Supporting Information

Odd–even effect of repeating aminoethylene units in the side chain of N-substituted polyaspartamides on gene transfection profiles

Hirokuni Uchida, Kanjiro Miyata, Makoto Oba, Takehiko Ishii, Tomoya Suma, Keiji Itaka, Nobuhiro Nishiyama, and Kazunori Kataoka

Experimental Section

Materials. β-Benzyl-L-aspartate *N*-carboxyanhydride (BLA-NCA) was purchased from Chuo Kasei Co. Ltd. (Osaka, Japan). *N*-Methyl-2-pyrrolidone (NMP) was purchased from Nakalai Tesque Inc. (Kyoto, Japan). *N*,*N*-Dimethylformamide (DMF), dichloromethane (CH_2Cl_2), *n*-butylamine, ethylenediamine (EDA), diethylenetriamine (DET), triethylenetetramine (TET), tetraethylenepentamine (TEP), tris(hydroxymethyl)aminomethane (Tris), 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES), and 2-morpholinoethanesulfonic acid monohydrate (MES) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). NMP, DMF, CH_2Cl_2, EDA, DET, TET, and TEP were used after conventional distillation. Dulbecco's modified eagle's medium (DMEM) was purchased from Sigma-Aldrich Co. (St. Louis, MO). A

plasmid DNA (pDNA) coding for luciferase with a CAG promoter was provided by RIKEN Bioresource Center (Tsukuba, Japan), amplified in competent DH5α *Escherichia coli*, and subsequently purified using a NucleoBond Xtra Maxi Plus purchased from Clontech Laboratories, Inc. (Mountain View, CA). A transfection reagent, ExGen 500 (linear polyethylenimine), was purchased from Fermentas LLC (Harrington Court, Canada). A Label IT Cy5 Labeling Kit was purchased from Mirus Bio Corporation (Madison, WI). 24- and 96-well culture plates were purchased from Becton Dickinson Labware (Franklin Lakes, NJ). A human hepatoma cell line, Huh-7, and a human lung adenocarcinoma epithelial cell line, A549, were obtained from RIKEN Bioresource Center (Tsukuba, Japan). A human umbilical vein endothelial cell, HUVEC, and the endothelial cell growth medium-2 bullet kit (EGM-2 bullet kit) were obtained from Lonza Ltd. (Basel, Switzerland). Fetal bovine serum (FBS) was purchased from MP Biomedicals, Inc. (Illkirch, France). The Luciferase Assay System (Luminescent Cell Viability Assay) Kit was purchased from Promega Co. (Madison, WI). The Micro BCA Protein Assay Reagent Kit was purchased from Pierce Co., Inc. (Rockford, IL). The Cell Counting Kit-8 was purchased from Dojindo Laboratories (Kumamoto, Japan).

Synthesis of poly(β-benzyl-L-aspartate) (PBLA). PBLA was synthesized by the ring-opening polymerization of BLA-NCA initiated by *n*-butylamine. BLA-NCA (1.2 g, 5 mmol) was dissolved in DMF (2 mL) and then diluted with CH_2Cl_2 (20 mL). *n*-Butylamine diluted with CH_2Cl_2 (0.045 mL,

0.045 mmol) was added to the BLA-NCA solution. The reaction solution was stirred for 2 days at 35 °C. All the procedures were performed under dry Ar atmosphere. The resultant PBLA was precipitated in an excess amount of *n*-hexane/AcOEt (6:4), filtrated, and dried *in vacuo* (0.90 g, 95%). The polydispersity (M_w/M_n) of PBLA was determined to be 1.06 by using a gel permeation chromatography (GPC) system (HLC-8220, TOSOH Co., Tokyo, Japan) equipped with TSKgel columns (SuperAW4000 and SuperAW3000 × 2, TOSOH Co., Tokyo, Japan) and an internal refractive index (RI) detector at 40 °C. NMP containing 50 mM LiBr was used as an eluent at a flow rate of 0.35 ml/min and linear PEG standards were used for the calibration (data not shown). The ^{1}H NMR spectrum of PBLA in d_6-DMSO at 80 °C was recorded on a JEOL EX300 spectrometer (JEOL, Tokyo, Japan), and the polymerization degree was confirmed to be 102 from the peak intensity ratio of methyl protons of the *n*-butyl terminus ($C\boldsymbol{H}_3CH_2CH_2CH_2$-, δ = 0.9 ppm) to aromatic protons of the benzyl esters ($C_6\boldsymbol{H}_5CH_2$-, δ = 7.3 ppm) (data not shown). Tetramethylsilane was used as an internal standard, and chemical shifts were expressed in ppm.

Synthesis of poly[*N*-(2-aminoethyl)aspartamide] (PAsp(EDA)), poly{*N*-[*N'*-(2-aminoethyl)-2-aminoethyl]aspartamide} (PAsp(DET)), poly(*N*-{*N'*-[*N''*-(2-aminoethyl)-2-aminoethyl]-2-aminoethyl}aspartamide) (PAsp(TET)), and poly[*N*-(*N'*-{*N''*-[*N'''*-(2-aminoethyl)-2-aminoethyl]-2-aminoethyl}-2-aminoethyl)aspartamide]

(PAsp(TEP)). Typically, PAsp(EDA) was synthesized as follows: lyophilized PBLA (200 mg) was dissolved in NMP (2 mL) and cooled to 0 °C. In another reaction tube, EDA (5 mL, 50-fold molar excess with respect to benzyl ester units) was diluted two-fold with NMP and cooled to 0 °C. The PBLA solution was added dropwise into the EDA solution. The reaction mixture was stirred for 1 h at 0 °C and then added dropwise in 1 N HCl aqueous solution (5 mL, equimolar concentration to the added amino groups) in ice-water cooling bath. The mixture solution was dialyzed (MWCO: 6-8000) at 4 °C against a 0.01 N HCl aqueous solution for 1 day and then against deionized water for an additional day. The final solution was lyophilized to obtain PAsp(EDA) as a chloride salt form (110 mg, yield 49%). PAsp(DET) (143 mg, yield 48%), PAsp(TET) (157 mg, yield 42%), and PAsp(TEP) (121 mg, yield 27%) were synthesized in a similar manner using DET, TET, and TEP, respectively, instead of EDA. In each reaction, quantitative conversion of benzyl ester groups to -CO-$(NHCH_2CH_2)_{1\text{-}4}$-$NH_2$ groups was confirmed by ^{1}H NMR measurement (polymer concentration: 10 mg/mL, solvent: D_2O, temperature: 70 °C).

Potentiometric titration. PAsp(EDA) (965 mg), PAsp(DET) (680 mg), PAsp(TET) (585 mg), and PAsp(TEP) (538 mg) were separately dissolved in 0.1 N HCl (50 mL) containing 50 mM NaCl to obtain a solution with 100 mM amine, and then titrated with 0.1 N NaOH containing 50 mM NaCl at 37 °C. An automatic titrator (TS-2000, Hiranuma, Kyoto, Japan) was used for the titration. In this

experiment, the titrant was added in quantities of 31.5 μL after the pH values were stabilized (minimal interval: 30 s).

Dynamic light scattering (DLS) and zeta potential measurements of polyplexes. DLS and zeta potential measurements were performed using a Zetasizer nanoseries (Malvern Instruments Ltd., Worcestershire, UK) at a detection angle of 173° and a temperature of 37 °C, and a He-Ne Laser (λ= 633 nm for the incident beam). Each polymer was dissolved in 10 mM Tris–HCl buffer (pH 7.4). The polymer solution at varying concentrations was added to a two-fold excess volume of 50 μg/mL pDNA solution (10 mM Tris–HCl (pH 7.4)) to prepare the polyplexes with different compositions, followed by 10 min incubation at ambient temperature. The final pDNA concentration was adjusted to 33.3 μg/mL. The mixing ratio was defined by the N/P ratio, which is the residual molar ratio of amino groups in polyaspartamides to phosphate groups in pDNA. DLS measurements were performed using a small glass cuvette (volume: 12 μL), ZEN2112 (Malvern Instruments Ltd.). The data obtained from the rate of decay in the photon correlation function were analyzed by the cumulant method and the corresponding hydrodynamic diameter of the polyplexes was then calculated by the Stokes–Einstein equation.[1] In the case of zeta potential measurements, the polyplex solutions (600 μL) were set in a folded capillary cell, DTS 1060 (Malvern Instruments Ltd.). From the obtained electrophoretic mobility, zeta potential was calculated by the Smoluchowski equation:

$$\zeta = 4\pi\eta\upsilon/\varepsilon,$$

where η is the viscosity of the solvent, υ is the electrophoretic mobility, and ε is the dielectric constant of the solvent.

Hemolysis assay. Murine erythrocytes collected in heparin solution (1×10^8 units/mL) were centrifuged at 100 × g for 5 min. The resultant pellet was washed several times with phosphate-buffered saline (PBS) by centrifugation at 600 × g for 10 min, and finally resuspended in 20 mM HEPES (pH 7.4) or 20 mM MES (pH 5.5) containing 150 mM NaCl for pH adjustment. The PAsp(EDA), PAsp(DET), PAsp(TET), PAsp(TEP), and ExGen 500 polymer solutions were added to the erythrocyte solutions at the polyaspartamide amine concentration of 5 mM and incubated at 37 °C in a shaking container (DeepWellMaximizer, Taitec, Saitama, Japan) for 2 h. After centrifugation (600 × g for 5 min), the liberated hemoglobin was determined by colorimetric analysis of the supernatant at 575 nm with a NanoDrop (ND-1000 Spectrophotometer, NanoDrop Technologies Inc, Wilmington, DE). The value for 100% hemolysis was set from the erythrocytes treated with 0.2 wt% Tween20. The results are presented as the mean and standard error of the mean obtained from four samples.

Transfection efficiency and cytotoxicity of polyplexes. Huh-7, A549, and HUVEC were cultured in DMEM 10% FBS (or the medium supplemented with the kit for HUVEC) and the cells were grown

at 37 °C in a humidified atmosphere containing 5% CO_2. Huh-7, A549, or HUVEC (10,000/well) were seeded onto 24-well culture plates and incubated overnight in DMEM 10% FBS (500 μL). The medium was replaced with a fresh, equal medium volume and then each polyplex solution (N/P = 5, 10, and 15) was applied to each well (1 μg of pDNA/well). After 24 h incubation, the medium was again replaced followed by an additional 24 h incubation. The luciferase gene expression was then evaluated on the basis of the photoluminescence intensity using the Luciferase Assay System Kit and a Lumat LB9507 luminometer (Berthold Technologies, Bad Wildbad, Germany). The amount of protein in each well was concomitantly determined using the Micro BCA Protein Assay Reagent Kit. The results were presented as the mean and standard error of the mean obtained from four samples. For the evaluation of cytotoxicity, the Cell Counting Kit-8 was used according to the manufacturer's protocol. The cell viability was evaluated on the basis of the absorbance of formazan from each well, where 100% cell viability was calculated from the wells without the polyplexes. The results are presented as the mean and standard error of the mean obtained from four samples.

Flow cytometry. The Label IT Cy5 Labeling Kit was used to label pDNA with Cy5 according to the manufacturer's protocol. Huh-7 cells (10,000/well) were seeded onto 24-well culture plates and incubated for 24 h in DMEM 10% FBS (500 μL). The medium was replaced with a fresh, equal medium volume and then each polyplex solution (N/P = 5, 10, and 15) was applied to each well (1 μg

of Cy5-labeled pDNA/well). After 24 h incubation, the cells were washed thrice with cold PBS and collected by trypsinization. The collected cells were centrifuged at 100 × g for 2 min and resuspended in cold PBS. The Cy5 fluorescence intensity of each cell was detected by a flow cytometer, BD LSR II instrument (BD Biosciences, Franklin Lakes, NJ) equipped with FACSDiva Software (BD Biosciences) using a 633-nm He-Ne laser for excitation and a 660/20-nm bandpass filter for emission.

Confocal laser scanning microscope (CLSM) observation. Huh-7 cells (50,000/dish) were seeded on 35-mm glass-based dishes (Iwaki, Tokyo, Japan) and incubated overnight in 1 mL of DMEM 10% FBS. The medium was replaced with fresh DMEM 10% FBS (1 mL) and then 90 μL of the polyplex solution containing 3 μg of Cy5-labeled pDNA (N/P = 10) was applied to the glass-based dishes. From 3 h to 48 h, the intracellular distribution of Cy5-labeled pDNA transfected by the PAsp(EDA), PAsp(DET), PAsp(TET), and PAsp(TEP) polyplexes was observed by CLSM after staining acidic late endosomes and lysosomes with LysoTracker Green (Molecular Probes, Eugene, OR) and nuclei with Hoechst 33342 (Dojindo Laboratories, Kumamoto, Japan). At 24 h, the medium was removed and the cells were washed thrice with PBS, followed by 24 h incubation in the absence of the polyplexes. The CLSM observations were performed using LSM 510 (Carl Zeiss, Oberlochen, Germany) with a 63× objective (C-Apochromat, Carl Zeiss, Germany) at excitation wavelengths of 488 (Ar laser), 633

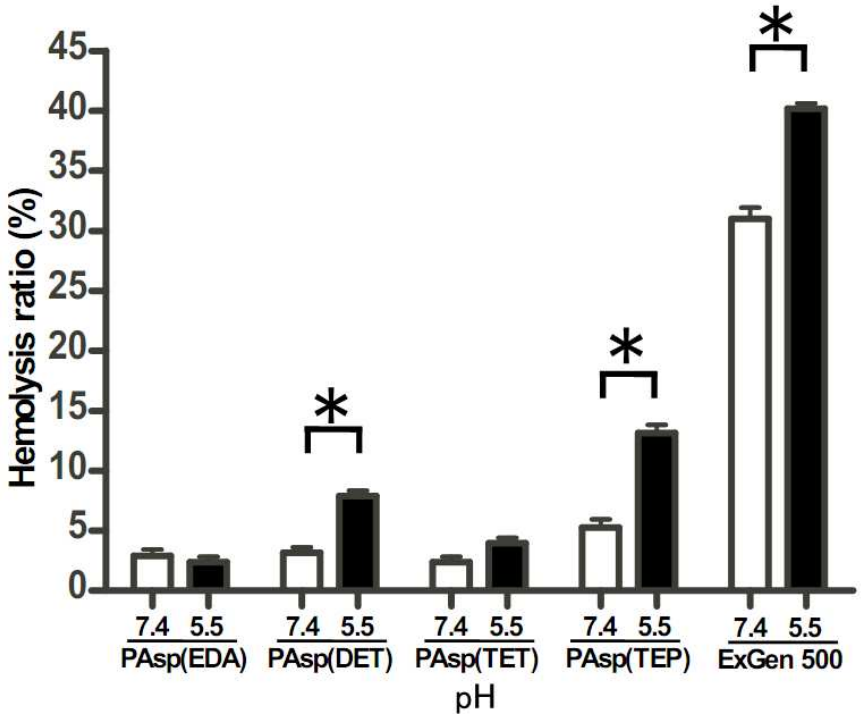
(He-Ne laser), and 710 nm (MaiTai laser for 2-photon imaging) for LysoTrakcer Green (green), Cy5 (red), and Hoechst 33342 (blue), respectively.

Co-localization of Cy5-labeled pDNA with the late endosomes and lysosomes was quantified as follows:

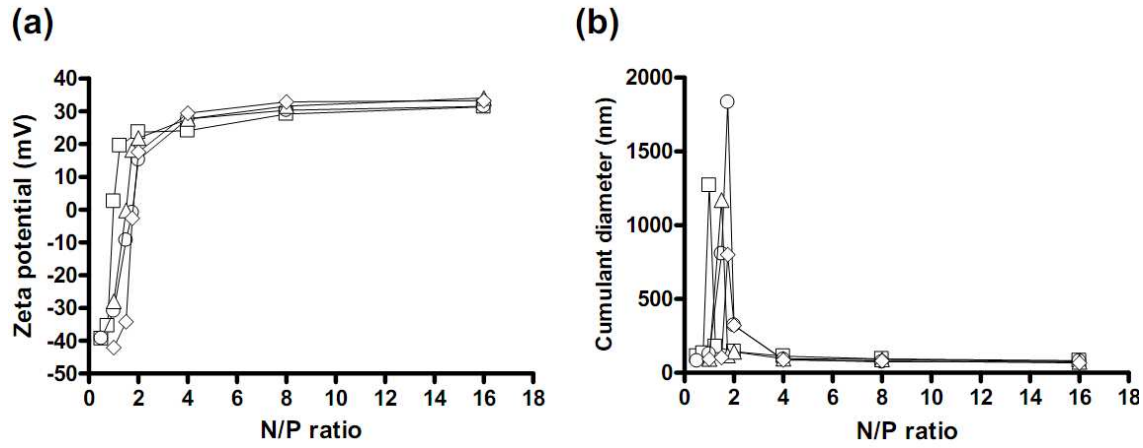
Co-localization ratio = Cy5 $pixels_{co\text{-}localization}$/Cy5 $pixels_{total}$,

where Cy5 $pixels_{co\text{-}localization}$ represents the number of Cy5 pixels co-localizing with LysoTracker Green and Cy5 $pixels_{total}$ represents the number of all the Cy5 pixels in cells. The results are presented as the mean and standard error of the mean obtained from 10 cells.

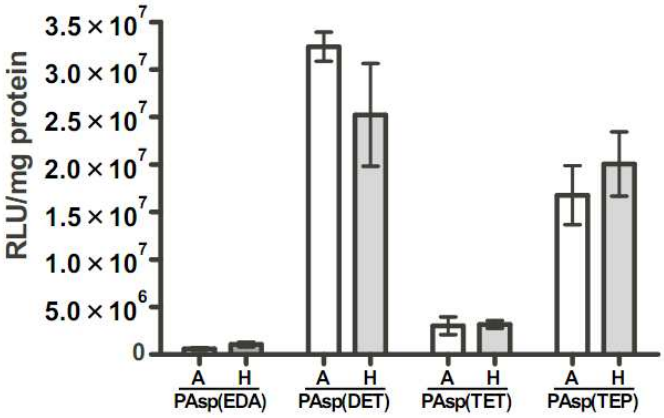
Statistics. Significant differences between two groups were evaluated by Student's *t*-test.



Supplement Figure 1. Hemolytic activity of the polyplexes from PAsp(EDA), PAsp(DET), PAsp(TET), PAsp(TEP), and ExGen 500 ([Amine] = 5 mM) against murine erythrocytes at pH 7.4 and 5.5 at N/P ratio = 10. Results are expressed as mean ± SEM (N = 4). *P < 0.05.



Supplement Figure 2. Change in (a) the zeta potential and (b) the size of PAsp(EDA) (square), PAsp(DET) (circle), PAsp(TET) (triangle), and PAsp(TEP) (diamond) polyplexes with N/P ratios.



Supplement Figure 3. In vitro transfection efficiency of PAsp(EDA), PAsp(DET), PAsp(TET), and PAsp(TEP) polyplexes at an N/P ratio of 10 with A549 (A) and HUVEC (H) determined by luciferase assay. Results are expressed as mean ± SEM (N = 4).

Reference

[1] Harada, A.; Kataoka, K. *Macromolecules.* **1995**, 28, 5294–5299.