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ORIGINAL ARTICLE

Potential Use of Folate-polyethylene glycol (PEG)-Appended Dendrimer (G3) Conjugate with α-Cyclodextrin as DNA Carriers to Tumor Cells

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Running title: Potential use of Fol-PαC as DNA Carriers to Tumors

ABSTRACT

We previously reported that polyamidoamine STARBURST™ dendrimer (generation 3, G3) (dendrimer) conjugate with α-cyclodextrin (α-CyD) having an average degree of substitution of 2.4 of α-CyD (α-CDE) provided remarkable aspects as a novel carriers for DNA and siRNA. To develop novel α-CDE derivatives with tumor cell specificity, we prepared folate-appended α-CDEs (Fol-α-CDEs) and folate-polyethylene glycol (PEG)-appended α-CDEs (Fol-PαCs) with the various degrees of substitution of folate (DSF), and evaluated *in vitro* and *in vivo* gene transfer activity, cytotoxicity, cellular association and physicochemical properties. *In vitro* gene transfer activity of Fol-α-CDEs (G3, DSF 2, 5 or 7) was lower than that of α-CDE (G3) in KB cells, folate receptor (FR)-overexpressing cancer cells. Of the three Fol-PαCs (G3, DSF 2, 5 or 7), Fol-PαC (G3, DSF 5) had the highest gene transfer activity in KB cells. The activity of Fol-PαC (G3, DSF 5) was significantly higher than that of α-CDE (G3) in KB cells, but not in A549 cells, FR-negative cells. Negligible cytotoxicity of the pDNA complex with Fol-PαC (G3, DSF 5) was observed in KB cells or A549 cells up to a charge ratio of 100/1 (carrier/pDNA). The cellular association of the pDNA complex with Fol-PαC (G3, DSF 5) could be mediated by FR on KB cells, resulting in its efficient cellular uptake. Fol-PαC (G3, DSF 5) had higher binding affinity with folate binding protein (FBP) than α-CDE (G3), although the physicochemical properties of pDNA complex with Fol-PαC (G3, DSF 5) were almost comparable to that with α-CDE (G3), although the onset charge ratio and the compaction ability of Fol-PαC (G3, DSF 5) were slightly different. Fol-PαC (G3, DSF 5) tended to show higher gene transfer activity than α-CDE (G3) 12 h after intratumoral administration in mice. These results suggest that Fol-PαC (G3, DSF 5), not Fol-α-CDEs, could be potentially used as a FR-overexpressing cancer cell-selective DNA carrier.

Keywords: PAMAM dendrimer; α-cyclodextrin; folate; gene transfer

Introduction

Polyamidoamine STARBURST™ dendrimers (dendrimers) are biocompatible, non-immunogenic and water-soluble, and possess terminal modifiable amine functional groups for bearing various targeting or guest molecules.[1] Unlike classical polymers, dendrimers have a high degree of molecular uniformity, narrow molecular weight distribution, specific size and shape characteristics, and a highly-functionalized terminal surface.[2] Dendrimers can form complexes with nucleic acid drugs such as plasmid DNA (pDNA), short-hairpin RNA (shRNA) and small-interfering RNA (siRNA) through the electrostatic interaction and bind to glycosaminoglycans (heparan sulfate, hyaluronic acid and chondroitin sulfate) on cell surface,[3, 4] and have been shown to be more efficient and safer than either cationic liposomes or other cationic polymers for *in vitro* gene transfer. [5, 6]

Cyclodextrins (CyDs) are cyclic (α-1,4)-linked oligosaccharides of α-D-glucopyranose containing a hydrophobic central cavity and hydrophilic outer surface, and they are known to be able to act as host molecules.[7-9] CyDs have recently been applied to gene transfer and oligonucleotide delivery.[10-13] We previously reported that of various dendrimer conjugates with α-CyD (α-CDE), α-CDE (G3) with the degree of substitution (DS) of 2.4 was revealed to have the highest transfection efficiency *in vitro* and *in vivo* with low cytotoxicity.[14-16] Moreover, we previously reported the potential use of α-CDEs bearing galactose (Gal-α-CDE), mannose (Man-α-CDE) or lactose (Lac-α-CDE) with the various DS values of these sugar moieties as gene delivery carriers.[17-20]

The targeted gene delivery using bioconjugates is exploring to increase the efficiency of drug delivery to specific tissues as well as to decrease the minimum effective dose of the drug as well as its side effects.[21] Strategies to develop tumor-cell specific bioconjugates are multimodal, but all attempts to selectively deliver therapeutics to cells use nano- and submicron-scale carriers such as dendrimers, liposomes, polymers, emulsions, or viruses including active and/or passive targeting moieties.[22] Folic acid (FA) has been shown to be one of the most promising ligands for targeting a range of human carcinomas, including breast, ovary, endometrium, kidney, lung, head and neck, brain and myeloid cancers, which are known to express folate receptors (FR).[23, 24] Moreover, FA is a relatively small

molecule (MW 441 Da) which consequently has only limited effects on the dimensions of the carrier system, high stability, compatibility with both organic and aqueous solvent, low-cost, non-immunogenic character and the ability to conjugate with a wide variety of molecules, so it has attracted wide attention as a targeting agent.[25] So far some papers regarding folate-appended dendrimers have been published. For example, Konda et al. reported the novel folate-dendrimer MRI contrast agents to the high affinity folate receptor (FR) expressed in ovarian tumor xenografts.[26] Shukla et al.[27] demonstrated the FR-targeted boronated PAMAM dendrimers as potential agents for neutron capture therapy. In addition, Singh et al. reported that folate-PEG-dendrimer conjugate was significantly safe and effective in tumor targeting for 5-fluorouracil, compared to a non-PEGylated formulation.[28]

In the subsequent study, therefore, we prepared folate-appended α-CDEs (Fol-α-CDE) and folate-PEG-appended α-CDEs (Fol-PαC) with various degrees of substitution of folate (DSF) as novel DNA carriers to clarify the effect of PEG and the DSF values, and examined *in vitro* and *in vivo* gene transfer activity, cytotoxicity, cellular uptake and the physicochemical properties.

Materials and methods

Materials

α-CyD was donated by Nihon Shokuhin Kako (Tokyo, Japan). Dendrimer (G3, the terminal amino groups=32, MW=6,909 Da) was purchased from Aldrich Chemical (Tokyo, Japan). Polyethyleneimines (PEIs, linear, 10 kDa and 25 kDa) were obtained from Wako Pure Chemical Industries (Osaka, Japan). *p*-Toluenesulfonyl chloride and FA were purchased from Nakalai Tesque (Kyoto, Japan). ω-Amino-α-carboxyl polyethylene glycol (PEG, MW=3,290 Da) was purchased from NOF corporation (Tokyo, Japan). Plasmid pRL-CMV-Luc vector encoding Renilla luciferase (pDNA) was obtained from Promega (Tokyo, Japan). The purification of pDNA amplified in bacteria was carried out using QIAGEN EndoFree plasmid maxi kit (< 0.1 EU/μg endotoxin). Other chemicals and solvents were of analytical reagent grade.

Preparation of Fol-α-CDEs (G3) and Fol-PαCs (G3)

Figure 1 shows the schemes for the preparation of Fol-α-CDEs (G3) and Fol-PαCs (G3). Fol-α-CDEs (G3) were prepared according to the method of Majoros et al.[29] and Oh et al.[30] Herein, α-CDE (G3, DS of α-CyD=2.4) was prepared as previously reported.[16] In brief, FA in DMF/DMSO containing 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was mixed at room temperature (r.t.) for 1 h. Then, α-CDE (G3) was added to the solution and incubated at r.t. for 48 h. In addition, Fol-PαCs (G3) were prepared as follows: FA in DMSO containing N,N-dicyclohexylcarbodiimide (DCC) and N-hydroxysuccinimide (NHS) was mixed at r.t. for 30 min. Then, ω-amino-α-carboxyl polyethylene glycol (PEG, MW=3,290 Da) and pyridine were added into the solution and incubated at r.t. for 2 h. After removal of intact FA by a precipitation method with water and a purification by gel-filtration (TOSOH TSKGel HW-40S, Tokyo, Japan), Fol-PEG-COOH was activated with 0.2 M boric acid solution containing EDC and NHS, and then mixed at r.t. for 2 h. Then, α-CDE (G3) was added to the solution and incubated for 48 h. Fol-α-CDEs (G3) and Fol-PαCs (G3) were purified by a dialysis and/or a gel-filtration.

Cell Culture

KB cells, a human carcinoma of the nasopharynx, were grown in a RPMI-1640 culture medium (FA-free) containing penicillin (1 x 10^5 mU ml^{-1}) and streptomycin (0.1 mg ml^{-1}) supplemented with 10% FCS at 37°C in a humidified 5% CO_2 and 95% air atmosphere. A549 cells, a human lung epithelium cell line, were cultured as reported previously.[15, 16]

In Vitro Gene Transfer

In vitro transfection of the pDNA complexes with carriers was performed utilizing the Renilla luciferase gene system in the various cells as reported previously.[15, 16] The Tris-EDTA (TE) buffer containing pDNA (2.0 μg) was generally mixed with 200 μl of serum-free medium containing α-CDE (G3), Fol-α-CDEs (G3, DSF 1, 2 or 3) or Fol-PαCs (G3, DSF 2, 5 or 7) mildly agitated, and then allowed to stand at r.t. for 15 min. These pDNA

complexes were prepared at a charge ratio of 50/1 (carrier/pDNA), where the optimal points for the gene transfer activity were in all of the pDNA complexes. The cells (2×10^5 cells $well^{-1}$) were seeded 6 h before transfection, and then washed twice with serum-free medium. Four hundred microliters of culture medium containing the complexes with various carriers supplemented with 10% FCS were added to each well, and then incubated at 37°C for 24 h. After transfection, the luciferase gene expression was measured as reported previously [15, 16].

Cytotoxicity

The effects of pDNA complex with α-CDE (G3), Fol-PαCs (G3, DSF 5) or PEIs (10 kDa and 25 kDa) on cell viability were measured as reported previously.[15, 16] In brief, the transfection was performed as described in the transfection section. After washing twice with HBSS (pH 7.4) to remove pDNA and/or various carriers, 270 μl of fresh HBSS and 30 μl of WST-1 reagent were added to the plates and incubated at 37°C for 30 min. The absorbance of the solution was measured at 450 nm, with referring absorbance at 655 nm, with a Bio-Rad Model 550 microplate reader (Bio-Rad Laboratories, Tokyo, Japan).

Cellular Association

Cellular association of the complex was determined by a flow cytometry. Two micrograms of Alexa488-labeled pDNA (Alexa-pDNA) were mixed with α-CDE (G3) or Fol-PαCs (G3, DSF 5) at a charge ratio of 50 (carrier/pDNA). After transfection with the complexes of Alexa-pDNA/carrier for 1 h in KB cells and A549 cells, the cells were washed with PBS (pH 7.4) twice and immediately scraped with 1 ml of PBS (pH 7.4). The cells were collected and filtered through nylon mesh. Data were collected for 1×10^4 cells on a FACSCalibur flow cytometer using a CellQuest software (Becton-Dickinson, Mountain View, CA).

Confocal Laser Scanning Microscopy (CLSM)

To observe the cellular association of Alexa-pDNA complex with α-CDE (G3) or Fol-PαCs (G3, DSF 5), KB cells (2 x 10^5 cells $dish^{-1}$) were incubated with the complexes of Alexa-pDNA/carrier for 3 h. After incubation, the cells were rinsed with PBS (pH 7.4) twice and fixed in methanol at 4˚C for 5 min prior to microscopy. Cells were observed by a CLSM (Olympus FV300-BXCarl Zeiss LSM-410, Tokyo) with an argon laser of 350-550 nm after fixation. Here, the intracellular distribution of Alexa-pDNA was observed in a single plane, not a z-section.

Interaction between pDNA and Carriers

Electrophoretic mobility of the pDNA complexes with α-CDE (G3) or Fol-PαC (G3, DSF 5) was measured using a gel electrophoresis system. Various amounts of α-CDE (G3) or Fol-PαC (G3, DSF 5) were mixed with 0.2 μg of pDNA in HBSS (pH 7.4). Gel electrophoresis was carried out at r.t. in TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0) in 1% agarose gel including 0.1 μg/ml of ethidium bromide using the Mupid™ system (Cosmo Bio, Tokyo, Japan) at 100 V for 40 min. The pDNA bands were visualized using an UV illuminator.

Particle Size and ζ-Potential

The solution containing α-CDE (G3) or Fol-PαC (G3, DSF 5) at various charge ratios was added to Tris-HCl buffer (10 mM, pH 7.4) containing 5 μg of pDNA. Then, the solution was incubated for 15 min. The particle size and ζ-potential of the pDNA complex of α-CDE (G3) or Fol-PαC (G3, DSF 5) were determined by dynamic light scattering using a Zetasizer Nano (Malvern Instruments, Worcestershire, UK). The dynamic light scattering was analyzed by the general purpose mode. The measurements were carried out at least in triplicates.

DNA Condensed Assay

pDNA (0.5 μg) and α-CDE (G3) or Fol-PαC (G3, DSF 5) were added to 350 μl of HBSS (pH 7.4) at the various charge ratios. The solutions were incubated at 25°C for 15 min, and then 1.75 μl of Picogreen® dsDNA reagent and 348.25 μl of HBSS were added to the solutions and incubated at 25°C for 30 min. The fluorescence (λ_{ex}=495 nm, λ_{emi}=525 nm) was measured by fluorescence spectrometer Hitachi F-4500 (Tokyo, Japan). Samples containing pDNA (0.5 μg) and Picogreen® dsDNA reagent (1.75 μl) were used to calibrate the apparatus to 100% fluorescence against a background of Picogreen® dsDNA reagent (1.75 μl).

Surface Plasmon Resonance (SPR) Optical Biosensor

The molecular interaction of FBP with Fol-PαC (G3, DSL 5) was examined using an optical biosensor "IAsys" based on SPR (Affinity Sensor, Cambridge, UK). The immobilization of FBP on the sensor cuvette was carried out by the reaction of a reactive linker molecule with the cuvette surface. After activation by washing with 8 M urea solution containing 10 mM $MnCl_2$, the interaction curves were measured at the concentrations of carriers (10^{-8} to 10^{-6} M) in 10 mM acetate buffer (pH 5.3) with 1 mM $CaCl_2$ and 100 mM NaCl at 25°C. The association constant was obtained by measuring the change in the refractive index according to the usual procedure. The computational results were derived using a software FAST-fit equipped in the IAsys.

In Vivo Gene Transfer

Murine colon-26 adenocarcinoma cells (5 x 10^5 cells 100 μl^{-1}) were inoculated subcutaneously in male four-weeks-old BALB/c mice (ca. 20 g). After 10 days, the tumor-bearing mice were intratumoraly injected with 500 μl of a 5% mannitol solution containing the pDNA complex of α-CDE (G3) or Fol-PαC (G3, DSF 5) at a charge ratio of 20 (carrier/pDNA) at the amount of 20 μg of pDNA in 30 sec under anesthesia with ether. Twelve h after intratumoral administration, the mice were sacrificed, and tumor tissues were isolated. The tissues were washed twice with ice-cold saline and were added to 2 ml of the Promega cell lysis buffer (Tokyo, Japan) containing the Roche protease inhibitor,

Complete® (Tokyo, Japan). The tissues were homogenized with a Polytron tissue grinder (Ultra-Turrax T25 Basic S1, IKA Works, Wilmington, NC). After three cycles of freezing and thawing, the homogenate was centrifuged for 10 min at 10,000 g (4°C), and 20 μl of the supernatant was added to 100 μl of the Renilla luciferase assay buffer (Promega, Tokyo, Japan). Luminescence was immediately measured for 10 sec (Lumat LB9506, EG&G Berthhold Japan, Tokyo, Japan). Total protein content of the supernatant was determined by Bio-Rad DC protein assay kit (Tokyo, Japan). The luciferase activity in the tumor cells was determined as described above.

Data Analysis

Data are given as the mean ± S.E.M. Statistical significance of mean coefficients for the studies was performed by analysis of variance followed by Scheffe's test. *p*-Values for significance were set at 0.05.

RESULTS AND DISCUSSION

Preparation of Fol-α-CDEs and Fol-PαCs

The preparations of Fol-α-CDEs (G3) and Fol-PαCs (G3) were carried out according to the method of Majoros et al.[29] and Oh et al.[30]. Fol-α-CDEs (G3) were prepared by direct conjugation of FA to the α-CDE (G3, DS of α-CyD = 2.4) by using EDC and NHS in DMF/DMSO, and then the resulting conjugates were purified by a dialysis (MWCO = 3,500) and a gel-filtration (Fig. 1). In the case of Fol-PαCs (G3), Fol-PEG-COOH was firstly prepared by using ω-amino-α-carboxyl polyethylene glycol (PEG, MW = 3,290 Da), and the preparation of Fol-PEG-COOH was confirmed by the MALDI-TOF-Mass spectrum (Supplementary Fig. 1). Next, a carboxyl group of the Fol-PEG-COOH was condensed with primary amino group of α-CDE (G3, DS of α-CyD=2.4), and then the resulting conjugates were purified by a dialysis (MWCO = 15,000) (Fig. 1). The DSF values of Fol-α-CDEs (G3) and Fol-PαCs (G3) were controlled by adjusting the additive amounts of FA and Fol-PEG-COOH, respectively. All of the resulting conjugates included no unreacted

FA or Fol-PEG-COOH. In the ^{1}H-NMR spectra, Fol-α-CDEs (G3) and Fol-PαCs (G3) gave peaks of each component, such as dendrimer, α-CyD and FA or Fol-PEG (Supplementary Figs. 2, 3). The DSF values of these conjugates were accurately determined by measuring peak areas of the anomeric proton of α-CyD and benzoic proton of FA. The product yields of conjugates were 28% (DSF 1), 61% (DSF 2) and 54% (DSF 3) in the Fol-α-CDEs (G3) system, and 72% (DSF 2), 79% (DSF 5) and 89% (DSF 7) in the Fol-PαCs (G3) system, respectively.

In Vitro Gene Transfer Activity

To investigate whether Fol-α-CDEs (G3) and Fol-PαCs (G3) have tumor cell-specific and efficient gene transfer activity and which carrier has the highest gene transfer activity, Renilla luciferase activity after transfection of pDNA complexes at a charge ratio of 50/1 (carrier/pDNA) in various cells was determined (Figs. 2, 3). Here, we confirmed that FR expresses in KB cells, but not in A549 cells, using a RT-PCR method (Supplementary Fig. 4), which is consistent with the results previously reported.[31] In KB cells, α-CDE (G3) showed efficient gene transfer activity, compared to dendrimer (G3), possibly due to the enhancement of endosomal escape of pDNA as reported by Arima et al.[14-16] Gene transfer activity of Fol-α-CDE (G3, DSF 2) and Fol-α-CDEs (G3, DSF 1 and 3) was almost comparable to α-CDE (G3) and significantly lower than α-CDE (G3), respectively, in the KB cells (Fig. 2). The insufficient gene transfer activity of Fol-α-CDEs (G3) may result from the low receptor binding activity to FR on the KB cells. Meanwhile, the previous reports demonstrated that an introduction of PEG as a spacer between FA and carriers increases FR-dependent gene transfer activity of liposomes[32] and poly-L-lysine.[33] To improve the FR binding activity of Fol-α-CDE (G3), therefore, we prepared Fol-PαCs (G3), which have a PEG spacer between dendrimer and FA (Fig. 1), and examined FR-selective gene transfer activity of Fol-PαCs (G3, DSF 2, 5 or 7) in KB cells and A549 cells (Fig. 3). In KB cells, Fol-PαC (G3, DSF 5) showed higher gene transfer activity than α-CDE (G3) and Fol-PαCs (G3, DSF 2 and 7) (Fig. 3A). Meanwhile, in A549 cells, all of

Fol-PαCs (G3, DSF 2, 5 or 7) showed markedly lower gene transfer activity than α-CDE (G3) (Fig. 3B). The activity of Fol-PαC (G3, DSF 5), not Fol-α-CDE (G3, DSF 2), in KB cells was 3.7-fold higher than that in A549 cells, although the activity of α-CDE (G3) in KB cells was decreased 0.9-fold relative to that in A549 cells (Fig. 3C). These results suggest that gene transfer activity of Fol-PαC (G3, DSF 5) in KB cells is in a FR-dependent manner.

Cytotoxicity

Cytotoxicity is often associated with less transfection efficiency of non-viral vectors.[34] Therefore, we evaluated cytotoxicity of Fol-PαC (G3, DSF 5) by the WST-1 method (Fig. 4). No cytotoxicity of pDNA complexes with Fol-PαC (G3, DSF 5) was observed in KB cells (Fig. 4A) and in A549 cells (Fig. 4B) up to a charge ratio of 100/1 (carrier/pDNA). Meanwhile, cytotoxicity of the pDNA complexes with α-CDE (G3) increased as the charge ratio increased in the both cells, indicating that the pDNA complex with Fol-PαC (G3, DSF 5) possesses low cytotoxicity rather than that with α-CDE (G3), which has been reported as a safe carrier.[16] As described above, an introduction of PEG between dendrimer and FA in the Fol-PαC (G3, DSF 5) molecule can be improved its binding activity to FR. In addition, an introduction of a PEG chain to drug carriers is generally acknowledged to improve its half-life in blood and biocompatibility.[35] Therefore, an introduction of PEG is useful to improve not only binding activity to FR but also low cytotoxicity. Meanwhile, the pDNA complexes with PEIs (10 and 25 kDa) markedly lowered cell viability, i.e. it decreased to about 50% even at a charge ratio of 20/1 (PEI/pDNA) (Fig. 4). These results suggest that Fol-PαC (G3, DSF 5) has negligible cytotoxicity under the present experimental conditions.

Cellular Association and Intracellular Distribution

As FR, which likely increases local folate concentrations at the plasma membrane to allow its efficient uptake via folate transporters, is a GPI-linked protein, which is entered by the

clathrin-independent carrier (CLIC)/GPI-AP-enriched early endosomal compartment (GEEC) pathway.[36] Therefore, we hypothesized that pDNA complex with Fol-PαC (G3, DSF 5) is entered by CLIC/GEEC endocytosis pathway. To verify this hypothesis, we examined the cellular association of Alexa-pDNA 1 h after transfection of the complexes of Alexa-pDNA/carriers with or without FA, a competitor for FR, in KB cells by a flow cytometric analysis (Fig. 5). The cellular association of Alexa-pDNA in the α-CDE (G3) system was not changed by addition of FA (Fig. 5A). Meanwhile, the competitive effect of FA on the cellular association of pDNA complex with Fol-PαC (G3, DSF 5) was observed in KB cells (Fig. 5B). These results suggest that cellular association of the pDNA complex with Fol-PαC (G3, DSF 5) could be mediated by FR on KB cells.

Next, we investigated the intracellular distribution of Alexa-pDNA after transfection of the complexes of Alexa-pDNA/carriers using a CLSM (Fig. 6). The complex of Alexa-pDNA with dendrimer or α-CDE (G3) gave a moderate fluorescence in KB cells. Meanwhile, more intense fluorescence was observed in the pDNA/Fol-PαC (G3, DSF 5) complex system, suggesting FR-dependent and efficient cellular uptake of pDNA complex with Fol-PαC (G3, DSF 5) in KB cells. This cellular association and intracellular distribution of pDNA complex with Fol-PαC (G3, DSF 5) is likely to be accordance to its gene transfer activity (Fig. 2). These results suggest that FR-mediated cellular uptake of pDNA complex with Fol-PαC (G3, DSF 5) is strongly involved in its cell-specific and efficient gene transfer activity.

Physicochemical Properties

To make sure whether pDNA/Fol-PαC (G3, DSF 5) complex has adequate physicochemical properties, we examined the complex formation between pDNA/α-CDE (G3) and pDNA/Fol-PαC (G3, DSF 5) using an agarose electrophoresis. As shown in Fig. 7, the intensity of the band derived from pDNA decreased as the charge ratio of pDNA/α-CDE (G3) increased, and at a charge ratio of 1 (carrier/pDNA) the band disappeared. In the case of Fol-PαC (G3, DSF 5), the bands vanished at a charge ratio of 2. These results

suggest that Fol-PαC (G3, DSF 5) can form the complex with pDNA at a charge ratio of more than 2, although the complexation ability of Fol-PαC (G3, DSF 5) with pDNA could be slightly lower than that of α-CDE (G3), owing to a decrease in the number of the positively charged primary amino groups in the molecule and/or steric hindrance by an introduction of Fol-PEG moieties to dendrimer molecule. Meanwhile, Fol-PαC (G3, DSF 5) provided the highest gene transfer activity at a charge ratio of more than 50 (supplementary Fig. 5). Herein, we calculated molar raios (carrie/pDNA) at the charge ratio (carrie/pDNA) of 50, i.e. the molar ratios of pDNA complexes with Fol-PαC (G3, DSF 5) and α-CDE (G3) calculated were 1.66×10^4 and 1.43×10^4 under the optimal *in vitro* transfection conditions. Thus, the free extents of Fol-PαC (G3, DSF 5) and α-CDE (G3) must be raised, when the charge ratios increased, because the molar ratios were so high that these carriers may be unable to bind to pDNA. Therefore, the enhancement of gene transfer activity may be ascribed to the additional free Fol-PαC (G3, DSF 5) and α-CDE (G3) at the higher charge ratios, causing enhancing endosomal escape of pDNA complexes with Fol-PαC (G3, DSF 5) and α-CDE (G3) as previously reported.[14-16]

Next, we determined the particle sizes and ζ-potential values of the pDNA complexes with α-CDE (G3) and Fol-PαC (G3, DSF 5) (Table 1). The mean diameters of the complexes with these carriers were around 100 nm, and the ζ-potential values were negative values at a charge ratio of 1 (carrier/pDNA). Meanwhile, the particle sizes and ζ-potential values of the pDNA complexes increased at a charge ratio of 50 in the both systems. It should be noted that ζ-potential values of the pDNA complex with Fol-PαC (G3, DSF 5) at a charge ratio of 50 were close to neutral, compared to that of α-CDE (G3), because of an introduction of Fol-PEG moieties to dendrimer molecule. Leamon et al. reported that a neutral complex is desirable to incorporate into a ligand-targeted gene transfer carrier, because it eliminates the opportunity for the non-specific adsorptive binding to non-target cells.[33] Reddy et al. demonstrated that folate-linked carriers of diameters less than 150 nm are efficiently bound and internalized by FR-expressing cells.[37,

[38] Thus, the low ζ-potential values of the pDNA complex with Fol-PαC (G3, DSF 5) at a charge ratio of 50 may be associated with its cell-selective gene transfer activity.

Cationic non-viral vectors such as cationic polymers and cationic lipids are acknowledged to exert pDNA compaction through electrostatic interaction, leading to the enhancing gene transfer activity.[39-41] To examine the effects of α-CDE (G3) and Fol-PαC (G3, DSF 5) on pDNA condensation, fluorescence intensity of Picogreen® dsDNA reagent was determined (Fig. 8). The relative fluorescence intensity decreased to 15% and 20% in the α-CDE (G3) system and the Fol-PαC (G3, DSF 5) system at a charge ratio of 100, respectively (Fig. 8). These results suggest that the compaction ability of Fol-PαC (G3, DSF 5) to pDNA was lower than that of α-CDE (G3). Nathan et al. reported that the release of pDNA from complexes with cationic polymers such as PEI in cellular nucleus is crucial for higher gene transfer activity.[42] Therefore, low compaction ability of Fol-PαC (G3, DSF 5) may accelerate the release of pDNA from the complex in cells. However, low compaction ability of carriers has also the possibility of decreasing in a stability of pDNA complex *in vivo*. Thereafter, we are currently investigating the release behavior of pDNA from the complex with Fol-PαC (G3, DSF 5) and the stability of the complex in cells, tissues and blood circulation.

To confirm whether Fol-PαC (G3, DSF 5) actually binds to FR, the association constant of Fol-PαC (G3, DSF 5) with FBP was determined using the SPR method (Table 2). Herein, we confirmed that the data showed fairly reproducibility. The association constant of Fol-PαC (G3, DSF 5) showed 35,000-fold and 317-fold increase greater than that of FA alone and α-CDE (G3), respectively, indicating the strong interaction of Fol-PαC (G3, DSF 5) with FBP. In this experiment, however, the association constant is derived from carrier alone, not from pDNA complex. Therefore, the association constant of pDNA complex with Fol-PαC (G3, DSF 5) may increase, compared to that of the carrier alone, possibly due to intense multivalent interaction. Thereafter, the association constant of the complex with Fol-PαC (G3, DSF 5) should be measured. Anyhow, these results indicate that Fol-PαC (G3, DSF 5) had a strong binding affinity with FBP, compared with α-CDE (G3).

In Vivo Gene Transfer

We examined *in vivo* gene transfer activity of pDNA complexes with Fol-PαC (G3, DSF 5) in mice bearing Colon-26 tumor cells. Figure 9 shows gene transfer activity 12 h after intratumoral administration of the solution containing the pDNA/α-CDE (G3) complex or pDNA/Fol-PαC (G3, DSF 5) complex at a charge ratio of 25 to mice bearing tumor cells. *In vivo* gene transfer activity of Fol-PαC (G3, DSF 5) in the tumor cells was higher than that of α-CDE (G3). However, there was not statistically significant difference among the both carriers. As described above, the interaction of pDNA with Fol-PαC (G3, DSF 5) was weaker than that of α-CDE (G3) (Figs. 7, 8). Therefore, the pDNA may rapidly dissociate from Fol-PαC (G3, DSF 5) under the *in vivo* condition, existing many biological compounds such as proteins and/or cellular matrixes. Thereafter, we should optimize the chemical structure of Fol-PαC for its *in vivo* application. Recently, Navarro et al. reported that polyplexes of dendrimer with pDNA allow targeted and extended transgene expression in tumors after systemic administration.[43, 44] Meanwhile, Fol-PαC (G3, DSF 5) has the PEG chain in the molecule, expecting to increase circulating half-life and tumor-selective accumulation by the EPR effects [45] after intravenous administration. Thereby, we should examine the *in vivo* gene transfer activity and antitumor effects of the pDNA complexes with optimized Fol-PαC after intravenous injection.

CONCLUSION

The present results suggest that Fol-PαC (G3, DSF 5) could be potentially used as a FR-overexpressing cancer cell-selective gene transfer carrier, because of its FR-mediated gene delivery, the extremely low cytotoxicity, endosomal escaping ability and adequate physicochemical properties. These findings may provide useful information for design and evaluation of FR-overexpressing cancer cell-selective gene transfer carriers using cationic polymers *in vitro* and *in vivo*.

Conflict of interest

The authors declare no conflict of interest.

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Fig. 1

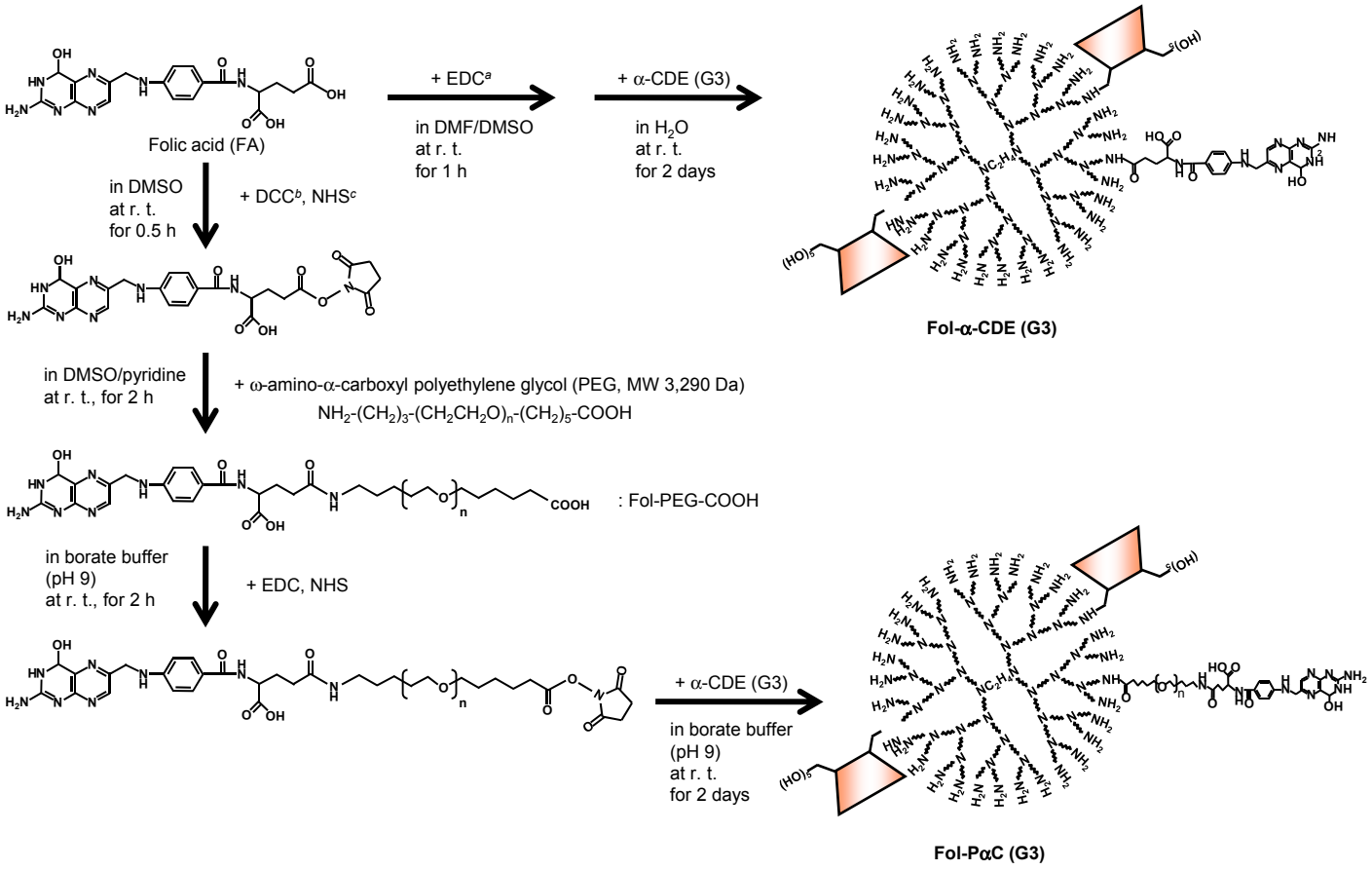
Folic acid (FA)
+ EDC[a]
in DMF/DMSO
at r. t.
for 1 h
+ α-CDE (G3)
in H2O
at r. t.
for 2 days
Fol-α-CDE (G3)
in DMSO
at r. t.
for 0.5 h
+ DCC[b], NHS[c]
in DMSO/pyridine
at r. t., for 2 h
+ ω-amino-α-carboxyl polyethylene glycol (PEG, MW 3,290 Da)
NH2-(CH2)3-(CH2CH2O)n-(CH2)5-COOH
: Fol-PEG-COOH
in borate buffer
(pH 9)
at r. t., for 2 h
+ EDC, NHS
+ α-CDE (G3)
in borate buffer
(pH 9)
at r. t.
for 2 days
Fol-PαC (G3)

Fig. 2

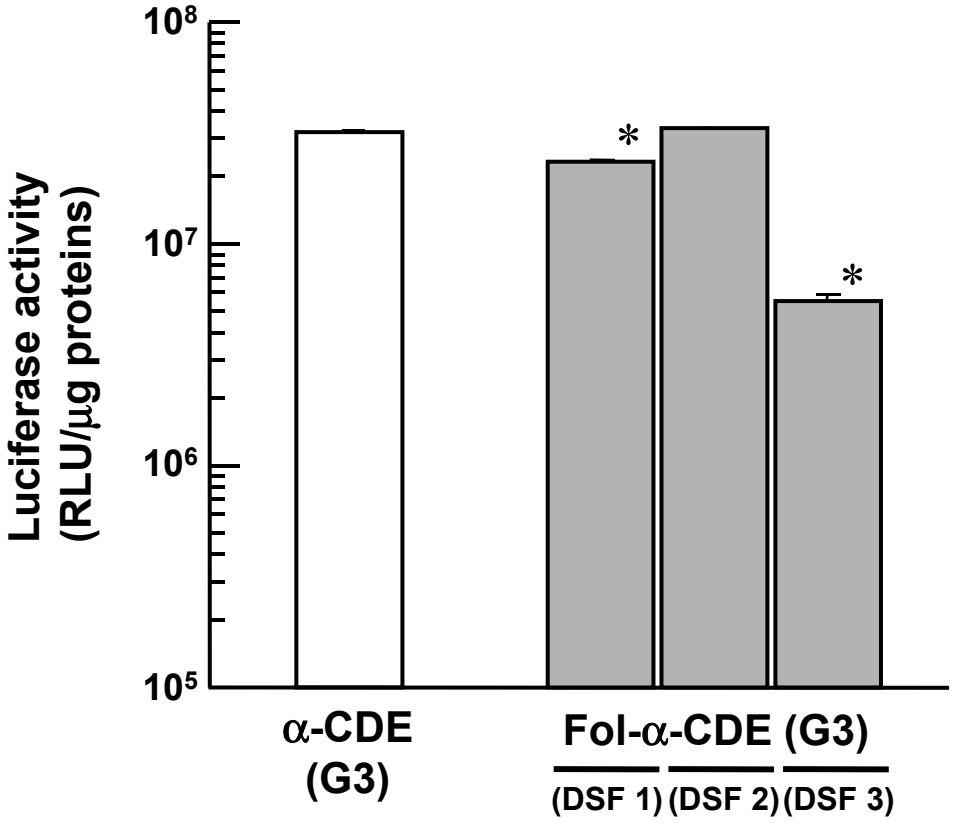
Luciferase activity
(RLU/μg proteins)
10^8
10^7
10^6
10^5
*
*
α-CDE
(G3)
Fol-α-CDE (G3)
(DSF 1)
(DSF 2)
(DSF 3)

Fig. 3

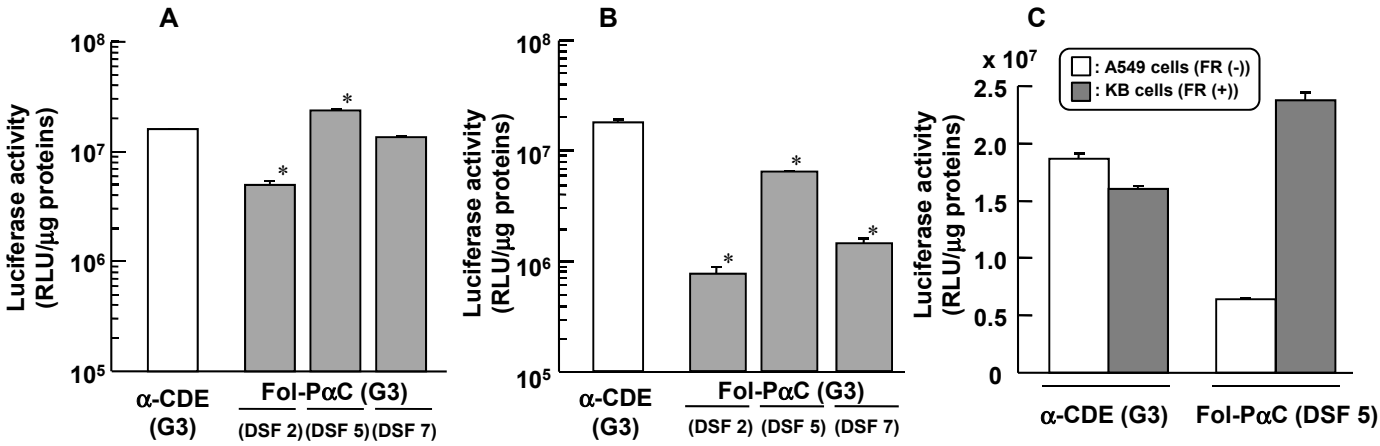
A
B
C
Luciferase activity (RLU/μg proteins)
10^8
10^7
10^6
10^5
α-CDE (G3)
Fol-PαC (G3)
(DSF 2)
(DSF 5)
(DSF 7)
x 10^7
2.5
2.0
1.5
1.0
0.5
0
: A549 cells (FR (-))
: KB cells (FR (+))
α-CDE (G3)
Fol-PαC (DSF 5)

Fig. 4

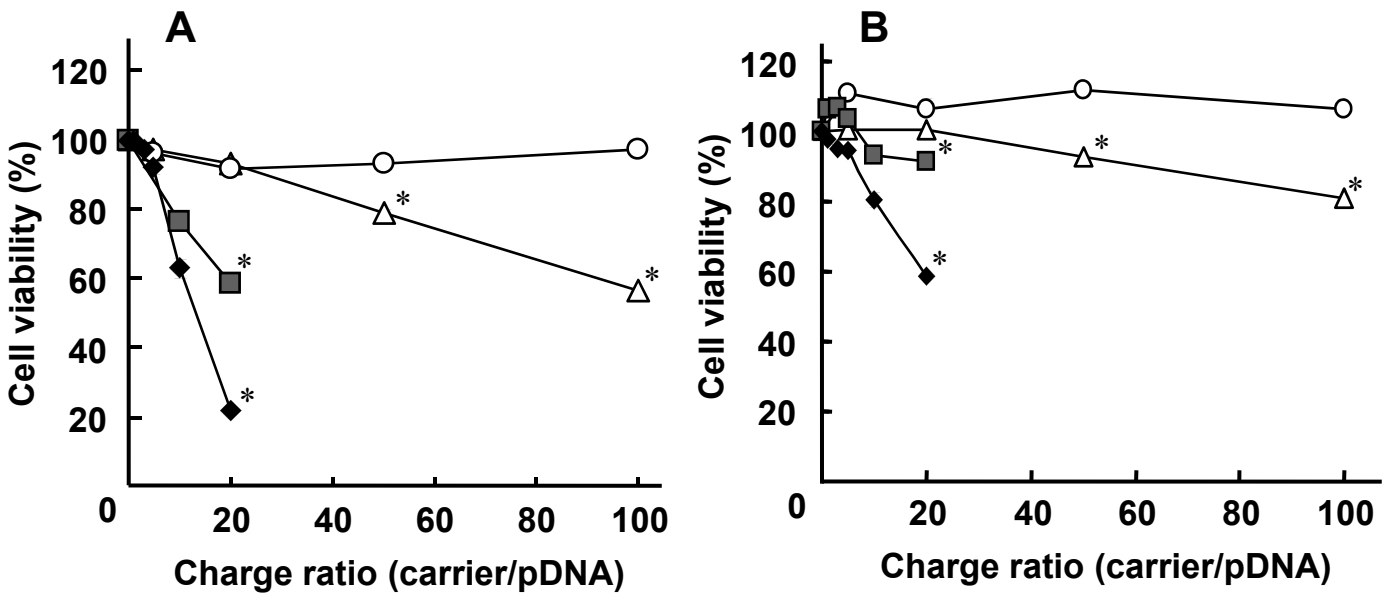
A
B
Cell viability (%)
Charge ratio (carrier/pDNA)
0
20
40
60
80
100
120
*

Fig. 5

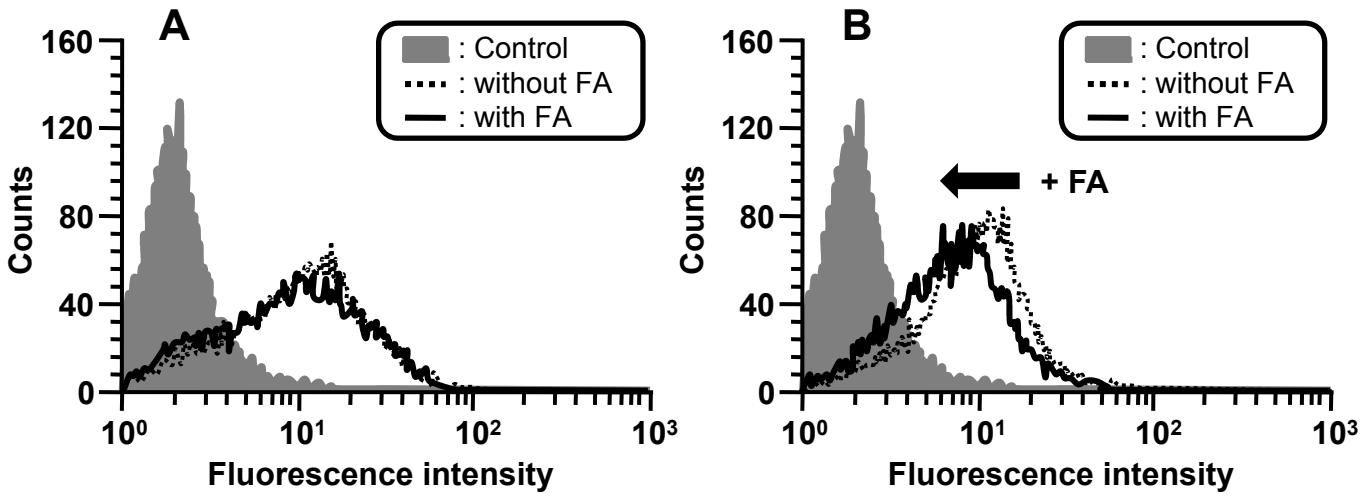
A
B
: Control
: without FA
: with FA
+ FA
Counts
Fluorescence intensity
0
40
80
120
160
10^0
10^1
10^2
10^3

Fig. 6

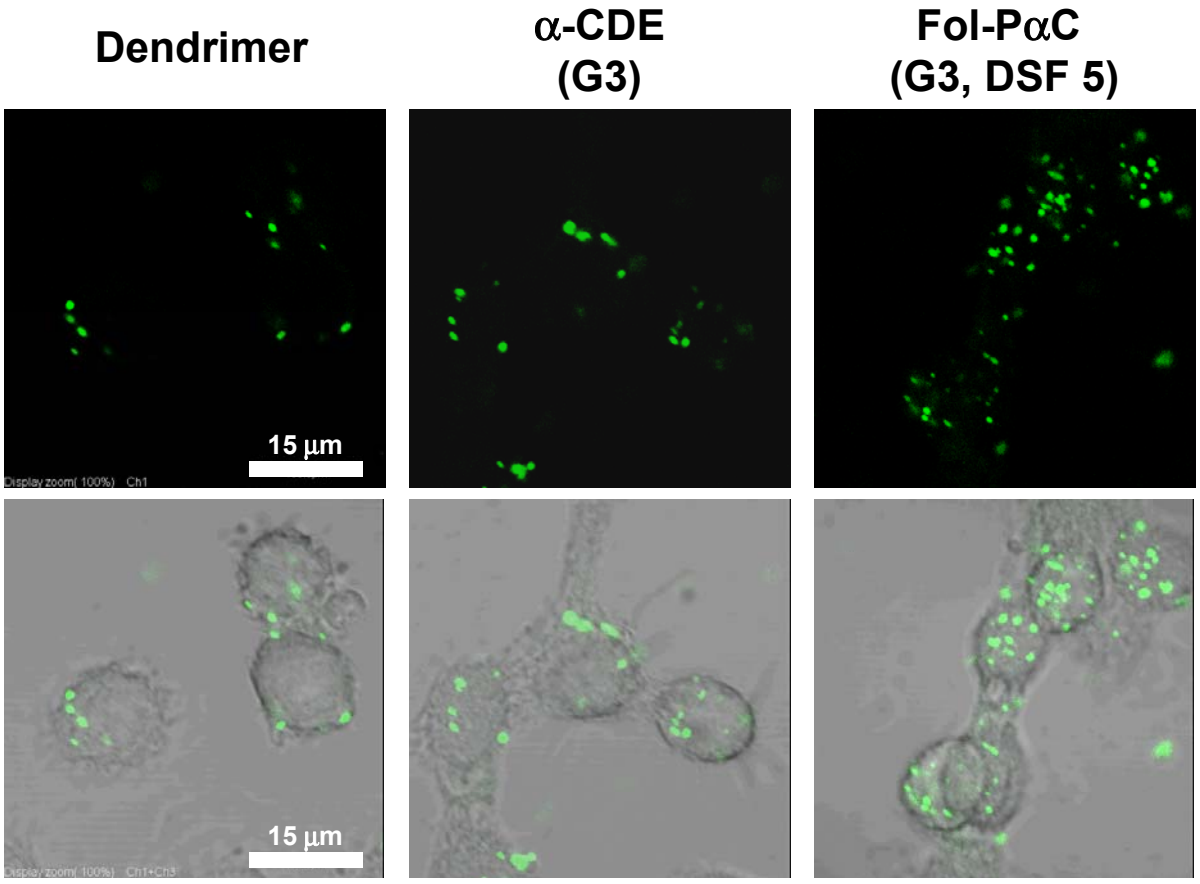

Fig. 7

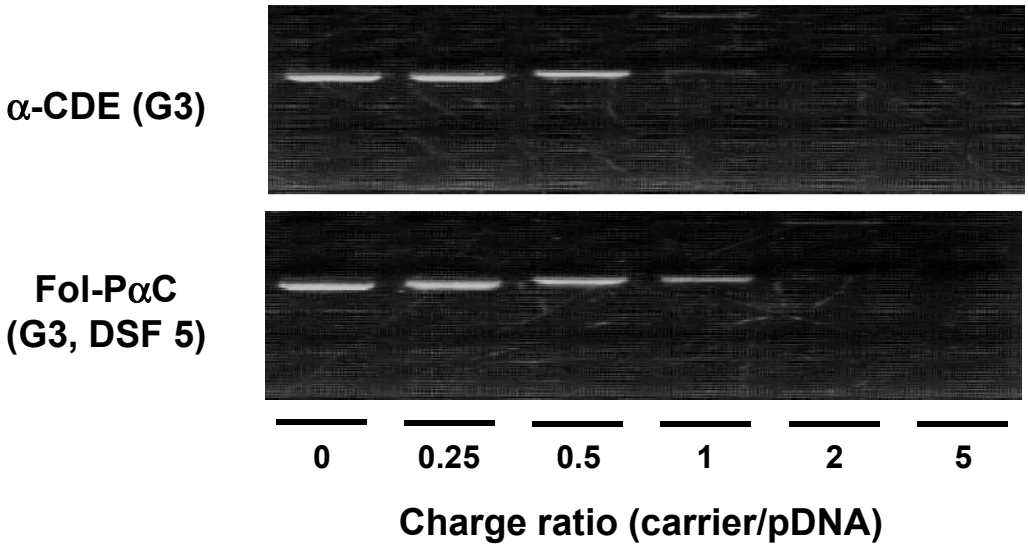

Fig. 8

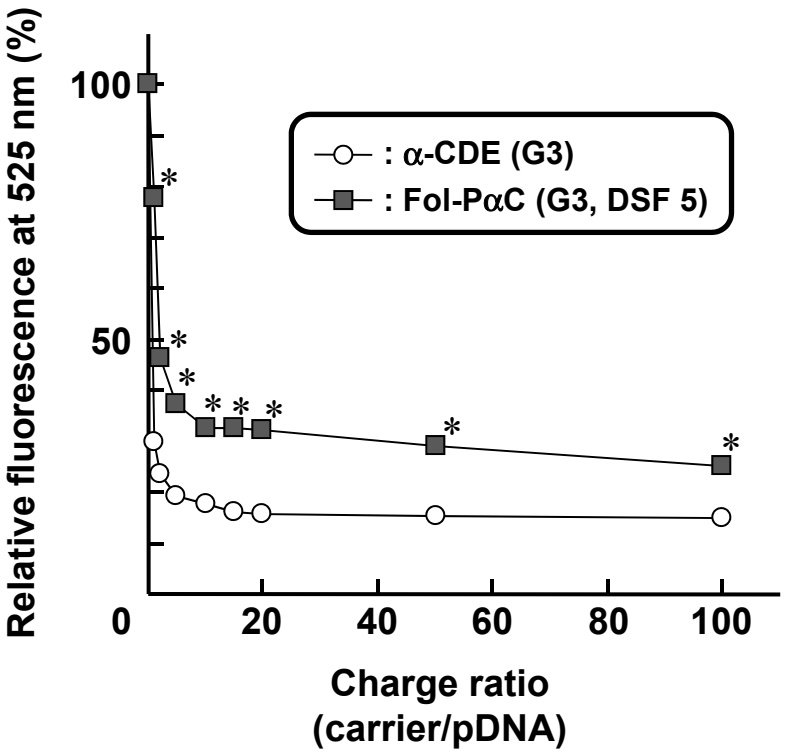
Relative fluorescence at 525 nm (%)
100
50
0
20
40
60
80
100
Charge ratio
(carrier/pDNA)
: α-CDE (G3)
: FoI-PαC (G3, DSF 5)

Fig. 9

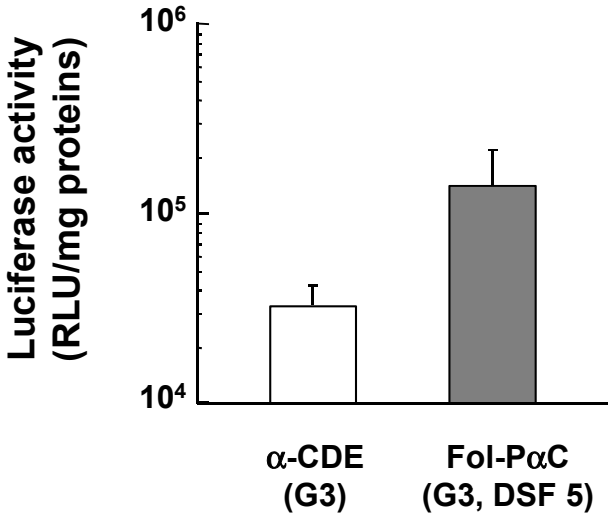
Luciferase activity
(RLU/mg proteins)
10^6
10^5
10^4
α-CDE
(G3)
FoI-PαC
(G3, DSF 5)

Table 1

Carrier	Charge ratio (carrier/pDNA)	Mean diameter (nm)	ζ-Potential (mV)
α-CDE (G3)	1	108.1 ± 9.5	-23.5 ± 2.7
	50	142.0 ± 0.6 *	31.3 ± 1.0 *
Fol-PαC (G3, DSF 5)	1	94.3 ± 4.3	-1.7 ± 0.6 *†
	50	140.3 ± 2.6 ‡	8.6 ± 2.0 *†

Table 2

Carrier	k_a [$M^{-1}s^{-1}$]^a	k_d [s^{-1}]^b	K_a [M^{-1}]^c
FA	**4.2**	**3.9×10^{-3}**	**1.1×10^{3}**
α-CDE (G3)	**2.4×10^{3}**	**2.0×10^{-2}**	**1.2×10^{5}**
Fol-PαC (G3, DSF 5)	**1.2×10^{5}**	**3.2×10^{-3}**	**3.8×10^{7}**