential change of the pAsp(DET) polyplex (●) and pAsp(EDA-Suc) (○) ternary polyplex at pH 5.5

7. In vitro transfection

Human Umbilical Vein Endothelial Cells (HUVEC) were seeded on the collagen-coated 24-well culture plates and incubated overnight in 400 μL of EBMTM-2 containing insulin, hEGF, GA-1000, hFGF-B, and FBS (5%). The medium was added by 40 μL of each sample solution (1 μg plasmid DNA/well). After 24 hr-incubation, the medium was changed to fresh medium without the samples, followed by an additional 24 hr-incubation. The cells were washed with 400 μL of Dulbecco's PBS, and lysed by 100 μL of cell culture Promega lysis buffer. The luciferase activity of the lysates was evaluated from the photoluminescence intensity using Mithras LB 940 (Berthold Technologies). The obtained luciferase was normalized with the amount of proteins in the lysates determined by the Micro BCATM Protein Assay Reagent Kit (Pierce). The observation of Venus (YFP) expression was performed using Biozero BZ-8000 (Keyence) at excitation wavelengths of 450-490 nm (emission filter : 510-560 nm).

8. Cell viability assay

For the cytotoxicity assay, HUVEC were incubated for 24 hr with the samples and the viability was evaluated by an MTT assay (Cell Counting Kit-9, Dojindo, Kumamoto, Japan). Each well was measured by reading the absorbance at 450 nm according to the protocol provided by the manufacturer. The results were expressed as the relative value (%) of the control cells, which were incubated in parallel with just Tris-HCl buffer (10 mM, pH 7.4).

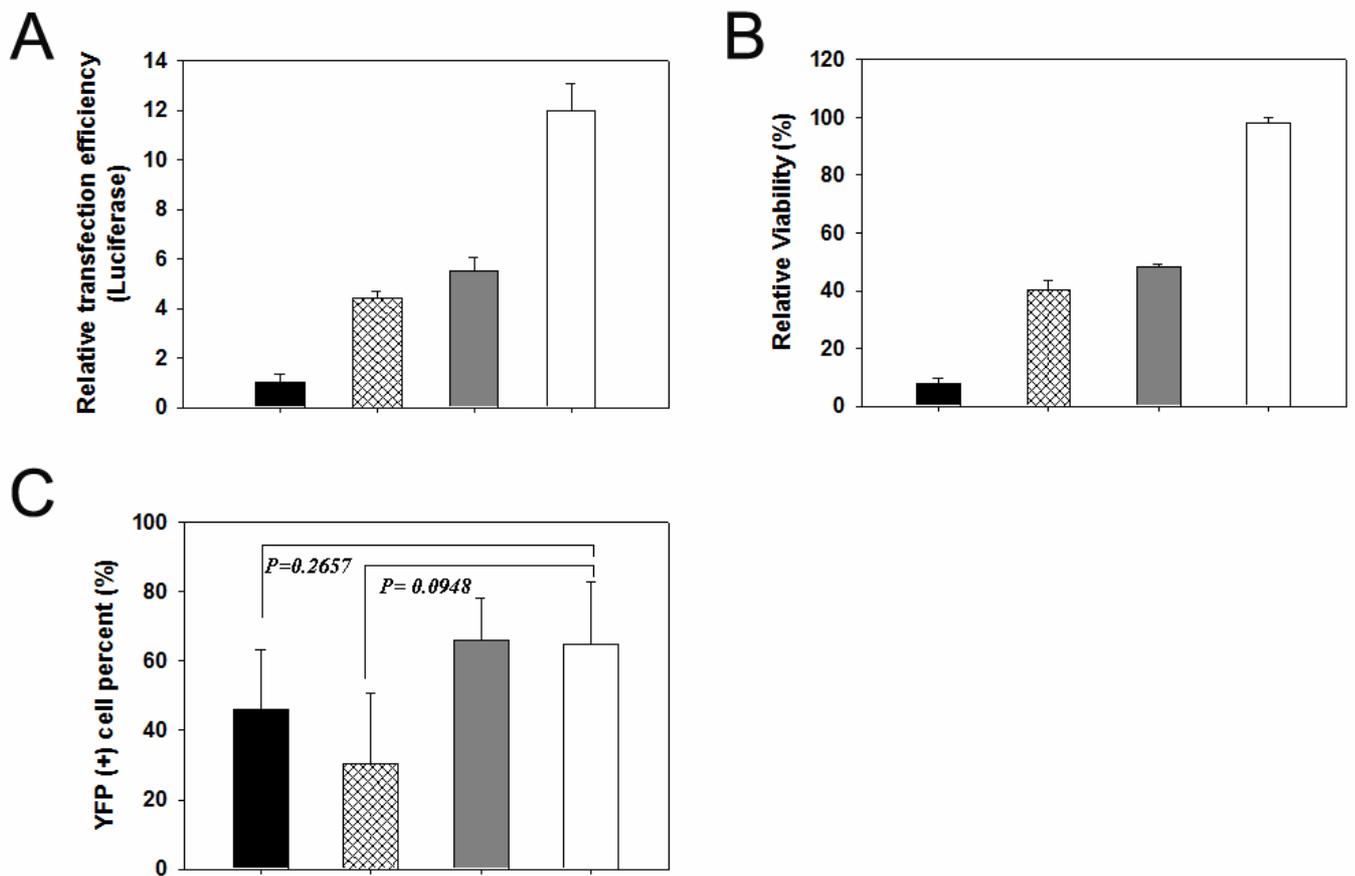


Figure S4. Comparison of transfection efficiency and toxicity by luciferase and Venus (YFP) transfection assay. (A) Relative transfection efficiency by luciferase activity (RLU/ μg protein), (B) Relative cell viability by MTT assay, and (C) Relative Venus (YFP) expression by calculating Venus (+) cell counting. (ExGen 500 (Fermentas, Canada)-black bars; Lipofectamine 2000 (Invitrogen, USA)-textile bars; pAsp(DET) polyplex-dark gray bars; pAsp(DET-Aco) ternary polyplex-white bars). Each error bar means standard deviation.

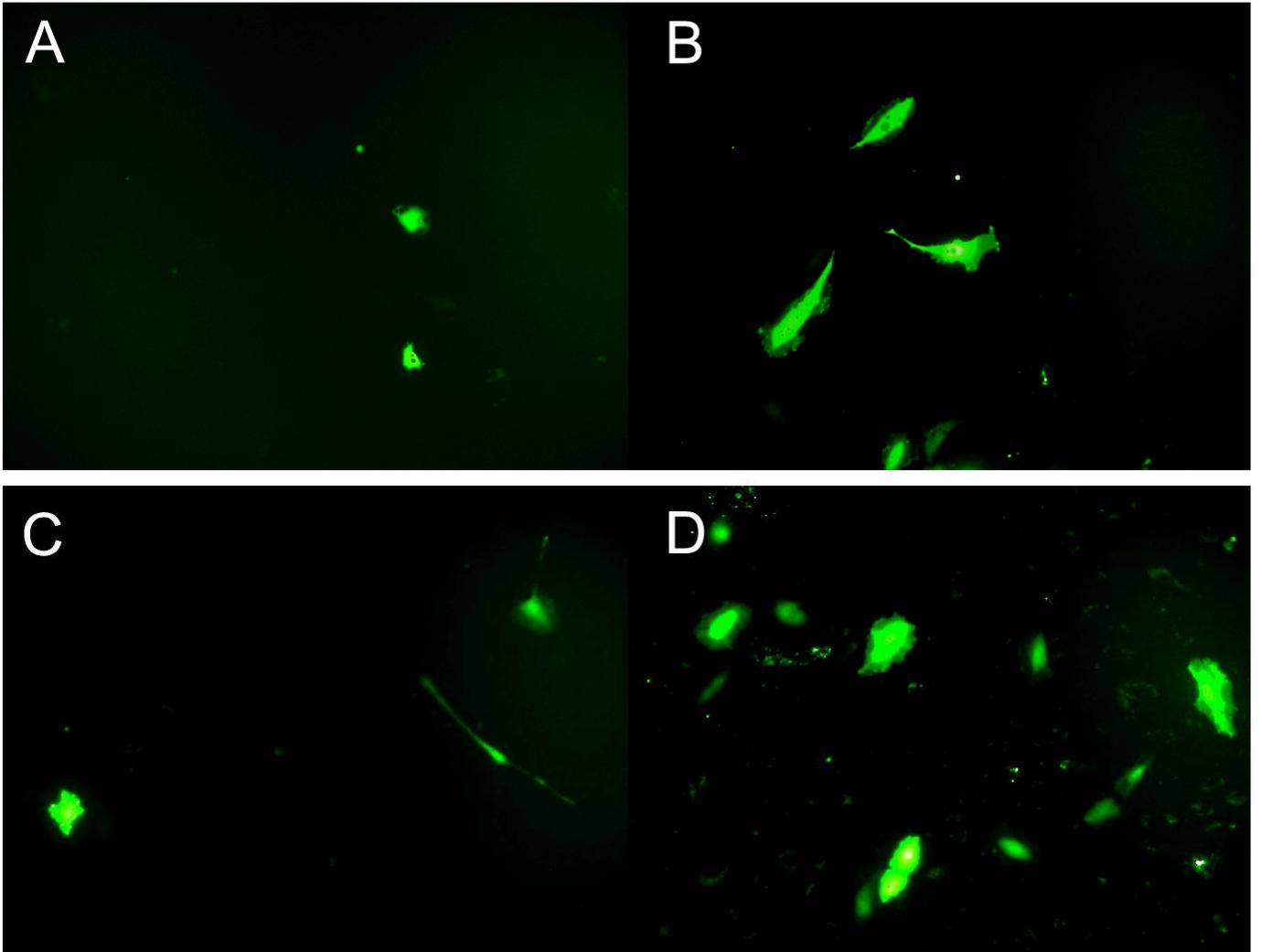


Figure S5. Venus (YFP) transfection on HUVEC by various transfection reagents. (A) ExGen 500, (B) Lipofectamine 2000, (C) pAsp(DET) polyplex, and (D) pAsp(DET-Aco) ternary polyplex.

9. Stability of the complex in the albumin solution

Each sample was diluted by the bovine serum albumin (BSA) solution in Tris-HCl buffer (10 mM, pH 7.4). The final plasmid concentration was 33 $\mu\text{g}/\text{mL}$ and the final BSA concentration was 2 mg/mL . After the incubation at 37°C, the size of the complex was measured by DLS as explained above.

10. Confocal Laser Scanning Microscope (CLSM) observation

pDNA was labeled with Cy5 using the Label IT Nucleic Acid Labeling Kit (Mirus, Madison, WI). HUVEC (30,000 cells) were seeded on a 35 mm glass base dish (Iwaki, Japan) and incubated overnight in 1mL of EBMTM-2 containing insulin, hEGF, GA-1000, hFGF-B, and FBS (5%). After the medium was replaced with fresh medium, 120 μL of polyplex or ternary complex solution containing 3 μg Cy 5-labeled pDNA (N/P =6; pAsp(DET-Aco) (or pAsp(EDA-Suc)/pAsp(DET) molar ratio = 2) was applied to a glass dish. After incubation, the medium was removed and the cells were washed twice with PBS. The intracellular distributions were observed by CLSM following acidic late endosome and lysosome staining with LysoTracker Green (Molecular Probes, Eugene, OR) and nuclear staining with Hoechst 33342 (Dojindo Laboratories, Japan). The CLSM observation was performed using LSM 510 (Carl Zeiss, Germany) with 63 \times objective (C-Apochromat, Carl Zeiss, Germany) at excitation wavelengths of 488 nm (Ar laser), 633 nm (He-Ne laser), and 710 nm (Mai Tai laser) for LysoTracker, Cy 5, and Hoechst 33342, respectively.

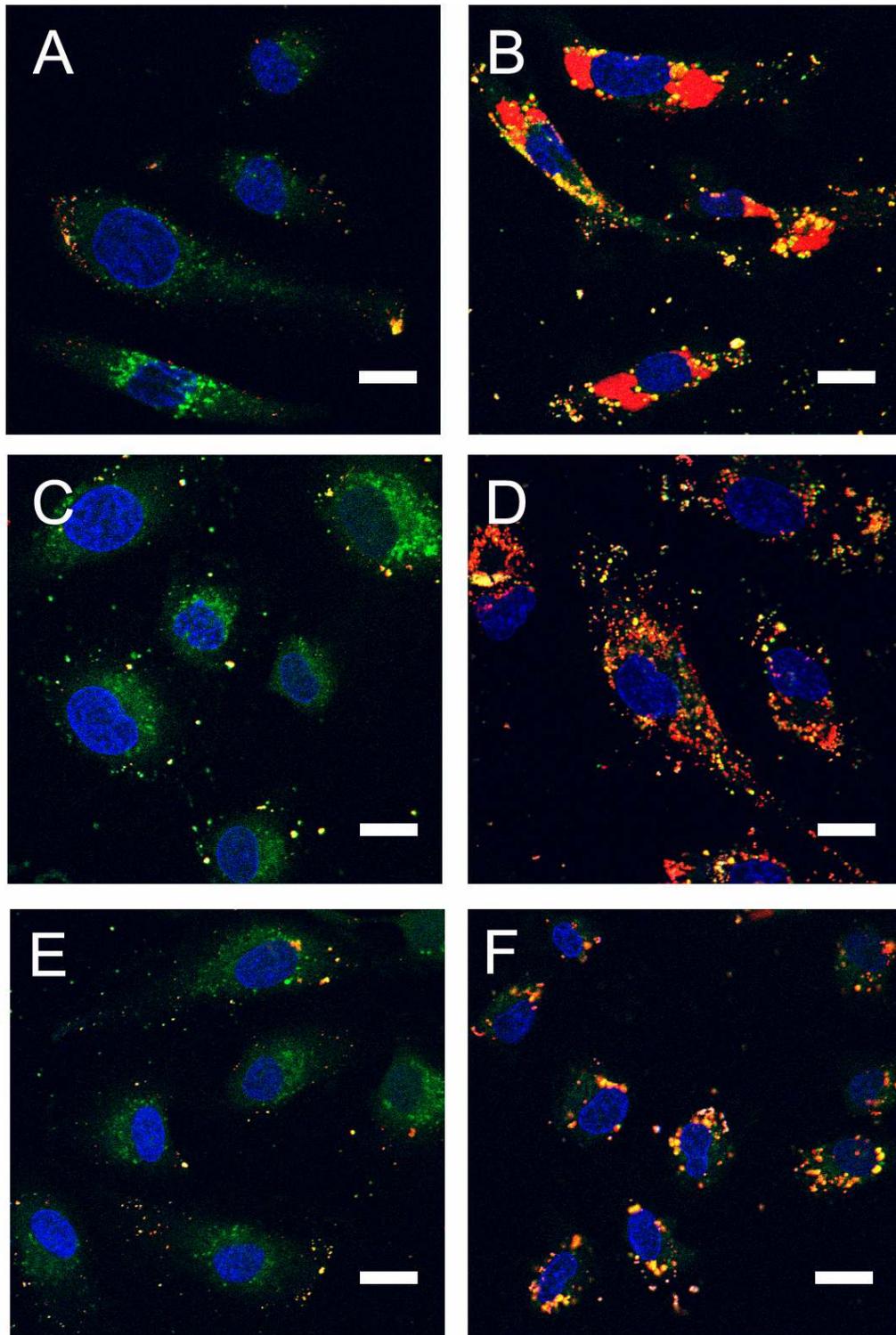


Figure S6. The CLSM images of the HUVEC transfected with pAsp(DET) polyplex (A), (B) , pAsp(DET-Aco) ternary polyplex(C), (D), and pAsp(EDA-Suc) ternary polyplex (E), (F). (A), (C), and (E) are the images after 1 hr-transfection, and (B), (D), and (F) are after 7 hr-transfection. Cy5 (red) - labeled plasmid DNA was used. The cell nuclei were stained with Hoechst 33342 (blue), and late endosome and lysosome were stained with LysoTracker Green (green) . Each scale bar represents 20 μ m.