Supporting Information

Fine-Tuning of Hydrophobicity in Amphiphilic Polyaspartamide Derivatives for Rapid and Transient Expression of Messenger RNA Directed Toward Genome Engineering in Brain

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Materials and methods

1. Materials

N,*N*-Dimethylformamide (DMF), dichloromethane (DCM), *n*-butylamine, diethylenetriamine (DET), *N*-methyl-2-pyrrolidone (NMP), methanol (MeOH), hexane, ethyl acetate, triethylamine, pentylamine, nonylamine, 1,1,3,3,-tetramethybutylamine, phenylethylamine, Dulbecco's modified Eagle's medium (DMEM), and D-PBS(–) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Heptylamine, octylamine, decylamine, 1-octanol, and trypsin-EDTA were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). 2-Cyclohexylethylamine was obtained from Tokyo Chemical Industry Co., Ltd. (TCI, Tokyo, Japan). Diethyl ether was purchased from Showa-Ether (Tokyo, Japan). Lithium chloride was purchased from Nacalai Tesque (Kyoto, Japan). UltraPure™ Agarose, Lipofectamine® 2000, and Lipofectamine® 3000 were purchased from ThermoFisher Scientific (Waltham, MA, USA). Fetal bovine serum (FBS) was purchased from Biosera (Nuaille, France). HEPES (1 M, pH 7.3) was purchased from Amresco (Solon, OH, USA). DMF, *n*-butylamine, and DET were distilled with the conventional methods before use. C2C12 (mouse myoblast cell line), Neuro-2a (mouse neuroblastoma cell line), and HeLa (human cervical cancer cell line) cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Gaussia luciferase (GLuc) and firefly luciferase (FLuc) mRNAs were kindly donated by Dr. Satoshi Uchida (The University of Tokyo). Briefly, these mRNAs were synthesized by mMessage mMachine™ T7 ULTRA Transcription Kit (ThermoFisher Scientific) as previously reported [12]. Label IT Cy3 Labeling Kit and Label IT Cy5 Labeling Kit (Mirus Bio Corporation, Madison, WI, USA) were used to attach the fluorescent dyes to GLuc-mRNA and sgRNA. A STOP cassette-targeting sgRNA was synthesized by TriLink BioTechnologies, Inc. (San Diego, CA, USA): (5´-aag UAA AAC CUC UAC AAA UGG UUU UAG AGC UAG AAA UAG CAA GUU AAA AUA AGG CUA GUC CGU UAU CAA CUU GAA AAA GUG GCA CCG AGU CGG UGC uuu U-3´) [34, 35]. Uppercase letters denote RNA. Lowercase letters and underlines indicate phosphorothioate and 2´-O-methyl-modified RNA.

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

β-Benzyl-L-aspartate *N*-carboxy-anhydride (BLA-NCA) was purchased from Chuo Kaseihin Co., Inc. (Tokyo, Japan). PBLA was synthesized by the ring-opening polymerization of

the BLA-NCA initiated by *n*-butylamine as previously reported [11]. Briefly, *n*-butylamine (6.56 μL, 0.0664 mmol) in DCM (660 μL) was added to BLA-NCA (410 mg, 1.66 mmol) dissolved in 6 mL of DCM/DMF (9:1 v/v). The polymerization proceeded for 48 h at 37 °C under argon atmosphere. The resulting polymer was precipitated in hexane/ethyl acetate (6:4 v/v) and dried overnight under reduced pressure. The prepared PBLA was characterized by gel permeation chromatography (GPC) and ^{1}H NMR (400 MHz, ECS-400, JEOL, Tokyo, Japan). The GPC system (HLC-8220, TOSOH CORPORATION, Tokyo, Japan) equipped with two TSK gel columns (TSK-gel Super AW4000 and Super AW3000) was eluted with DMF containing lithium chloride (10 mM) at 0.8 mL/min. A molecular weight distribution (M_w/M_n) of PBLA was determined to be 1.18 from the GPC chart (Figure S1A). A degree of polymerization of benzyl-L-aspartate (BLA) unit in PBLA was calculated to be 26 from the peak intensity ratio of the phenyl protons ($COOCH_2C_6H_5$, δ = 7.2–7.3) to the methyl protons ($CH_3(CH_2)_3NH$, δ = 0.8) in the ^{1}H NMR spectrum (Figure S1B). The PBLAs with DP = 63 and 121 were similarly synthesized to evaluate the effect of DP of polyaspartamide derivatives and their M_w/M_n values were determined to be 1.19 and 1.34, respectively.

3. Synthesis of amphiphilic polyaspartamide derivatives with diethylenetriamine moieties (PAsp(DET/R))

A series of PAsp(DET/R)s were prepared by the simultaneous aminolysis reaction of PBLA with DET and varying aliphatic amines [11]. For the synthesis of PAsp(DET/CHE) (DP = 26), PBLA (DP = 26, 30 mg) was dissolved in NMP (2 mL) and cooled to 4 °C. The PBLA solution was dropwised to the mixture of DET (790 μL) and 2-cyclohexylethylamine (2.14 mL) (molar ratio = 1:2). The solution was stirred for 1 h at 4 °C under argon atmosphere. The reaction mixture was dropwised to cold 5 M HCl (5 mL) for neutralization. The polymer product was purified by dialysis against 0.01 M HCl and then distilled water at 4 °C. The dialyzed solution was lyophilized to obtain a solid powder. The powder was dissolved in MeOH (4 mL) with a small amount of triethylamine and then precipitated into an excess amount of diethyl ether to completely remove unreacted 2-cyclohexylehtylamine. The precipitate was filtered and dissolved in distilled water. The polymer was further purified by dialysis against 0.01 M HCl and then distilled water at 4 °C. The dialyzed solution was lyophilized to obtain the final product. Quantitative conversion of benzyl ester groups to diethylenetriamine (DET) and 2-

cyclohexylethy (CHE) moieties in side chains was confirmed in the ^{1}H NMR spectrum (10 mg/mL, 80 °C) (Figure S2). The other PAsp(DET/R)s were further prepared according to the similar procedures. The quantitative conversions of benzyl ester groups to aliphatic moieties, *e.g.*, pentyl (PEN), heptyl (HEP), octyl (OCT), nonyl (NON), and decyl (DEC), were confirmed from the peak intensity ratio between alkyl protons in hydrophobic moieties (-C*H*$_2$- or C*H*$_3$-, δ = 0.9–1.5 ppm) and methine protons in polymer backbone (-COC*H*NH-, δ = 4.6–4.8 ppm) in the ^{1}H NMR spectra (Figure S3 for PAsp(DET/OCT)). An introduction rate of 2-phenylethyl (PHE) moieties was calculated from the peak intensity ratio between phenyl protons (*Ph*-, δ = 7.0–7.5 ppm) and methine protons in polymer backbone (-COC*H*NH-, δ = 4.6–4.8 ppm) in the ^{1}H NMR spectrum (Figure S4). PAsp(DET/CHE)s with lower introduction rates of CHE moieties were prepared by reacting PBLA (DP = 26) with the mixtures at diethylenetriamine:2-cyclohexylethylamine = 4.5:1 and 1.2:1 in molar ratio. Also, PBLAs with DP = 63 and 121 were reacted with the mixture at DET:2-cyclohexylethylamine = 1:2. The introduction rates of CHE and DET moieties in these PAsp(DET/CHE)s were similarly determined from the peak intensity ratio in the ^{1}H NMR spectra (data not shown).

4. Octanol-water partition coefficient measurement

Octanol-water partition coefficients (log *P*) of PAsp(DET/R) series were determined in the 1:1 mixture of 1-octanol/HEPES buffer (10 mM, pH 7.3) [39, 40]. PAsp(DET/R)s were labeled with Alexa Fluor® 647 dye NHS ester (Alexa647) (ThermoFisher Scientific) in MeOH with a small amount of triethylamine. The obtained Alexa647-labeled PAsp(DET/R)s (100 μM, 50 μL) dissolved in 10 mM HEPES buffer (pH 7.3) were mixed with 1-octanol (50 μL) and the mixtures were vigorously agitated for 30 min (2900 rpm, Eppendorf MixMate, Hamburg, Germany). The biphasic mixtures were shortly centrifuged and placed at 4 °C for 1 h. The 1-octanol phase was carefully collected and further placed at 4 °C for 1 h. The remaining HEPES buffer phase was centrifuged at 3300 g for 5 min and placed at 4 °C for 1 h. The fluorescence intensities of Alexa647 in 1-octanol and HEPES buffer phases were measured by Spectrofluorometer (FP-8300, Jasco, Tokyo, Japan).

5. Preparation and characterization of IVT mRNA-loaded polyplexes with PAsp(DET/R)s

PAsp(DET/R)s were dissolved in 10 mM HEPES buffer (pH 7.3) and then mixed with IVT mRNA solution (100 ng/μL mRNA in 10 mM HEPES buffer, pH 7.3) to prepare polyplexes (20 ng/μL mRNA) at desired molar ratios of amino groups in DET unit to phosphate groups in mRNA (N/P). Polyplex size (cumulant diameter) and size distribution (polydispersity index (PDI)) were determined by dynamic light scattering using a Zetasizer (Malvern Instruments, Worcestershire, UK) equipped with a He–Ne Laser (λ = 633 nm) at a temperature of 25 °C and a detection angle of 173°. The size measurement was performed in a low volume quartz cuvette (ZEN2112, Malvern Instruments). The data obtained from the rate of decay in the photon correlation function were analyzed by the cumulant method to obtain the corresponding cumulant diameter and PDI. Zeta potential of polyplex was determined with the same apparatus using a folded capillary cell (Malvern Instruments). The zeta potential was calculated from the measured electrophoretic mobility based on the Smoluchowski equation.

6. Gel retardation analysis.

PAsp(DET/R) and GLuc-mRNA were mixed at N/P ratios of 0, 1, 2, 3, 4, and 5, as aforementioned. Glycerol was added to the mixed solutions (final glycerol concentration: 8 vol%, final mRNA amount: 1 μg). The samples were electrophoresed on an agarose gel (1 wt% agarose gel, 1 × TBE buffer, 135 V, 15 min). The mRNA on the gel was stained with ethidium bromide and visualized using a Molecular Imager FX (Bio-Rad, Hercules, CA). To observe stability of polyplexes against PBS containing 10% FBS (PBS/FBS), polyplexes (N/P = 5, 50 ng, 2.5 μL) or naked mRNA were mixed with PBS/FBS (100 μL) for 1 h at 37 °C. The mixture was purified with a RNeasy Mini Kit (Qiagen). The samples were electrophoresed on a Novex TBE gel (4–20%, Invitrogen, 1 × TBE buffer, 200 V, 50 min). The mRNA on the gel was stained with SYBR Green II (Lonza, Basel, Switzerland) and visualized using a Molecular Imager FX.

7. Transfection of IVT mRNA-loaded polyplexes in cultured cells.

C2C12 or Neuro-2a cells were seeded into a 96-well plate at a density of 8000 cells/well in DMEM containing 10% FBS (DMEM/FBS). On the next day, the medium was replaced with fresh DMEM/FBS. Polyplex solutions prepared from GLuc- or FLuc-mRNA were then added to each well (50 ng mRNA/well) and incubated for 24 h. The expression levels of FLuc and GLuc

were determined from the photoluminescence intensity of cell lysates and supernatants, respectively, using the Luciferase Assay System (Promega, Madison, WI) for FLuc and the Renilla Luciferase Assay System (Promega) for GLuc. The photoluminescence intensities were measured with a luminescence microplate reader (Mithras LB 940, Berthold technologies, Bad Wildbad, Germany).

8. Cell viability assay

Cell viability was evaluated using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). C2C12 cells were seeded into a 96-well plate (8000 cells/well) in DMEM/FBS. On the next day, polyplex samples prepared from PAsp(DET/CHE) (CHE-polyplexes) and GLuc-mRNA at N/P = 5 were added to the cell at varying concentrations and the cells were incubated for 24 h. The medium was exchanged with a fresh medium (100 μL) containing the kit solution (10 μL) and the cells were further incubated for 1 h. The absorbance of the medium was measured at 450 nm using a Spark 20M Multimode Microplate Reader (TECAN, Switzerland).

9. Flow cytometric analysis

C2C12 cells were seeded into a 12-well plate at a density of 100,000 cells/well in DMEM/FBS. On the next day, the medium was replaced with fresh DMEM/FBS. Polyplex solutions (N/P = 5) containing Cy5-labeled GLuc-mRNA (Cy5-mRNA) were then added to each well (500 ng mRNA/well). After 4-h incubation, the medium was removed and the cells were washed with 1 mL of PBS. The cells were treated with a trypsin-EDTA solution for 1 min and suspended in PBS. The fluorescence intensity of Cy5-mRNA derived from the cells was measured using a BD™ LSR II flow cytometer (BD Biosciences), by which the cells were excited with a 633 nm laser. To monitor release profiles of polyplexes, Cy3-labeled GLuc-mRNA (Cy3-mRNA) was mixed with Alexa647-labeled PAsp(DET/R)s (non-labeled polymer:Alexa647-labeled polymer = 4:1) to prepare polyplex samples (N/P = 5). The polyplex samples were transfected to the cells in a 12-well plate at 500 ng mRNA/well. After 6- and 12-h incubation, the medium was removed and the cells were washed with 1 mL of PBS. The cells were treated with a trypsin-EDTA solution for 1 min and suspended in PBS. Fluorescence intensities of Alexa647 and Cy3 from the cells were monitored by a BD™ LSR II flow cytometer equipped with a 488 nm laser. The emission profiles of Alexa647 and Cy3 were

detected using 695/40 and 575/26 nm band-pass filters, respectively. The FRET ratio was calculated based on the following equation: FRET ratio = mean fluorescence intensity (MFI) from 695/40 nm filter / MFI from 575/26 nm filter.

10. Confocal laser scanning microscopic (CLSM) observation

C2C12 cells were seeded into a 35-mm glass-based dish (Iwaki, Tokyo, Japan) at a density of 50,000 cells/well in DMEM/FBS. To observe endosome-escaping ability of polyplexes, Cy5-mRNA was mixed with PAsp(DET/R)s to prepare polyplex samples (N/P = 5). The polyplex samples were transfected at 500 ng mRNA/well. The cells were stained with LysoTracker Green (Molecular Probes, Eugene, OR, USA) for 1 h and Hoechst 33342 (Dojindo, Japan) for 5 min. Each dish was observed using a CLSM (ZEISS LSM 780, Carl Zeiss, Oberkochen, Germany) equipped with a C-Apochromat 40× objective (Carl Zeiss).

11. Homology-directed repair (HDR) efficacy by luciferase reporter system

HeLa cells were seeded into a 96-well black-walled plate at a density of 10,000 cells/well in DMEM/FBS. On the next day, the medium was replaced with fresh DMEM/FBS. Reporter components, *i.e.*, a full-length luciferase donor DNA and two pDNAs encoding sgRNA or StopFluc reporter [28], were transfected into the cells using Lipofectamine 3000 (Thermo Fischer Scientific) according to the manufacturer's protocol. The cells were incubated for 24 h. CHE-polyplex (DP = 26, N/P = 5), $DET_{0/121}$-polyplex (DP = 121, N/P = 5), and Lipofectamine 3000 loaded with SpCas9 mRNA (Miltenyi Biotec Inc., CA, USA) were transfected at 50 ng mRNA/well, and the cells were incubated for 24 h. As a positive control, a pDNA encoding codon-optimized SpCas9 was also transfected into the cells simultaneously with the reporter components using Lipofectamine 3000 and the cells were incubated for total 48 h. Thereafter, the medium was replaced with 100 μL of 1× Hank's Balanced Salt Solution (Life technologies) containing 500 μM of D-luciferin (Wako Pure Chemical Industries) as a substrate and the cells were further incubated for 30 min at 37 °C. Luminescence measurements were performed using a luminescence microplate reader (Mithras LB 940).

12. Intracerebroventricular and intrathecal administrations

For intracerebroventricular injection, 6 to 8-week-old female BALB/c mice (Charles River Laboratories Japan, INC., Yokoyama, Japan) were anesthetized by inhalation of isoflurane (Pfizer, NY, USA), and were held in the prone position on a stereotaxic instrument (Narishige Group, Tokyo, Japan) [41]. The scalp was sagittally incised in the lengths of 1.0–1.5 cm and the calvarium was exposed by blunt dissection. A small parietal hole was perpendicularly created on the sagittal suture of the skull at the 0.5 mm posterior to the bregma by a 1-mm diameter diamond plated points. A polyplex solution (10 μL) containing 4.5 μg FLuc-mRNA was administered to the 3rd ventricle at the depth of 3.0 mm ventral to the surface of the brain with a Hamilton syringe connected to a stereotaxic micromanipulator (Narishige Group) at a rate of ~2.0 μL/min. Then, the incision was closed using sutures. Lipofectamine 2000 (Thermo Fischer Scientific) was used as a control. The luminescence intensity from the brain was measured with an IVIS instrument (PerkinElmer, Waltham, MA, USA) equipped with a Living Image Software (PerkinElmer). For histological examination, a CHE-polyplex solution (10 μL, N/P = 5) containing 3.0 μg Cy5-mRNA was administered to the 3rd ventricle as described above. The brains were fixed with 4% paraformaldehyde in PBS, immersed in 20% sucrose, and embedded in OCT Compound (Sakura Finetek Japan, Tokyo, Japan) for frozen sectioning. Tissue slices were stained with ProLong™ Diamond Antifade Mountant with DAPI (Thermo Fisher Scientific) and observed using ZEISS LSM 780 equipped with a Plan-Apochromat 10× objective (Carl Zeiss). For intrathecal injection, mice were anesthetized with inhalation of isoflurane and placed in a prone position. The skin was incised to dissect the muscle tissue and the thoracic and lumbar laminae were exposed. A catheter (Alzet, Cupertino, CA, USA) was inserted into the subarachnoid space at L1 and a polyplex solution (10 μL) containing 3.0 μg FLuc-mRNA was administered at Th10 with a Hamilton syringe for 60 sec. The tip of the catheter was kept at the injection site for 5 min to prevent leakage of cerebrospinal fluid. The luminescence intensity from the mouse was measured with an IVIS instrument. For gene editing via double-stranded breaks in B6.Cg-*Gt(ROSA)26Sor*$^{tm9(CAG\text{-}tdTomato)Hze}$/J (Ai9, The Jackson Laboratory, Bar Harbor, ME, USA), a CHE-polyplex solution(10 μL, N/P = 5) containing 1.5 μg SpCas9 mRNA and 1.5 μg sgRNA was intracerebroventricularly administered to the 3rd ventricle. The brain was processed to tissue slices as described above and observed by using ZEISS LSM 780 with a Plan-Apochromat 10× objective or C-Apochromate 40× objective (Carl Zeiss).

Supplemental References

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Table S1. Compositions and log *P* of a series of PAsp(DET/R)s with DP = 26.

Polymer	R moiety	Introduction number [a]		Introduction rate of R moiety (%)	Log *P* [b]
		DET moiety	R moiety		
PAsp(DET/PEN)	Pentyl	13	13	50	–2.45 ± 0.03
PAsp(DET/HEP)	Heptyl	15	11	42	–2.15 ± 0.02
PAsp(DET/OCT)	Octyl	16	10	38	–2.06 ± 0.05
PAsp(DET/NON)	Nonyl	16	10	38	–1.92 ± 0.02
PAsp(DET/DEC)	Decyl	17	9	35	–2.09 ± 0.02
PAsp(DET/CHE)	2-Cyclohexylethyl	15	11	42	–2.31 ± 0.04
PAsp(DET/PHE)	2-Phenylethyl	15	11	42	–2.45 ± 0.01
PAsp(DET)	–	26	0	0	–

[a] Values were determined from ^{1}H NMR spectra. [b] Values are expressed as mean ± SD (n = 3).

Table S2. Various PAsp(DET/CHE)s with different DPs and introduction rates of CHE moiety.

Polymer	DP	Introduction number [a]		Introduction rate of CHE moiety (%)	Log *P* [b]
		DET moiety	CHE moiety		
PAsp(DET/CHE$_2$)$_{26}$	26	24	2	8	–2.59 ± 0.03
PAsp(DET/CHE$_8$)$_{26}$	26	18	8	31	–2.45 ± 0.08
PAsp(DET/CHE$_{32}$)$_{63}$	63	31	32	51	–
PAsp(DET/CHE$_{47}$)$_{121}$	121	74	47	39	–

[a] Values were determined from ^{1}H NMR spectra. [b] Values are expressed as mean ± SD (n = 3).

Table S3. Nomenclature of polyplexes in this study.

Polyplex	PAsp(DET/R)	Polyplex	PAsp(DET/R)
DET-polyplex	PAsp(DET)$_{26}$ [a]	CHE$_{2/26}$-polyplex	PAsp(DET/CHE$_2$)$_{26}$
PEN-polyplex	PAsp(DET/PEN$_{13}$)$_{26}$ [a]	CHE$_{8/26}$-polyplex	PAsp(DET/CHE$_8$)$_{26}$
HEP-polyplex	PAsp(DET/HEP$_{11}$)$_{26}$ [a]	CHE$_{32/63}$-polyplex	PAsp(DET/CHE$_{32}$)$_{63}$
OCT-polyplex	PAsp(DET/OCT$_{10}$)$_{26}$ [a]	CHE$_{47/121}$-polyplex	PAsp(DET/CHE$_{47}$)$_{121}$
NON-polyplex	PAsp(DET/NON$_{10}$)$_{26}$ [a]	DET$_{0/63}$-polyplex	PAsp(DET)$_{63}$
DEC-polyplex	PAsp(DET/DEC$_9$)$_{26}$ [a]	DET$_{0/121}$-polyplex	PAsp(DET)$_{121}$
CHE-polyplex	PAsp(DET/CHE$_{11}$)$_{26}$ [a]		
PHE-polyplex	PAsp(DET/PHE$_{11}$)$_{26}$ [a]		

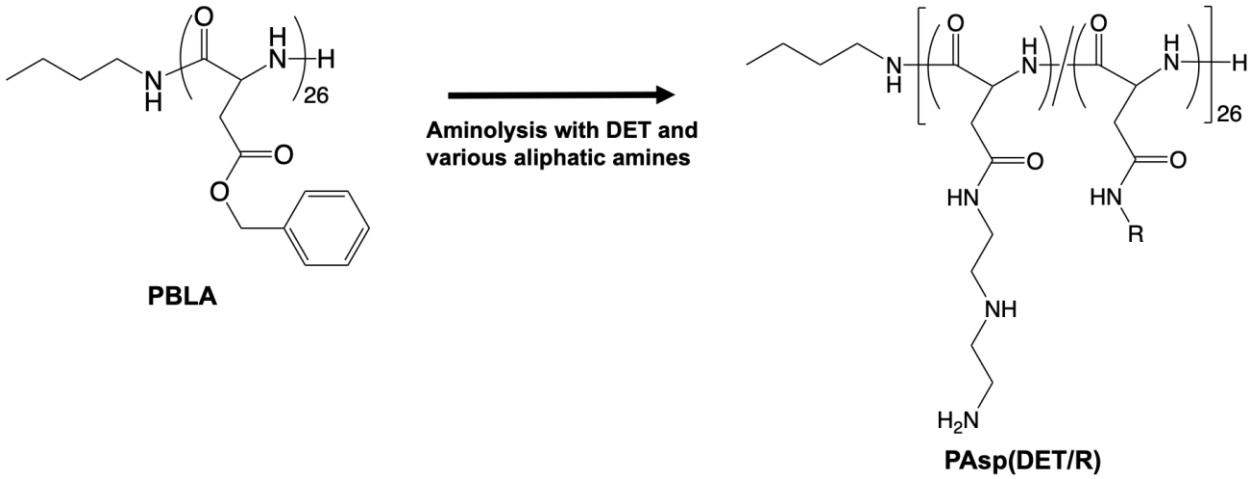
[a] In these polymers/polyplexes, the unit number of R moiety and DP were omitted in their nomenclatures for simplicity.

Table S4. D_H, PDI, and zeta potential of polyplexes prepared from varying PAsp(DET/R)s with DP = 26 (mean ± SD, n = 3).

Polyplex	N/P = 3			N/P = 5		
	D_H (nm)	PDI	Zeta potential (mV)	D_H (nm)	PDI	Zeta potential (mV)
DET-polyplex	129 ± 8	0.16 ± 0.04	6.8 ± 5.4	105 ± 2	0.27 ± 0.02	23.1 ± 6.1
PEN-polyplex	129 ± 3	0.11 ± 0.03	8.1 ± 4.3	96 ± 3	0.19 ± 0.01	19.4 ± 7.3
HEP-polyplex	109 ± 4	0.21 ± 0.02	9.7 ± 4.3	102 ± 6	0.24 ± 0.06	23.5 ± 7.0
OCT-polyplex	157 ± 32	0.31 ± 0.11	12.7 ± 4.1	113 ± 10	0.23 ± 0.03	21.3 ± 4.9
NON-polyplex	156 ± 11	0.25 ± 0.04	12.0 ± 5.0	117 ± 13	0.22 ± 0.02	21.8 ± 5.8
DEC-polyplex	138 ± 7	0.31 ± 0.02	17.0 ± 4.3	131 ± 37	0.29 ± 0.07	19.8 ± 5.6
CHE-polyplex	119 ± 17	0.21 ± 0.03	9.7 ± 6.1	103 ± 16	0.20 ± 0.04	24.4 ± 3.8
PHE-polyplex	153 ± 15	0.11 ± 0.03	6.9 ± 4.0	102 ± 3	0.16 ± 0.03	10.2 ± 12.0

Table S5. Hydrodynamic diameter, PDI, and zeta potential of varying DET- and CHE-polyplexes (mean ± SD, n = 3).

Polyplex	N/P = 3			N/P = 5		
	D_H (nm)	PDI	Zeta potential (mV)	D_H (nm)	PDI	Zeta potential (mV)
$CHE_{2/26}$-polyplex	158 ± 3	0.03 ± 0.01	6.8 ± 5.6	101 ± 5	0.15 ± 0.04	10.6 ± 7.6
$CHE_{8/26}$-polyplex	115 ± 1	0.10 ± 0.03	10.9 ± 7.9	108 ± 1	0.21 ± 0.01	21.0 ± 5.5
$CHE_{(11/26)}$-polyplex	119 ± 17	0.21 ± 0.03	9.7 ± 6.1	103 ± 16	0.20 ± 0.04	24.4 ± 3.8
$CHE_{32/63}$-polyplex	130 ± 5	0.21 ± 0.03	18.6 ± 4.0	98 ± 2	0.18 ± 0.02	27.9 ± 5.9
$CHE_{47/121}$-polyplex	112 ± 4	0.18 ± 0.04	20.8 ± 5.5	92 ± 4	0.14 ± 0.01	31.3 ± 9.0
$DET_{(0/26)}$-polyplex	129 ± 8	0.16 ± 0.04	6.8 ± 5.4	105 ± 2	0.27 ± 0.02	23.1 ± 6.1
$DET_{0/63}$-polyplex	89 ± 9	0.19 ± 0.04	29.7 ± 8.6	76 ± 3	0.18 ± 0.01	24.2 ± 12.1
$DET_{0/121}$-polyplex	95 ± 3	0.17 ± 0.02	34.5 ± 8.6	79 ± 1	0.18 ± 0.02	27.9 ± 9.2



Scheme S1. Synthetic procedure of amphiphilic polyaspartamide derivatives (PAsp(DET/R)s) through the aminolysis reaction of PBLA with DET and aliphatic amines.

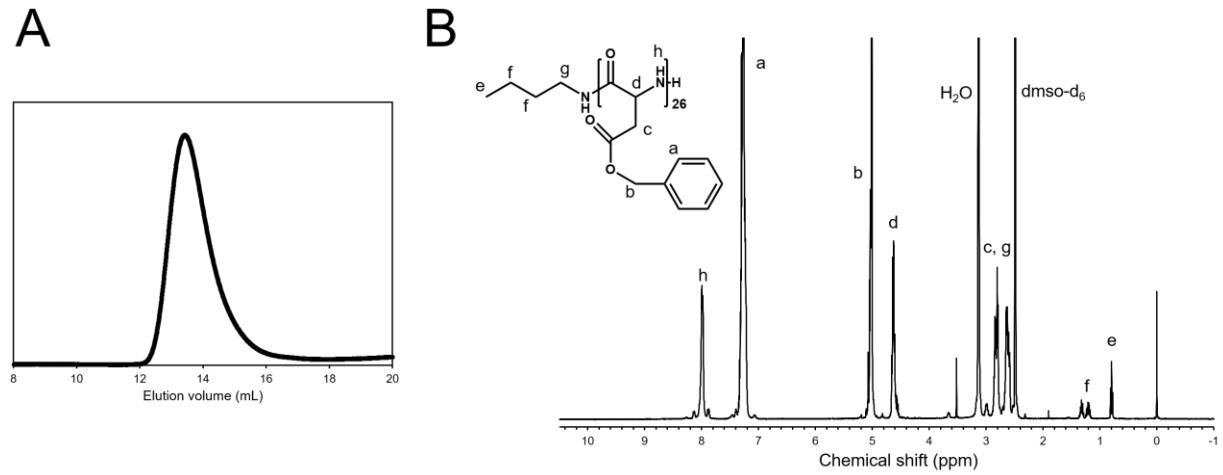


Figure S1. (A) SEC chart and (B) ^{1}H NMR spectrum of PBLA with DP = 26 (10 mg/mL, DMSO-d_6, 400 MHz, rt).

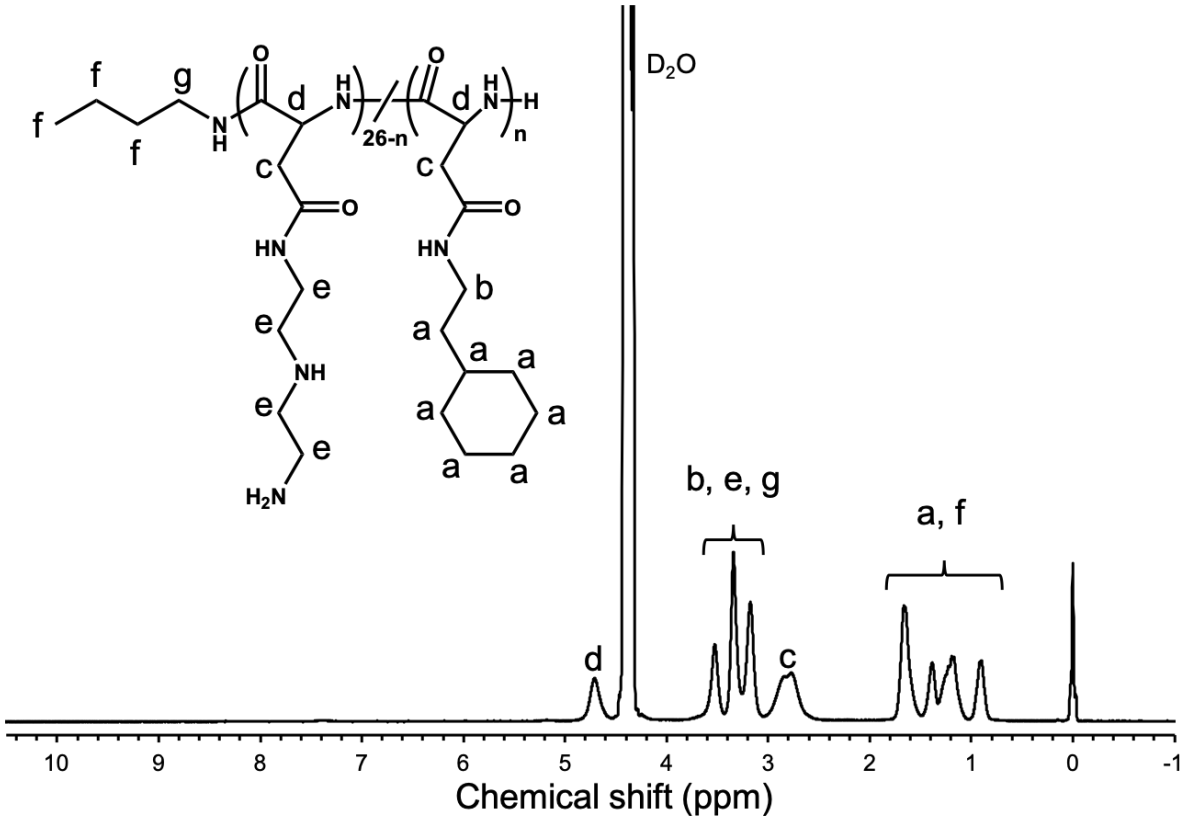


Figure S2. ^{1}H NMR spectrum of PAsp(DET/CHE) (DP = 26, CHE unit number = 11) (10 mg/mL, D_2O, 400 MHz, 80 °C).

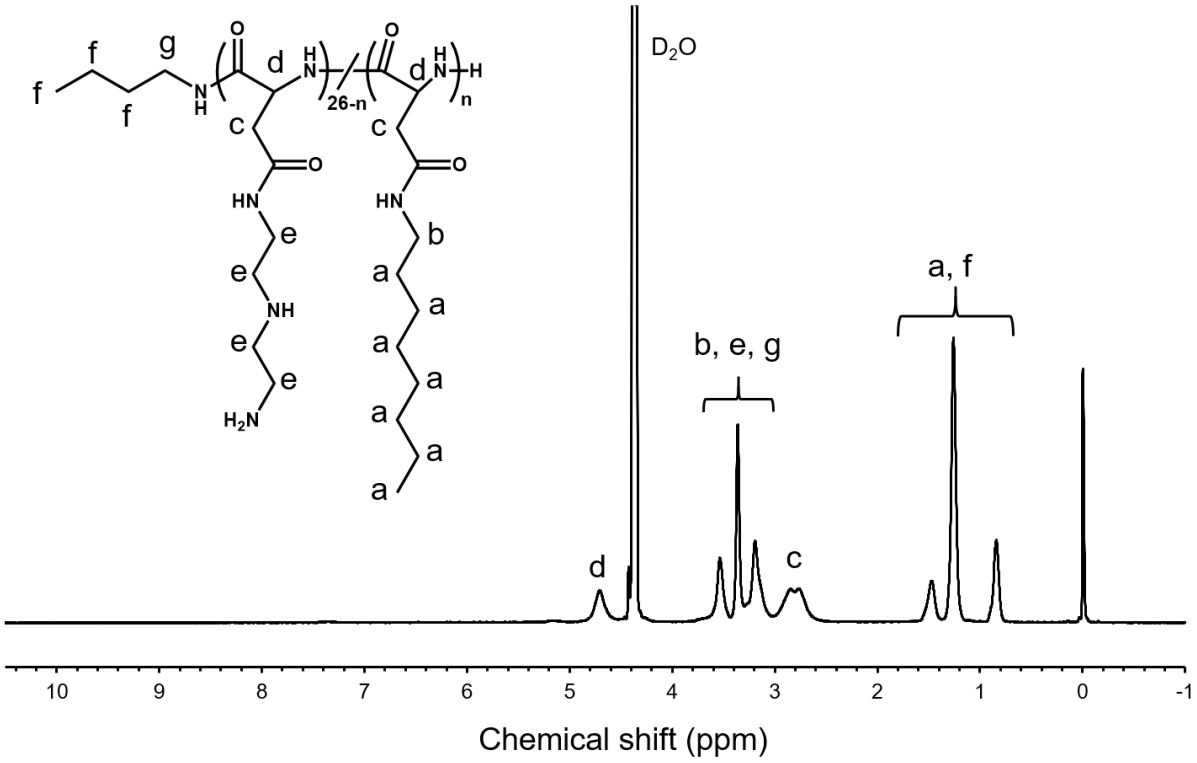


Figure S3. ^{1}H NMR spectrum of PAsp(DET/OCT) (DP = 26, OCT unit number = 10) (10 mg/mL, D_2O, 400 MHz, 80 °C).

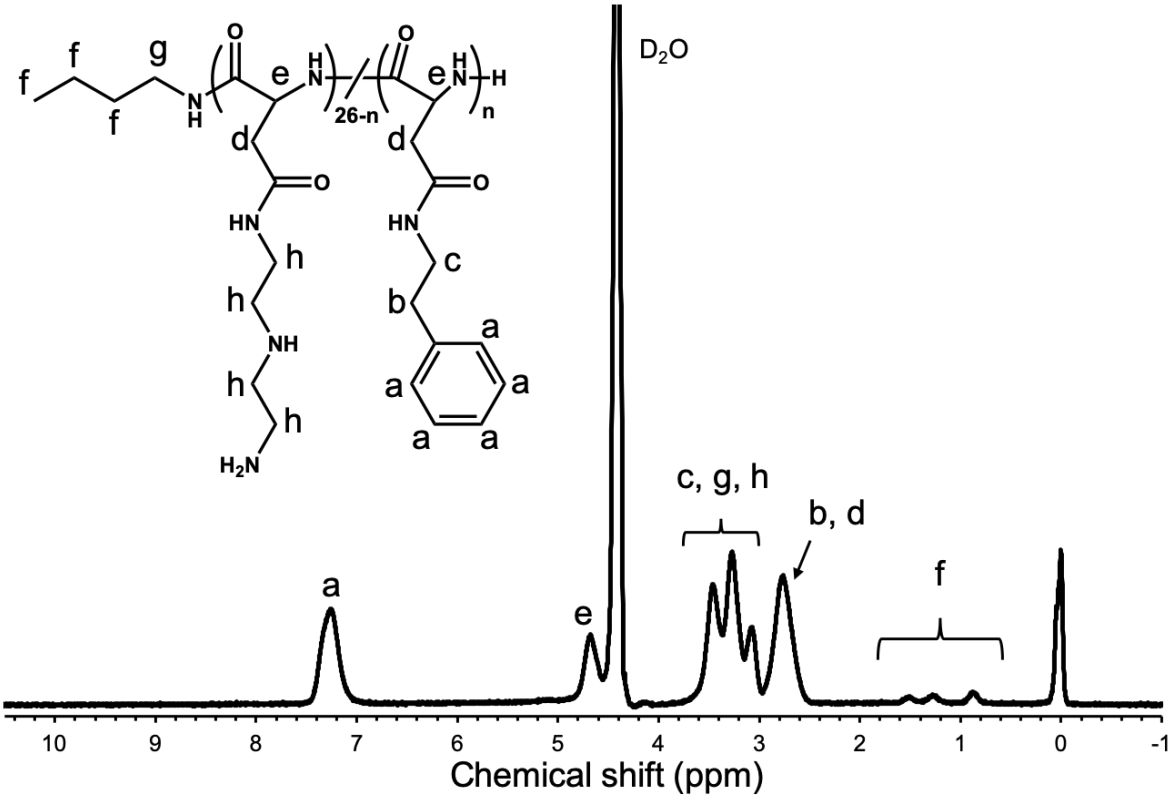


Figure S4. ^{1}H NMR spectrum of PAsp(DET/PHE) (DP = 26, PHE unit number = 11) (10 mg/mL, D_2O, 400 MHz, 80 °C).

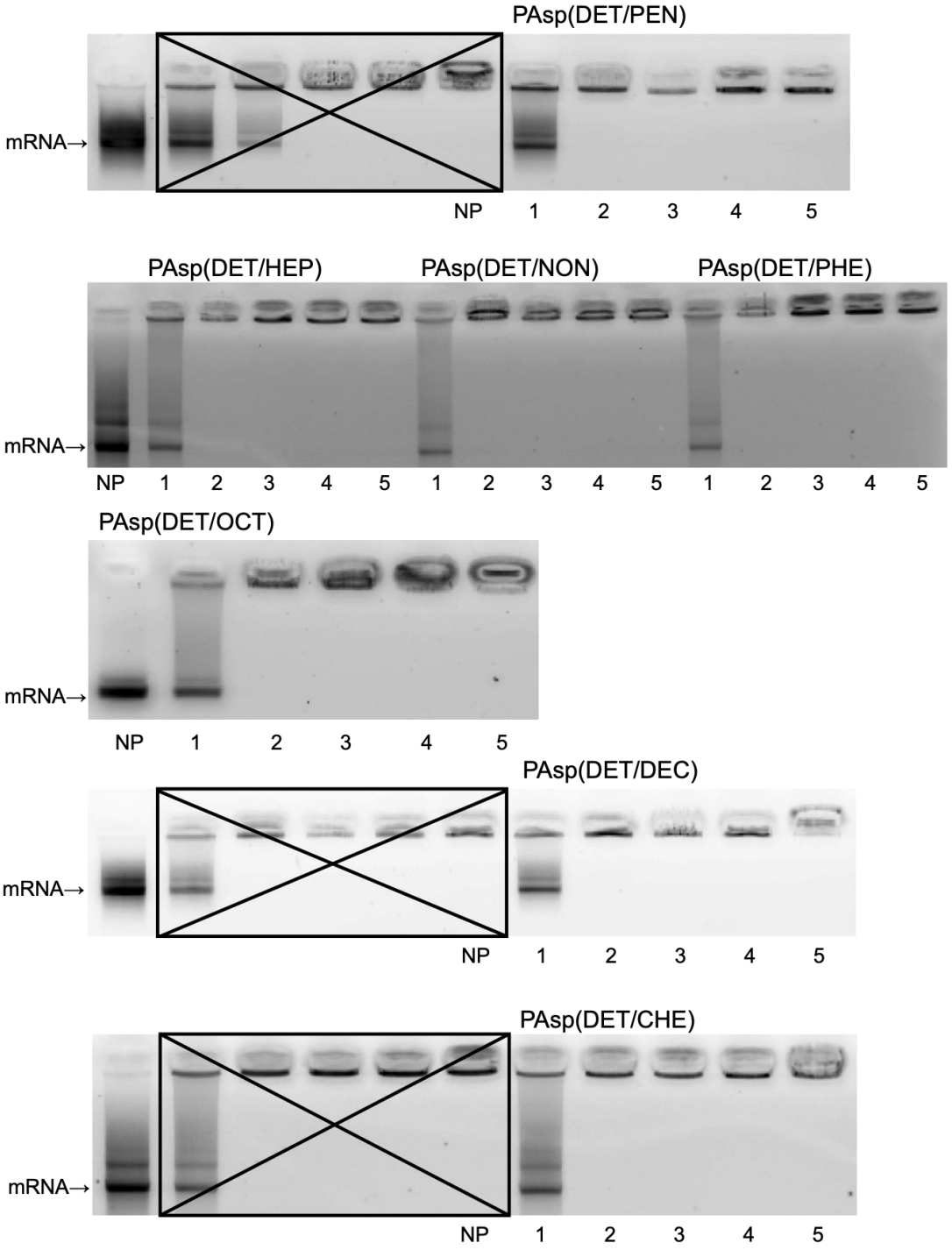


Figure S5. Agarose gel electrophoresis of polyplexes prepared at various N/P ratios between amphiphilic polyaspartamides (DP = 26) and GLuc-mRNA. The mRNA on the gel was stained with ethidium bromide. Unrelated lanes are marked with cross.

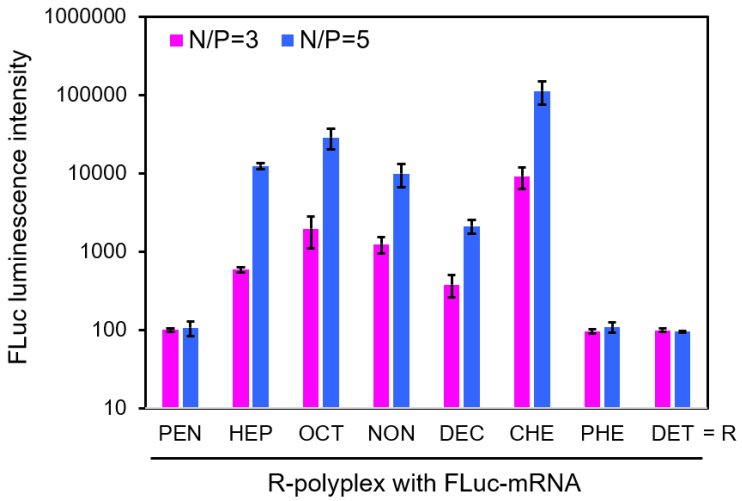


Figure S6. Luminescence intensities of cultured C2C12 cells transfected with FLuc-mRNA. The mRNA-loaded polyplex samples were prepared from varying PAsp(DET/R)s (DP = 26) at N/P ratios of 3 and 5. The cells were incubated with each polyplex (50 ng mRNA/well) for 24 h. All results are expressed as the mean ± SD (n = 4).

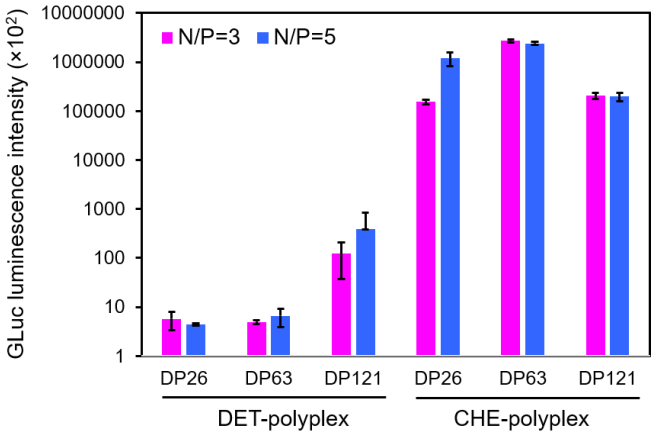


Figure S7. Luminescence intensities of cultured cells transfected with GLuc-mRNA. C2C12 cells were transfected by PAsp(DET) and PAsp(DET/CHE) with varying DPs. The cells were incubated with each polyplex (50 ng mRNA/well) for 24 h. All results are expressed as mean ± SD (n = 4).

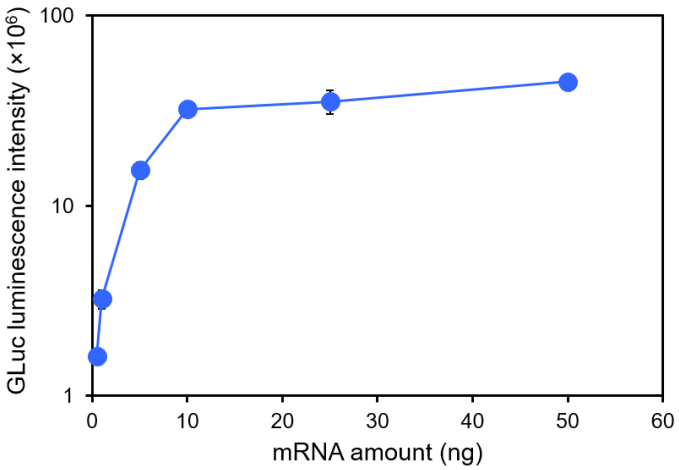


Figure S8. A dose-dependent GLuc expression profile of CHE-polyplex (DP = 26, N/P = 5) at 0.5–50 ng mRNA/well. The C2C12 cells were transfected for 24 h before luminescence measurement. All results are expressed as the mean ± SD (n = 4).

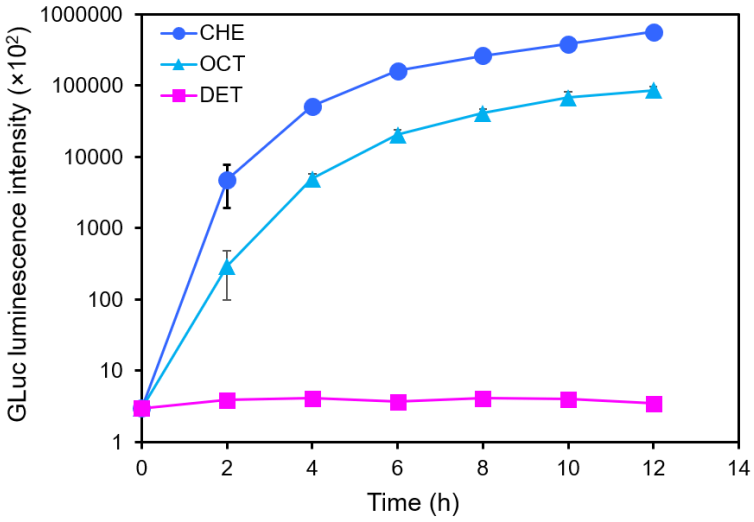


Figure S9. Time-dependent GLuc expression profiles of CHE-, OCT-, and DET-polyplexes (DP = 26, N/P = 5) at 50 ng mRNA/well. All results are expressed as the mean ± SD (*n* = 4).

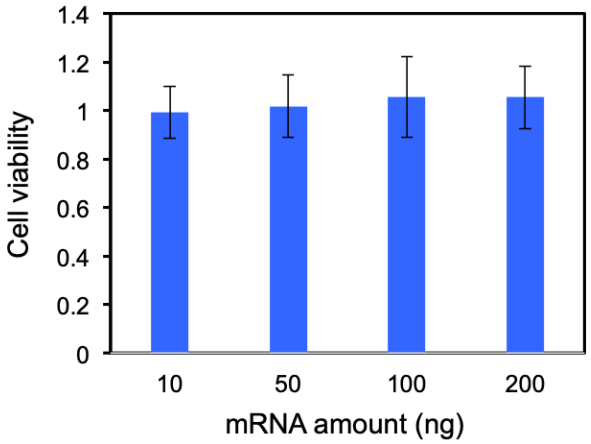


Figure S10. Dose-dependent viability of C2C12 cells treated with CHE-polyplexes (DP = 26, N/P = 5) for 24 h. Results are expressed as the mean ± SD (*n* = 4).

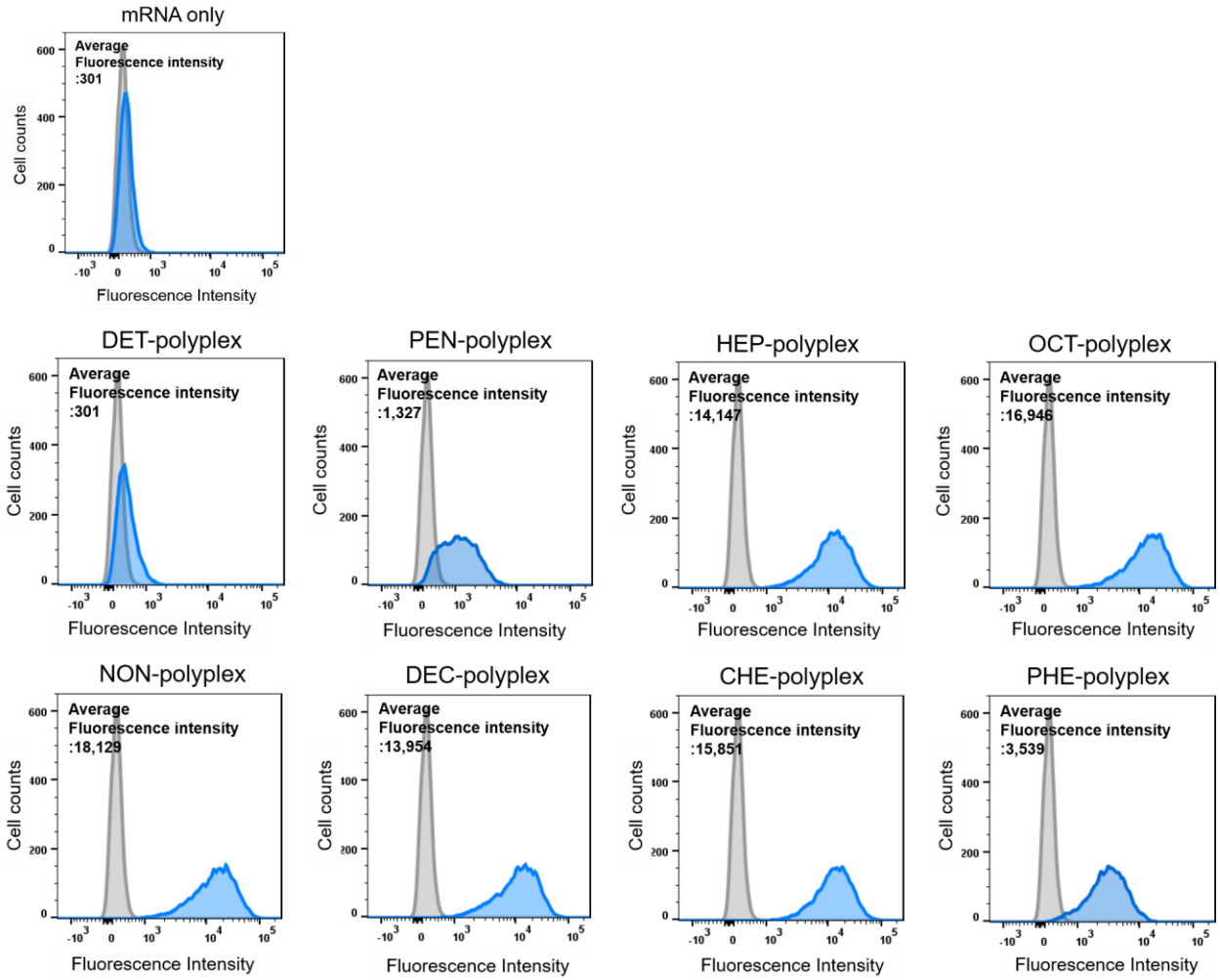


Figure S11. Cellular uptake profiles of Cy5-mRNA-loaded polyplexes (DP = 26, N/P = 5, 500 ng mRNA/well) in cultured C2C12 cells in 4-h incubation. Buffer-treated control cells are shown in gray color.

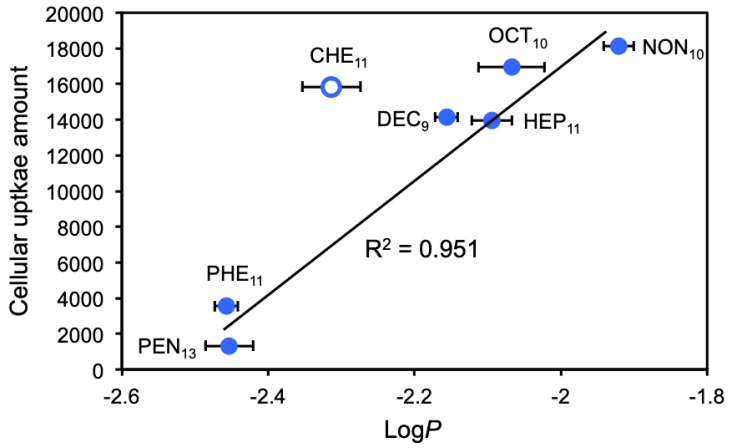


Figure S12. Cellular uptake efficiencies plotted against log *P*. The cellular uptake efficiency of Cy5-mRNA-loaded polyplexes (DP = 26, N/P = 5) in C2C12 cells was determined by flow cytometric analyses (Figure S11). The approximate straight line was drawn after omitting the value obtained from CHE-polyplex.

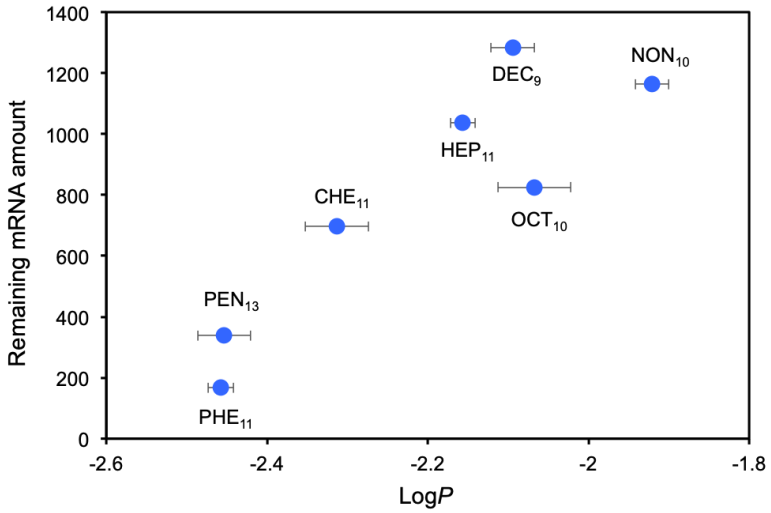


Figure S13. The remaining naked mRNA amounts plotted against log *P*. Polyplexes (N/P = 5) were incubated in 10% FBS for 1 h at 37 °C. The remaining mRNA amounts were quantitatively analyzed by Image Lab™ software (Bio-Rad) (Figure 3A).

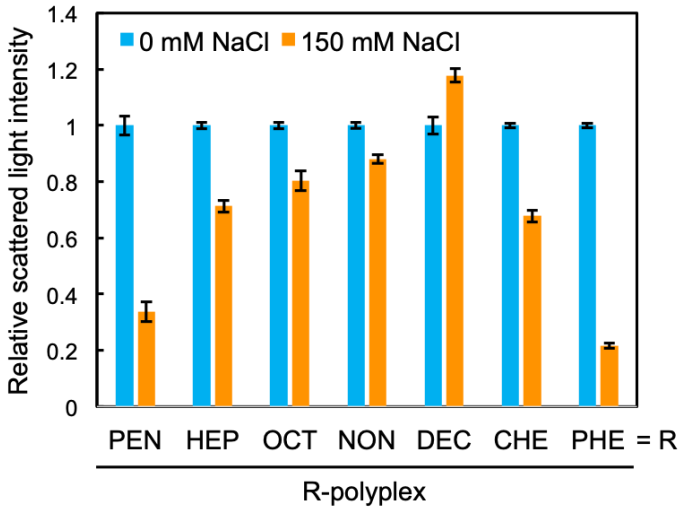


Figure S14. Relative scattered light intensity of polyplex samples (50 ng mRNA, N/P = 5) at 0 mM and 150 mM NaCl, determined by a Zetasizer. Polyplex solution was incubated for 4 h at room temperature before measurement. All results are expressed as mean ± SD (*n* = 3).

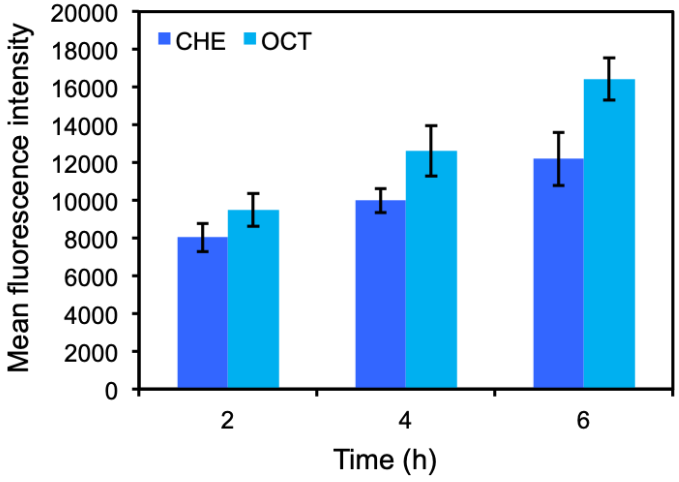


Figure S15. Time dependent cellular uptake of CHE- and OCT-polyplexes (N/P = 5), measured by flow cytometry. Results are expressed as mean ± SD (*n* = 3).

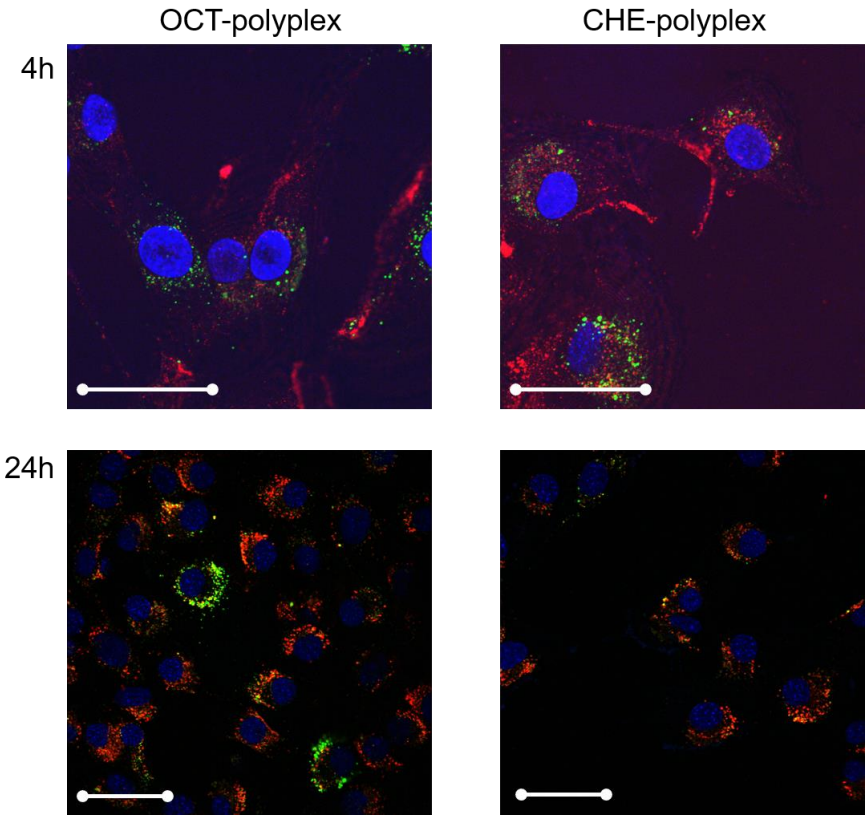


Figure S16. CLSM images showing intracellular distribution of OCT- and CHE-polyplexes (N/P = 5) in C2C12 cells. Cy5-mRNA and LysoTracker (a late-endosome and lysosome marker) are shown in red and green, respectively. The nuclei were stained with Hoechst 33342 in blue. Scale bars represent 50 μm.

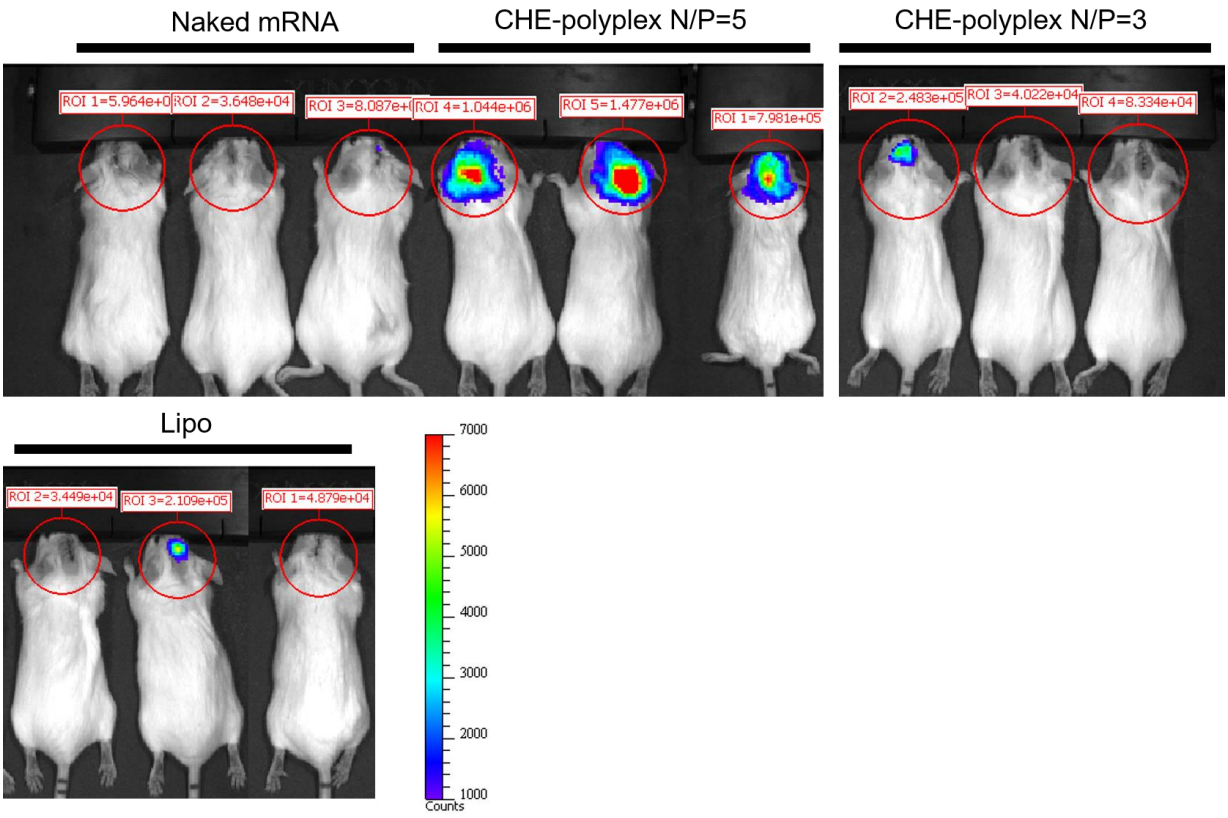


Figure S17. Bioluminescence images of mice treated with FLuc-mRNA and its polyplexes (DP = 26) and lipoplex. The FLuc expression was recorded at 4 h post-intracerebroventricular administration into mice (4.5 µg FLuc-mRNA/mouse).

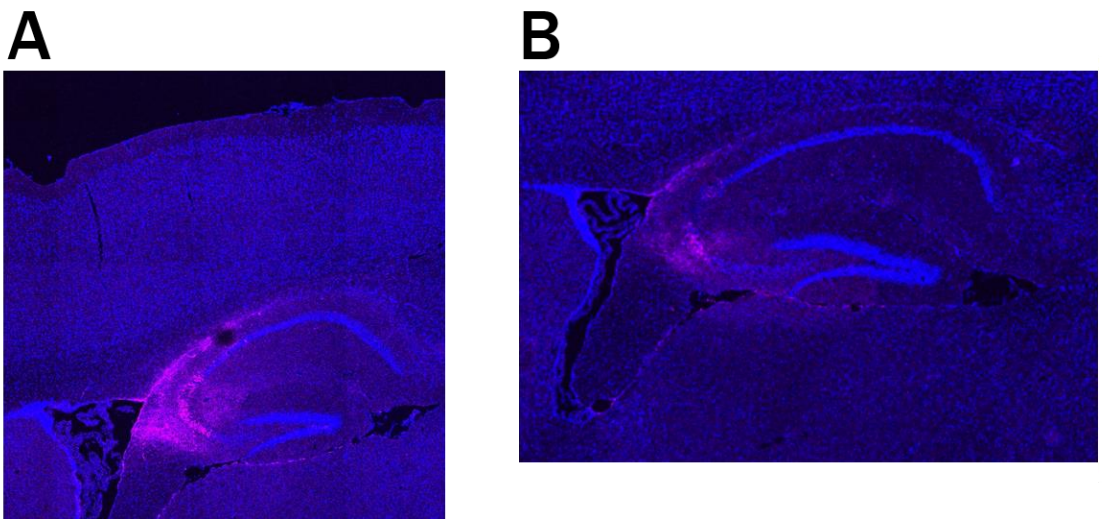


Figure S18. Histological evaluation of naked Cy5-mRNA at 4 h postinjection. Brain was serially sectioned from the center to the side. Section images were positioned at (A) ~1.2 mm and (B) ~1.4 mm from an injection site. DAPI-stained nucleus and Cy5 dye were shown in blue and red, respectively.

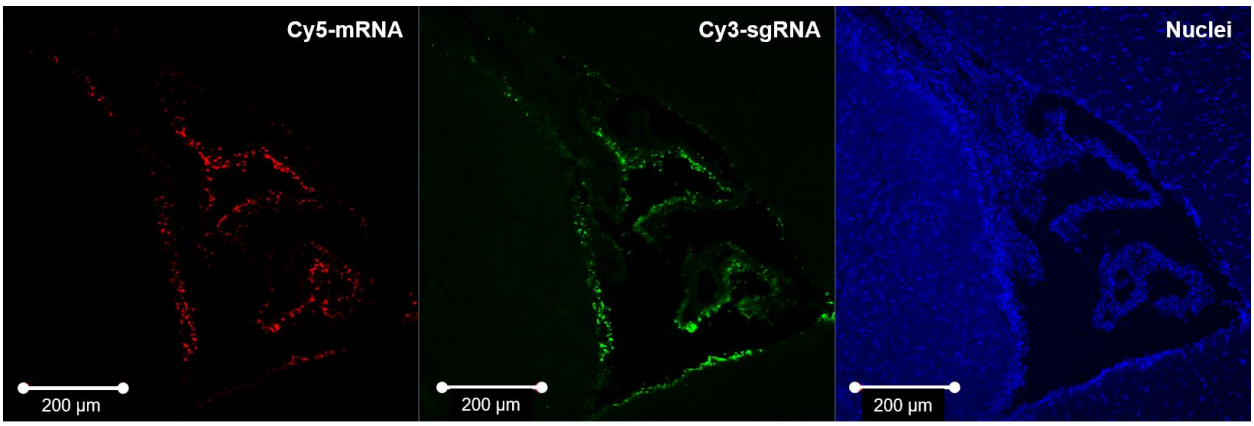


Figure S19. Histological evaluation of Cy5-mRNA/Cy3-sgRNA-loaded CHE-polyplexes (DP = 26, N/P = 5) 4 h after intracerebroventricular injection (1.5 μg Cy5-mRNA and 1.5 μg Cy3-sgRNA/mouse). Cy5-mRNA, Cy3-sgRNA, and DAPI-stained nuclei are shown in red, green, and blue, respectively.