Polypseudorotaxanes of Pegylated α-Cyclodextrin/Polyamidoamine Dendrimer Conjugate with Cyclodextrins as a Sustained Release System for DNA

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ABSTRACT

Nonviral gene delivery suffers from a number of limitations including short transgene expression times and low transfection efficiency. In this study, we examined whether polypseudorotaxanes (PPRXs) of polyethylene glycol (PEG, molecular weight: 2,000)-grafted α-cyclodextrin (α-CyD)/polyamidoamine dendrimer conjugate (PEG-α-CDE) with CyDs have the potential for the novel sustained release systems for plasmid DNA (pDNA). The PEG-α-CDE/pDNA complex formed PPRXs with α-CyD and γ-CyD solutions, but not with β-CyD solution. In the PEG-α-CDE/CyDs PPRX systems, 20.6 mole of α-CyD and 11.8 mole of γ-CyD were involved in the PPRXs formation with one PEG chain by α-CyD and γ-CyD, respectively, consistent with in the PEG-dendrimer/CyDs systems. PEG-α-CDE/pDNA/α-CyD PPRX and PEG-α-CDE/pDNA/γ-CyD PPRX formed hexagonal and tetragonal columnar channels in the crystalline phase, respectively. In addition, the CyDs PPRX provided the sustained release of pDNA from PEG-α-CDE complex with pDNA at least 72 h *in vitro*. The release of pDNA from CyDs PPRX retarded as the volume of dissolution medium decreased. Furthermore, the PEG-α-CDE/γ-CyD PPRX system showed sustained transfection efficiency after intramuscular injection to mice at least for 14 days. These results suggest that the PEG-α-CDE/CyD PPRX systems are useful for novel sustained DNA release systems.

Key words: cyclodextrin; polypseudorotaxane; dendrimer; DNA; sustained release

1. Introduction

Gene therapy has immense promise for the treatment of a wide variety of diseases and disorders. A wide variety of vectors to deliver therapeutic and genetic materials such as plasmid DNA (pDNA) or oligonucleotides into the desired target cells have been investigated.[1,2] There are two categories of gene therapy vectors, i.e., viral vectors and nonviral vectors. Recently, the potentials of nonviral vectors have come to attention due to easy preparation of vector/pDNA complexes, low cytotoxicity, and lack of immunogenicity. Starburst polyamidoamine (PAMAM) dendrimer (dendrimer) employed is a spherical, highly ordered, dendritic polymer with positively charged primary amino groups on the surface at physiological pH[3,4] and is widely used in nonviral vectors. Dendrimers are especially effective at condensing and protecting pDNA, with lower cytotoxicity and higher transfection efficiencies than the other cationic polymers. However, in most cases the nonviral systems cannot match the transfection efficiencies of viral vectors, nor do they allow long-term transfection.[5-7]

Over the last two decades, supramolecular assemblies have attracted much attention, due to their intriguing topologies and application in various fields such as nanodevices, sensors, molecular switches, and drug delivery systems (DDS). Macrocyclic compounds such as cyclodextrins (CyDs) are most often used as host molecules in supramolecular chemistry.[8] CyDs have been widely applied to DDS due to their good bioadaptability.[9,10] CyDs are a series of natural cyclic oligosaccharides composed of 6, 7, and 8 D-glucose units linked by α-1,4-linkages and named α-CyD, β-CyD and γ-CyD, respectively. Harada *et al*. first reported the supramolecular assemblies of polyethylene glycol (PEG) and α-CyD, in which a number of the cyclic molecules are spontaneously threaded onto the polymer chain.[11,12] These complexes are called

polypseudorotaxane (PPRX), because the CyDs can be dethreaded the polymer chain, when PPRXs were allowed to dissolve in water. This complexation displays a size dependency, i.e. the small cavity of α-CyD forms a PPRX with one PEG chain, while the middle cavity of β-CyD with polypropylene glycol, but not with PEG, and large cavity of γ-CyD forms a PPRX with two PEG chains.[11,12] Yui *et al*. prepared PEG/α-CyD polyrotaxanes end-capped with amino acids, oligopeptides and polypeptides, which work as biodegradable drug carriers or stimuli-responsive hydrogels.[13,14] Recently, we found that pegylated insulin and pegylated lysozyme form PPRXs with α-CyD and γ-CyD in a similar manner as PEG does, and the resulting PPRXs may be useful as a sustained drug delivery technique of pegylated proteins.[15-18] In spite of the many studies on the formation of PPRXs reported so far, little is known about the application of PPRXs of pegylated gene delivery carriers with CyDs to a controlled release system for pDNA or oligonucleotides. More recently, we reported that PPRXs of pegylated dendrimer (G2) (PEG-dendrimer (G2)) with α-CyD and γ-CyD showed a sustained release of pDNA at least 72 h *in vitro*.[19] However, PEG-dendrimer also has drawbacks due to its low gene transfer activity. On the other hand, we previously reported that the dendrimer (G2) conjugated with α-CyD (α-CDE) showed luciferase gene expression approximately 100 times higher than the unfunctionalized dendrimer through the cooperative effects of the proton sponge effect of dendrimer and the endosomal membrane-disruptive effects of α-CyD, having negligible cytotoxicity.[20,21] Therefore, to improve gene transfer activity of the PPRXs of PEG-dendrimer with CyDs, we newly prepared the PPRXs of pegylated α-CDE (PEG-α-CDE) with CyDs. In the present study, we evaluated the potential use of the PPRXs of PEG-α-CDE with CyDs as the novel sustained release systems for pDNA *in*

vitro and *in vivo*.

2. Materials and methods

2.1. Materials

α-CyD, β-CyD, and γ-CyD were obtained from Nihon Shokuhin Kako (Tokyo, Japan). Starburst® PAMAM dendrimer (ethylenediamine core, G2, the terminal amino groups = 16, molecular weight = 3,256) was purchased from Aldrich Chemical (Tokyo, Japan). Methoxypolyethylene glycol succinimidyl carboxymethyl ester (PEG-COO-NHS, SUNBRIGHT® ME-020AS, molecular weight = c.a. 2,000) was obtained from NOF (Tokyo, Japan). Fetal calf serum (FCS) was obtained from Nichirei (Tokyo, Japan). RPMI-1640 medium was purchased from Nissui Pharmaceuticals (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). All other chemicals and solvents were of analytical reagent grade and double distilled water was used throughout the study.

2.2. Preparation of PEG-α-CDE

α-CDE (G2, DS 1.5) was prepared as reported previously.[20] PEG-COO-NHS (50 mg, 2.5 x 10^{-5} mol) and α-CDE (30 mg, G2, DS 1.5) were dissolved in 0.5 mL of DMSO and stirred for 24 h at room temperature. Then, the reactant was dialyzed using a dialysis membrane, Spectra/pore (MWCO = 3,500), in water for 5 days. After dialysis, the sample was concentrated and lyophilized to obtain PEG-α-CDE. The average degree of substitution of PEG (DSP) was 4, determined by ^{1}H-NMR. (Supplementary

Fig.S1)

2.3. Preparation of PEG-α-CDE/pDNA/CyDs PPRXs

The solutions containing the pDNA complexes with PEG-α-CDE (2.91×10^{-9} mol, 41.48 μg) were incubated for 15 min at room temperature. Thereafter, the solution containing α-CDE/pDNA complex or PEG-α-CDE/pDNA complex was incubated with 0.5 mL of α-CyD (145 mg/mL), β-CyD (18.5 mg/mL) or γ-CyD (232 mg/mL) solution for 12 h at 4ºC. The amount of pDNA was 5 μg. The charge ratio of PEG-α-CDE/pDNA was 2, because the complete complexation of PEG-α-CDE/pDNA was observed at a charge ratio of 2 using electrophoresis. To determine the association ratio of pDNA in PEG-α-CDE/pDNA/CyDs PPRXs, the PPRXs suspensions were centrifuged (5,000 rpm, 10 min, 4ºC), and then the concentration of pDNA in supernatant was determined by measuring the absorbance at 260 nm (sample) and 280 nm (reference) using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). The association ratio was expressed by the subtraction of the concentration of pDNA in supernatant from the total concentration of pDNA.

2.4. Powder X-ray diffraction studies

Powder X-ray diffraction patterns were measured by a Rigaku Ultima IV X-ray diffractometer (Tokyo, Japan) with a Ni filtered Cu-Kα radiation, a voltage of 40 kV, a current of 20 mA, a divergent slit of 10 mm (0.5°), a scanning speed of 5°/min, opened scattering and receiving slits.

2.5. Interaction between pDNA and PEG-α-CDE

Electrophoretic mobility of the complex of pDNA/PEG-α-CDE was measured using a gel electrophoresis system. Various amounts of PEG-α-CDE were mixed with 0.2 μg of pDNA in Tris-EDTA buffer (TE buffer, pH 7.4). Gel electrophoresis was carried out at room temperature in TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0) in 1% agarose gel including 0.1 mg/mL of ethidium bromide in the Mupid^{TM} system (Cosmo Bio, Tokyo, Japan) at 100 V for 30 min. The pDNA bands were visualized using an UV illuminator.

2.6. Physicochemical properties of PEG-α-CDE/pDNA/CyDs PPRXs

The solutions containing pDNA complexes with PEG-α-CDE were added to Tris-HCl buffer (10 mM, pH 7.4). The particle sizes and ζ-potentials of the complexes with PEG-α-CDE with CyDs were determined by a Zetasizer Nano (Malvern Instruments, Worcestershire, UK).

2.7. Cell culture

Colon-26 cells, a mouse colon adenocarcinoma cell line, were obtained from Riken Bioresource Center (Tsukuba, Japan). Colon-26 cells were cultured in RPMI-1640 containing 1 x 10^5 mU/mL of penicillin, 0.1 mg/mL of streptomycin supplemented with 10% FCS at 37°C in a humidified 5% CO_2 and 95% air atmosphere.

2.8. In vitro gene transfer

In vitro transfection of the pDNA complex with PEG-α-CDE was performed utilizing luciferase expression of pDNA in Colon-26 cells. Two microliters of Tris-EDTA buffer (pH 7.4) containing pDNA (1 μg/μL) were mixed with 10 μL of Hanks' balanced

salt solutions (HBSS, pH 7.4) containing PEG-α-CDE at a charge ratio of 2 (carrier/pDNA). The pDNA complex with PEG-α-CDE was then allowed to stand for 15 min at room temperature. The cells (2 x 10^5 cells per 24 well plate) were seeded 6 h before transfection, and then washed twice with serum-free medium. After washing the cells with serum-free medium twice, 200 μL of medium containing 20% FCS (final concentrations of FCS was 10%) were added to each dish, and then incubated at 37°C for 21 h. After transfection, gene expression was measured as follows: Renilla luciferase content in the cell lysate was quantified using the Promega Renilla luciferase assay reagent (Tokyo, Japan) and a luminometer (Lumat LB9506, EG&G Berthold Japan, Tokyo, Japan). It was confirmed that PEG-α-CDE has no influence on the luciferase assay under the present experimental conditions. Total protein content of the supernatant was determined by Bio-Rad protein assay kit (Bio-Rad Laboratories, Tokyo, Japan).

2.9. Cytotoxicity

The effects of the pDNA complex with PEG-α-CDE or polyethyleneimine (PEI) on cell viability were measured as reported previously.[20] The transfection was performed as described in the transfection section. After washing twice with HBSS (pH 7.4) to remove the pDNA and/or 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).

2.10. Release of pDNA from PEG-α-CDE/pDNA/CyDs PPRXs

The release rate of the PEG-α-CDE/pDNA was measured by the modified dispersed-amount method,[16] i.e. 100-500 μL of phosphate buffer (pH 7.4) were added to the PPRXs suspensions at room temperature, and the suspensions were stirred at 100 rpm. The amount of pDNA was 30 μg. At appropriate intervals, an aliquot of the dissolution medium was withdrawn, centrifuged at 3,000 rpm for 3 min. The concentration of pDNA in the PEG-α-CDE/pDNA complex released from PPRXs was determined by measuring the absorbance at 260 nm (sample) and 280 nm (reference) using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE).

2.11. In vivo gene transfer

Four-weeks-old BALB/c male mice (ca. 20 g) were intramuscularly injected in the thigh muscle with 50 μL of γ-CyD saturated solution (232 mg/mL) containing the PPRX of PEG-α-CDE at a charge ratio of 2 (carrier/pDNA). At appropriate time after intramuscular administration, the mice were sacrificed, and the injection site was isolated. The isolated thigh muscle was added to 2 mL of the Promega cell lysis buffer (Tokyo, Japan) containing the Roche protease inhibitor, Complete® (Tokyo, Japan). The organs 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 5 min at 10,000 g (4°C), and 20 μL of the supernatant were 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).

2.12. Data analysis

Data are given as the mean±SEM. 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.

3. Results and discussion

3.1. Preparation of PPRXs of PEG-α-CDE with CyDs

To prepare PEG-α-CDE, the PEG moiety at the primary amino group of α-CDE (G2) was introduced, using PEG-COO-NHS in DMSO. After dialysis, PEG-α-CDE (Fig. 1) was obtained with approximately 50.5% product yield. The average degree of substitution of PEG (DSP) was determined to be about 4 by ^{1}H-NMR. The PPRXs of the PEG-α-CDE with CyDs were prepared by mixing their aqueous solutions. Figure 2 shows the solution after mixing α-CDE or PEG-α-CDE both in the absence and presence of pDNA with α-CyD, β-CyD or γ-CyD solutions, and then standing for 12 h at 4ºC. The addition of CyDs solutions to α-CDE and α-CDE/pDNA complex solutions failed to produce precipitate (Fig. 2A, C), indicating that PPRX was not formed in the non-PEG systems. Meanwhile, the addition of α-CyD and γ-CyD solutions, rather than β-CyD solution, to PEG-α-CDE solution showed precipitates (Fig. 2B), indicating that α-CyD and γ-CyD form PPRXs with PEG-α-CDE, like PPRX formation of PEG with these CyDs reported by Harada *et al*.[11,12] In order to form the PPRX with CyDs, hydrogen bonds between threaded CyDs molecules with PEG chain are necessary.[11,12] The α-CyD molecules in PEG-α-CDE do not appear to thread with PEG molecules, probably due to the steric hindrance of dendrimer. Therefore, the α-CyD molecules in PEG-α-CDE do not appear to affect the formation of the PPRX

with CyDs added. Importantly, we confirmed the PPRX formation of PEG-α-CDE even in the presence of pDNA (Fig. 2D). The stoichiometry of CyDs and PEG of the PPRXs was accurately determined by measuring integral values of the anomeric protons of CyDs and the ethylene protons of PEG in the PEG-α-CDE molecule in ^{1}H-NMR spectra after dissolving the solid PPRXs in DMSO. As a result, 20.6 and 11.8 moles of α-CyD and γ-CyD in the absence of pDNA system and 20.2 and 12.9 moles of α-CyD and γ-CyD in the presence of pDNA system were found to be involved in the PPRXs formation with one PEG chain by α-CyD or γ-CyD, respectively. Harada *et al*. reported that the depth of CyDs cavity corresponds to the length of two ethylene glycol units.[11,22] Therefore, it is estimated that PEG (MW: 2000) can form PPRX with the number of 22-23 CyDs molecules. These results suggest that α-CyD and γ-CyD formed PPRX with PEG-α-CDE by including one PEG chain and two PEG chains, respectively, in the absence and presence of pDNA.

Next, to examine the PPRX formation, we measured the powder X-ray diffraction patterns of the α-CyD PPRX and γ-CyD PPRX with PEG-α-CDE, and compared them to those with PEG. The diffraction patterns of the PEG-α-CDE/α-CyD PPRX and PEG-α-CDE/pDNA/α-CyD PPRX (Fig. 3C, D) were different from those of α-CyD alone (Fig. 3A) and physical mixture (Fig. 3B), but were the same as that of PEG/α-CyD PPRX (Fig. 3E).[22] Collectively, these results suggest that PEG-α-CDE and PEG-α-CDE/pDNA complex form PPRXs with α-CyD in the solid state. Meanwhile, the diffraction pattern of the PEG-α-CDE/γ-CyD PPRX (Fig. 3H) was so broad, and was different from that of γ-CyD alone (Fig. 3F) or physical mixture (Fig. 3G), although it showed a relatively similar pattern to that of PEG/γ-CyD PPRX (Fig. 3J). Thus, it is assumed that γ-CyD forms PPRXs with PEG-α-CDE having more

complicated structures, e.g. γ-CyD formed PPRXs with 1) two PEG chains derived from one PEG-α-CDE molecule, 2) two PEG chains derived from different PEG-α-CDE molecule, and 3) one bended PEG chain (equivalent to two PEG chains were threaded into γ-CyD). Importantly, the diffraction pattern of PEG-α-CDE/pDNA/γ-CyD PPRX (Fig. 3I) showed sharp diffraction peaks, compared to that of PEG-α-CDE/γ-CyD PPRX (Fig. 3H) and was similar to that of PEG/γ-CyD PPRX (Fig. 3J). These findings suggest that the crystallinity of PEG-α-CDE/pDNA/γ-CyD PPRX was higher than that of PEG-α-CDE/γ-CyD PPRX.

Generally, three types of crystal packing of CyD complexes are well known, *i.e.*, a channel type, a cage type and a layer structure.[23,24] Powder X-ray diffractograms can provide information to distinguish between the herringbone packing of free CyDs and the different packing of inclusion complexes.[25] The diffraction patterns of the PEG-α-CDE/pDNA/α-CyD PPRX (Fig. 3D) and PEG-α-CDE/pDNA/γ-CyD PPRX (Fig. 3I) were found to be hexagonal and tetragonal columnar channels of the linearly aligned α-CyD and γ-CyD cavities in the crystalline phase, respectively.[26-29] Therefore, these diffraction patterns were indexed on the basis of the two-dimensional hexagonal and tetragonal unit cells with dimensions $a=b$=27.46 Å and $a=b$=24.10 Å, respectively, as shown in Table 1. The *d*-spacings of the *hkl* (200) reflection were used to calculate the unit cell dimensions as shown in Fig. 3D and 3I. All values of the calculated *d*-spacings (d_{cal}) were in agreement with those of the observed *d*-spacings (d_{obs}), indicating that PPRXs of PEG-α-CDE with α-CyD and γ-CyD formed the hexagonal and tetragonal structures, respectively (Fig. 4).

3.2. Physicochemical properties of PEG-α-CDE as a gene transfer carrier

To clarify physicochemical properties of PEG-α-CDE as a gene transfer carrier, we examined the complex formation between PEG-α-CDE and pDNA using an agarose gel electrophoresis. As shown in Fig. 5, the intensity of the band derived from pDNA decreased as the charge ratio of PEG-α-CDE/pDNA increased, and at a charge ratio of 2 (carrier/pDNA) the band disappeared. These results suggest that pDNA completely forms complexes with cationic PEG-α-CDE at a charge ratio of 2 (carrier/pDNA).

Next, we determined the particle sizes and ζ-potential values of the PEG-α-CDE/pDNA/CyDs PPRXs at a charge ratio of 2 (carrier/pDNA) (Table 2). The mean diameters of the PEG-α-CDE alone and PEG-α-CDE/pDNA complex were significantly increased by the addition of α-CyD and γ-CyD, probably due to the formation of PPRXs in solution. There was no significant difference in the mean diameter between PEG-α-CDE/CyDs PPRXs and PEG-α-CDE/pDNA/CyDs PPRXs. Meanwhile, the ζ-potential values of PEG-α-CDE/pDNA/CyDs PPRXs were smaller than those of PEG-α-CDE/CyDs PPRXs. These results indicate that pDNA lowers the ζ-potential values and does not affect the particle sizes of the PEG-α-CDE/CyDs PPRXs.

The association ratio of pDNA with a gene delivery carrier is an important parameter for gene transfer activity. Therefore, we examined the association ratio of pDNA in PEG-α-CDE/CyDs PPRXs (Fig. 6). Both PEG-α-CDE/α-CyD PPRX and PEG-α-CDE/γ-CyD PPRX had an association ratio with pDNA greater than 90%. These results suggest that PEG-α-CDE/CyDs PPRXs can greatly increase encapsulation efficacy of pDNA.

3.3. In vitro gene transfer activity

To examine whether PEG-α-CDE has potent gene transfer activity, Renilla luciferase activity after transfection of pDNA complex with PEG-α-CDE at a charge ratio of 50 (carrier/pDNA) in Colon-26 cells was determined (Fig. 7). Herein, the charge ratio of 50 (carrier/pDNA) was chosen for *in vitro* gene transfer study, because PEG-α-CDE showed the highest gene transfer activity at this charge ratio (data not shown). When pDNA alone was transfected to cells, no luciferase activity was observed. In sharp contrast, PEG-α-CDE/pDNA complex showed significantly higher luciferase activity, compared to pDNA alone. In our previous report, luciferase activity at 24 h after transfection of pDNA complex with PEG-dendrimer, which lacks α-CyD, was about 6.6 x 10^5 relative light unit/mg proteins.[19] Therefore, transfection efficiency of PEG-α-CDE/pDNA complex (1.1 x 10^6 relative light unit/mg proteins, Fig. 7) was higher than that of PEG-dendrimer/pDNA complex, probably due to the acceleration of endosomal escaping of the pDNA complex through the disruption of endosomal membrane by α-CyD.[20] However, the extent of luciferase activity after transfection of PEG-α-CDE/pDNA complex was thought to be low, possibly due to "PEG dilemma".[30] Further investigation into the improvement of transfection efficiency of PEG-α-CDE/pDNA complex to overcome "PEG dilemma" is being done, based on the manipulation of cellular uptake and endosomal release using functional devices such as specific ligands, cleavable PEG systems and endosomal fusogenic/disruptive peptides. These results suggest that PEG-α-CDE/pDNA/CyDs PPRXs have the potential for transfection efficiency.

3.4. Cytotoxicity of complexes with carriers

From the viewpoint of safety, we examined the cytotoxicity of PEG-α-CDE/pDNA

complex by the WST-1 method (Fig. 8). No cytotoxicity of PEG-α-CDE/pDNA complex was observed in Colon-26 cells up to the charge ratio of at least 50 (carrier/pDNA). Meanwhile, severe cytotoxicity of the complex with polyethyleneimine (PEI) was observed even at a charge ratio of 20. These results indicate that PEG-α-CDE/pDNA complex has less cytotoxicity.

3.5. In vitro sustained release of pDNA from PEG-α-CDE/pDNA/CyD PPRXs

Figure 9 shows the release profiles of the pDNA from PEG-α-CDE/pDNA/CyDs PPRXs in phosphate buffer (pH 7.4). Both the α-CyD PPRX and γ-CyD PPRX systems showed an initial burst followed by slow release profiles at least up to 72 h (Fig. 9A). The release rate of the PEG-α-CDE/pDNA/γ-CyD PPRX system was higher than that of the PEG-α-CDE/pDNA/α-CyD PPRX system. The difference in the release rate between the α-CyD PPRX and γ-CyD PPRX may be ascribed to the distinct crystal structure, because γ-CyD PPRX with a tetragonal columnar channel crystal structure having more space between the γ-CyD molecules, compared to α-CyD PPRX with a hexagonal columnar channel crystal structure, could facilitate the penetration of water molecules into the γ-CyD PPRX, resulting in an acceleration of the dissolution rate of the pDNA from γ-CyD PPRX. This hypothesis is strongly supported by the fact that the dissolution rate of budenoside from its γ-CyD PPRX with a tetragonal columnar channel crystal structure was higher than that from the PPRX with a hexagonal columnar channel crystal structure reported by Toropainen.[29] In addition, the release of pDNA from α-CyD PPRX and γ-CyD PPRX retarded as the volume of the dissolution medium decreased in the order of 500 μL > 300 μL > 100 μL (Fig. 9B, C). This may be due to the enhancement of the dethreading of CyDs from PPRXs with

increasing the volume of dissolution medium.[22] This presumption is strongly supported by our previous data showing pegylated insulin or pegylated lysozyme gradually released from their PPRXs through the dethreading of PPRXs.[15-18] These results indicate that PEG-α-CDE/pDNA/CyD PPRXs can work as a novel sustained release system for pDNA and the release rate of pDNA from the PPRXs can be controlled by adjusting volume of dissolution medium.

3.6. In vivo gene transfer activity of PPRX of PEG-α-CDE with γ-CyD

Finally, we examined *in vivo* gene transfer activity of the PPRX of PEG-α-CDE with γ-CyD in mice. Herein, the γ-CyD PPRX was chosen for *in vivo* study, because of the safety profile of γ-CyD with its low hemolytic activity and high biodegradability, compared to those of α-CyD.[31,32] In this study, we injected the PEG-α-CDE/pDNA/γ-CyD PPRX intramuscularly to mice, because muscular cells readily uptake pDNA and provide the gene expression. Figure 10 shows gene transfer activity after intramuscular injection of the solution containing pDNA alone and suspension containing the PEG-α-CDE/pDNA/γ-CyD PPRX in the presence of γ-CyD in phosphate buffer (pH 7.4) to mice. When naked pDNA was administered, the maximum level of luciferase activity occurred at 3 days after injection, and thereafter luciferase activity rapidly decreased. On the other hand, after injection of PEG-α-CDE/pDNA/γ-CyD PPRX, the luciferase activity gradually increased for at least 14 days, indicating a delayed and sustained gene expression. However, it is still unclear how long the gene expression of PEG-α-CDE/pDNA/γ-CyD PPRX is maintained after intramuscular injection. Further investigation into the period of gene expression after intramuscular injection of PEG-α-CDE/pDNA/γ-CyD PPRX should be

performed. The proposed mechanism for delayed and sustained gene expression of PEG-α-CDE/pDNA/γ-CyD PPRX suspension after intramuscular injection to mice was shown in Fig. 11. Firstly, the γ-CyD PPRX was gradually diluted by the body fluid after intramuscular injection and was gently dethreaded from the PPRXs. This dilution process can be controlled not only by the volume of biological fluid at injection site but also by the molecular weight of appended PEG to dendrimer. The dethreaded γ-CyD is thought to enter systemic circulation from the muscular tissue and is rapidly eliminated via kidney, suggesting that the side effects caused by γ-CyD would be negligible in this system. Meanwhile, the PEG-α-CDE/pDNA complex released from the PPRX entered the cells via endocytosis and escaped from endosomes by the cooperative effects of the proton sponge effects by dendrimer and the interaction of α-CyD molecules in PEG-α-CDE with endosomal membrane lipids, eventually providing delayed and sustained gene expression. Thereafter, further elaborate studies on the intracellular trafficking or biological fate of PEG-α-CDE after sustained delivery of pDNA are required. These results suggest that the PEG-α-CDE/pDNA/γ-CyD PPRX system can provide delayed and sustained gene expression *in vivo*.

4. Conclusions

In the present study, we demonstrated the potential use of PEG-α-CDE/pDNA/CyDs PPRXs as novel sustained release systems for pDNA. CyDs PPRXs have several advantages in sustained gene delivery systems, 1) simple preparation without organic solvent, 2) high encapsulation of pDNA, 3) applicable for other nucleic acids such as siRNA, micro RNA and decoy DNA, and 4) utilizable for other pegylated carriers such as microsphere and microcapsules. These results suggest that the PEG-α-CDE/CyD

PPRX systems are useful for novel sustained DNA release systems.

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Figure legends

Figure 1. Chemical structure of PEG-α-CDE

Figure 2. Photographs of PEG-α-CDE/CyD PPRXs with or without pDNA. The solutions containing the pDNA complexes with PEG-α-CDE were incubated for 15 min at room temperature. Thereafter, solutions containing α-CDE/pDNA complex or PEG-α-CDE/pDNA complex were incubated with α-CyD (145 mg/mL), β-CyD (18.5 mg/mL) or γ-CyD (232 mg/mL) solution for 12 h. The amount of pDNA was 5 μg. The charge ratio of α-CDE/pDNA or PEG-α-CDE/pDNA was 2.

Figure 3. Powder X-ray diffraction patterns of PEG-α-CDE/CyD PPRX. (A) α-CyD alone, (B) PEG-α-CDE/α-CyD physical mixture, (C) PEG-α-CDE/α-CyD PPRX, (D) PEG-α-CDE/pDNA/α-CyD PPRX, (E) PEG/α-CyD PPRX, (F) γ-CyD alone, (G) PEG-α-CDE/γ-CyD physical mixture, (H) PEG-α-CDE/γ-CyD PPRX, (I) PEG-α-CDE/pDNA/γ-CyD PPRX, (J) PEG/γ-CyD PPRX.

Figure 4. Schematic representation of crystal packing structures of α-CyD (A) and γ-CyD (B) PPRXs

Figure 5. Agarose gel electrophoretic analysis of PEG-α-CDE/pDNA complex. The solution containing the pDNA complexes with carriers was incubated for 15 min at room temperature. The electrophoresis was performed at 100 V for about 30 min. The amount of pDNA was 0.2 μg.

Figure 6. Association ratios of pDNA in PEG-α-CDE/pDNA/CyDs PPRXs. The solution containing the pDNA complexes with PEG-α-CDE was incubated for 15 min at room temperature. Thereafter, α-CyD (145 mg/mL) or γ-CyD (232 mg/mL) solution was added. After 12 h, the association ratios of pDNA were calculated by measuring supernatant of pDNA as a ratio of total amount of pDNA. The amount of pDNA was 5 μg. The charge ratio of PEG-α-CDE/pDNA was 2. Each value represents the mean±S.E.M. of 3-4 experiments.

Figure 7. *In vitro* luciferase activity of the pDNA complexes with PEG-α-CDE in Colon-26 cells. Transfection was performed with culture medium without FCS for 3 h. The luciferase activity in cell lysates was determined 24 h after incubation. The charge ratio of PEG-α-CDE/pDNA was 50. The amount of pDNA was 2.0 μg. Each value represents the mean±S.E.M. of 4 experiments. * $p<0.05$ *versus* pDNA alone.

Figure 8. Cytotoxicity of the pDNA complexes with various carriers in Colon-26 cells. Transfection was performed with culture medium without FCS for 3 h. Cell viability was assayed by the WST-1 method. The amount of pDNA was 2.0 μg. Each point represents the mean±S.E.M. of 4 experiments. * $p<0.05$ *versus* PEG-α-CDE (G2, DSP4).

Figure 9. *In vitro* release profiles of pDNA from PEG-α-CDE/pDNA/CyD PPRXs in phosphate buffer (pH 7.4). (A) The volume of dissolution media was 500 μL. (B, C) Effects of volume of dissolution media on the release profiles of pDNA. The amount of pDNA was 30 μg. The charge ratio of PEG-α-CDE/pDNA was 2. The PPRX

suspensions were stirred at 100 rpm. Each point represents the mean±S.E.M. of 5-7 experiments. *p<0.05 *versus* γ-CyD PPRX with 500 μL of PBS (pH 7.4).

Figure 10. *In vivo* luciferase activity after intramuscular injection of solutions containing of pDNA with CyD PPRXs to BALB/c mice. Fifty μL of naked pDNA solution and PEG-α-CDE/pDNA/γ-CyD PPRX suspension were injected intramuscularly to mice. The dose of pDNA was 10 μg. The luciferase activity was determined 0, 3, 7 and 14 days after intramuscular injection. The charge ratio of PEG-α-CDE/pDNA was 2. Each point represents the mean±S.E.M. of 4 experiments.

Figure 11. Proposed scheme for gene transfer mechanism of PEG-α-CDE/γ-CyD PPRX suspension after intramuscular injection to mice

Table 1. Crystallographic characteristics of PEG-α-CDE/CyDs PPRXs

Table 2. Particle sizes and ζ-potentials of PEG-α-CDE/CyD PPRXs and PEG-α-CDE/pDNA/CyD PPRX in Tris-HCl buffer (pH 7.4).

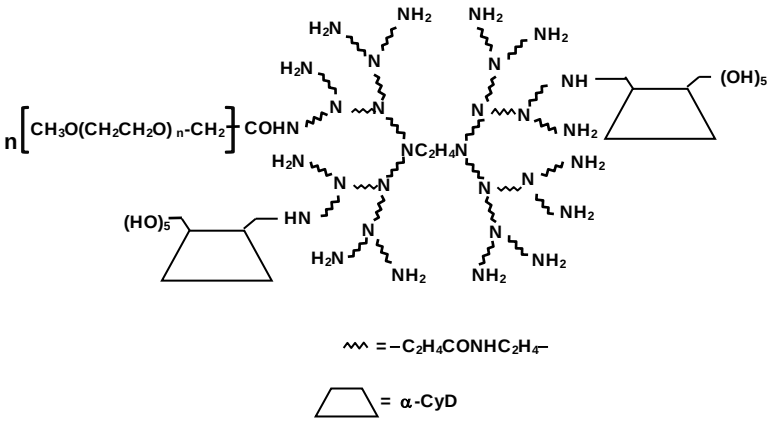
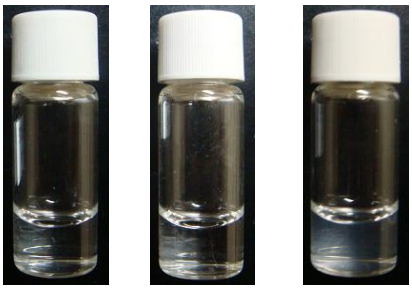

n
CH3O(CH2CH2O) n-CH2
COHN
NC2H4N
NH
(OH)5
HN
(HO)5
NH2
H2N
N
=–C2H4CONHC2H4–
= α-CyD

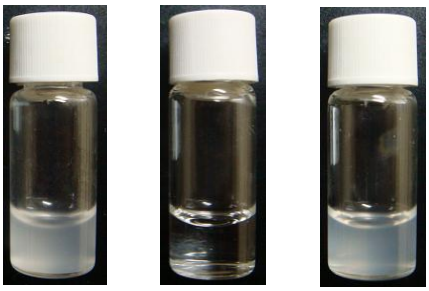
Figure 1

(A) α-CDE



+ α-CyD + β-CyD + γ-CyD

(B) PEG-α-CDE

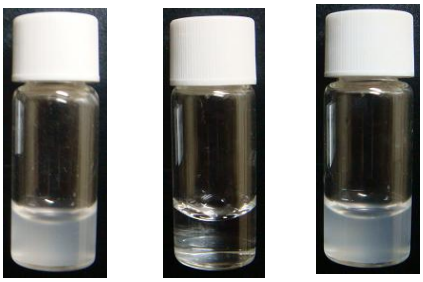


+ α-CyD + β-CyD + γ-CyD

(C) α-CDE/pDNA

+ α-CyD + β-CyD + γ-CyD

(D) PEG-α-CDE/pDNA



+ α-CyD + β-CyD + γ-CyD

Figure 2

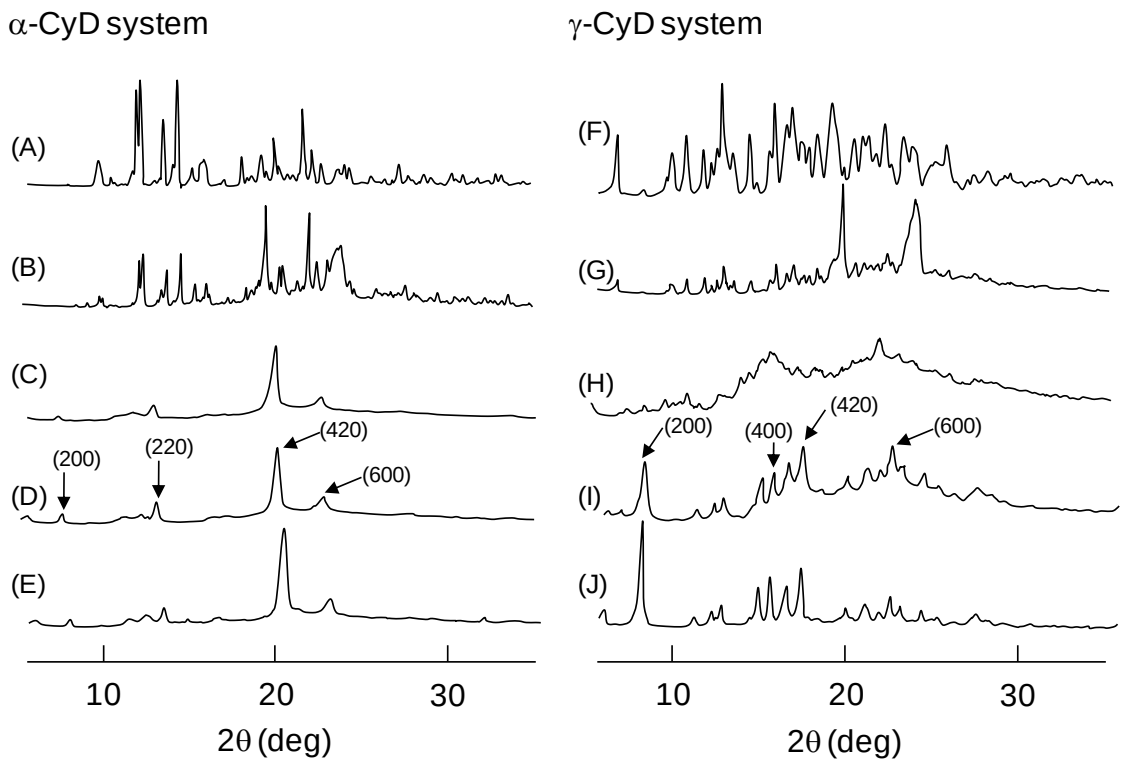
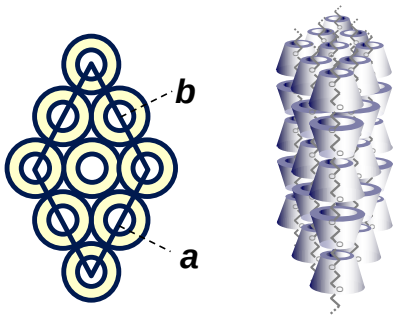

α-CyD system
γ-CyD system
(A)
(B)
(C)
(D)
(E)
(F)
(G)
(H)
(I)
(J)
(200)
(220)
(420)
(600)
(200)
(400)
(420)
(600)
10
20
30
2θ (deg)
10
20
30
2θ (deg)

Figure 3

(A) Hexagonal columnar channels



(B) Tetragonal columnar channels

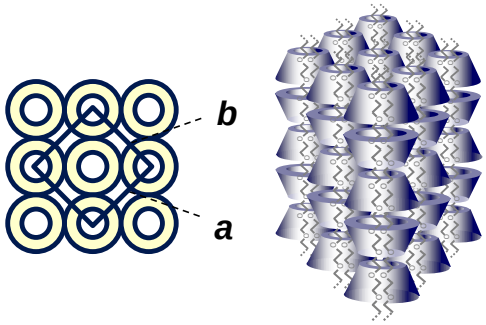


Figure 4

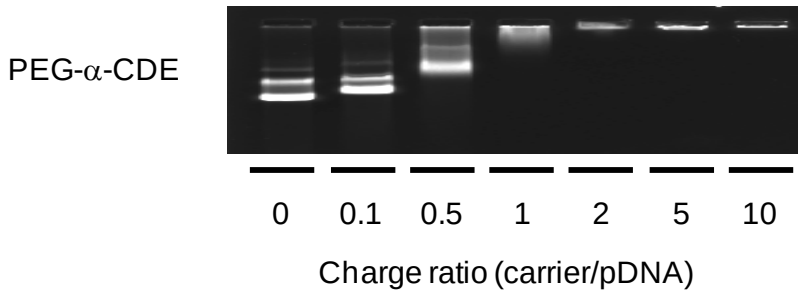


Figure 5

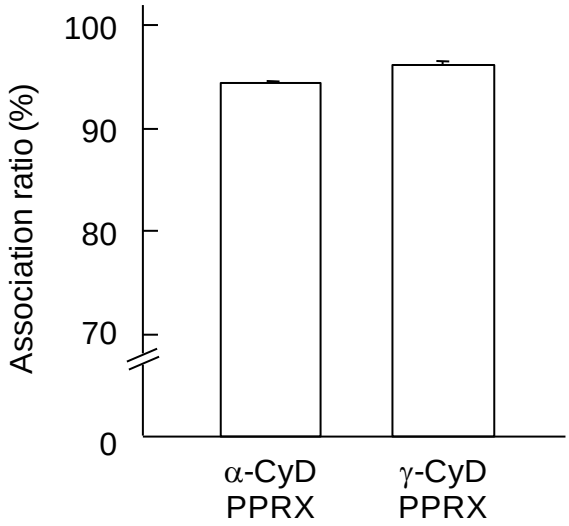


Figure 6

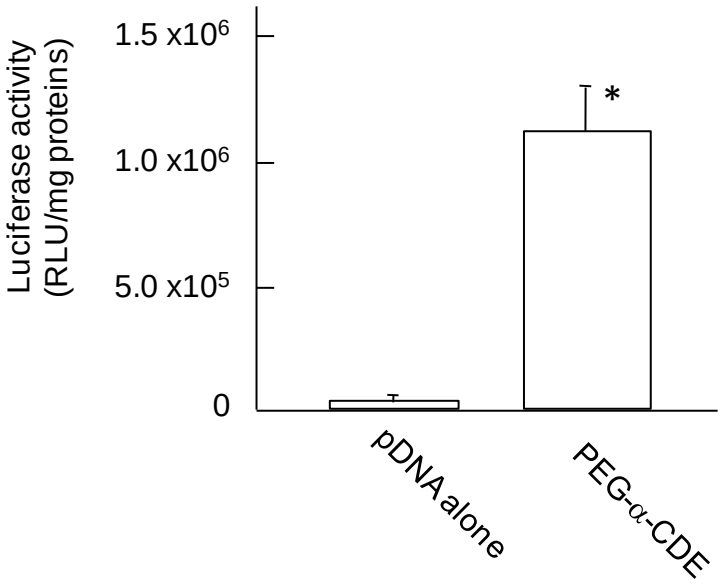

Luciferase activity
(RLU/mg proteins)
1.5 x10^6
1.0 x10^6
5.0 x10^5
0
*
pDNA alone
PEG-α-CDE

Figure 7

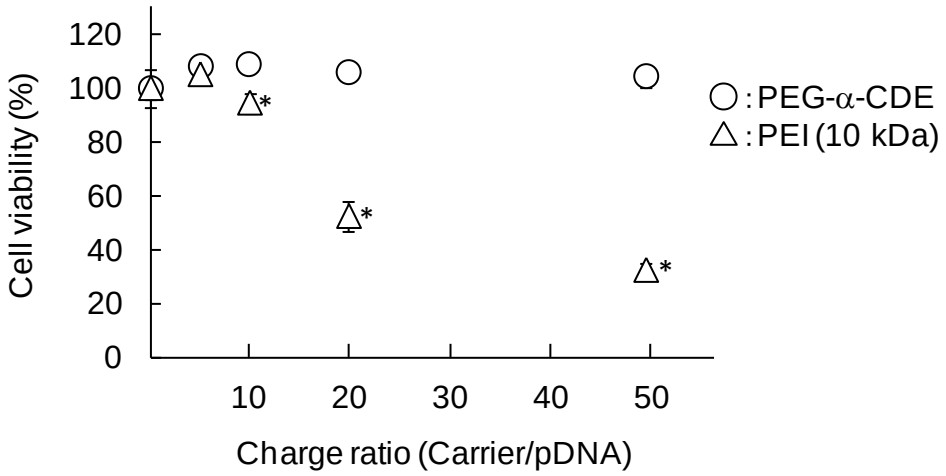
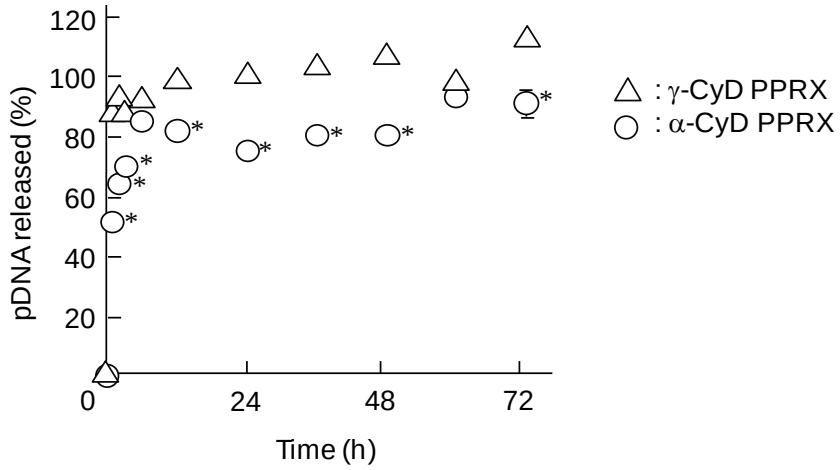
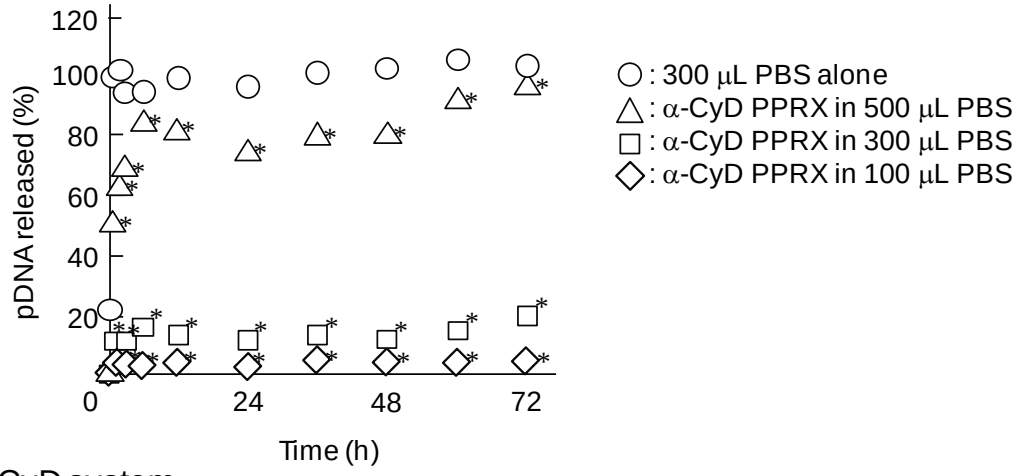

120
100
80
60
40
20
0
Cell viability (%)
10
20
30
40
50
Charge ratio (Carrier/pDNA)
○ : PEG-α-CDE
△ : PEI (10 kDa)
*
*
*

Figure 8

(A) α-CyD and γ-CyD systems



(B) α-CyD system



(C) γ-CyD system

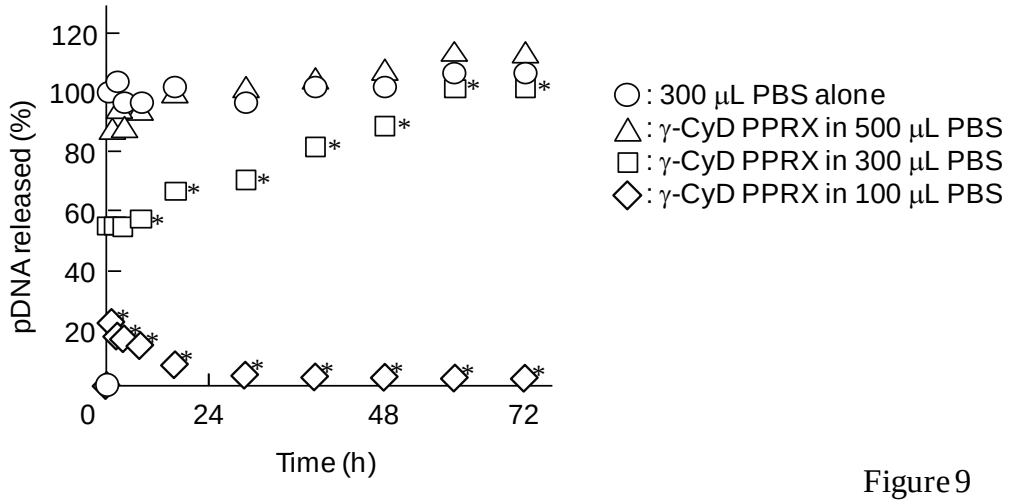


Figure 9

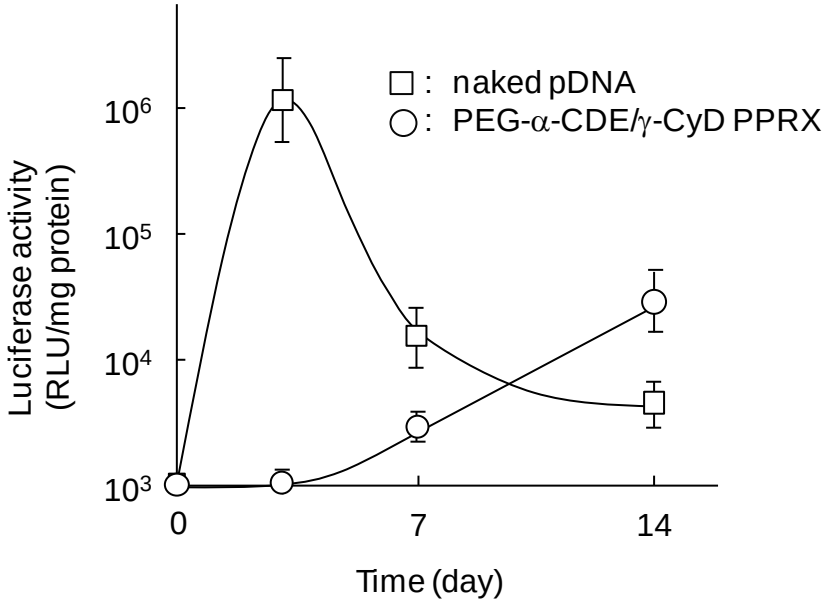

□ : naked pDNA
○ : PEG-α-CDE/γ-CyD PPRX
Luciferase activity
(RLU/mg protein)
10⁶
10⁵
10⁴
10³
0
7
14
Time (day)

Figure 10

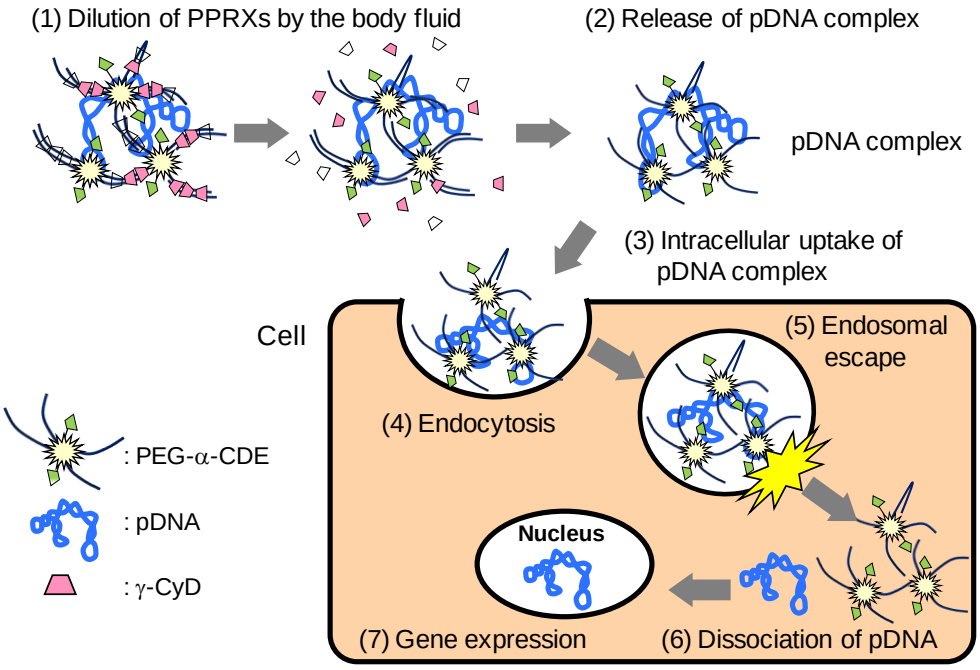

(1) Dilution of PPRXs by the body fluid
(2) Release of pDNA complex
pDNA complex
(3) Intracellular uptake of pDNA complex
Cell
(4) Endocytosis
(5) Endosomal escape
Nucleus
(6) Dissociation of pDNA
(7) Gene expression
: PEG-α-CDE
: pDNA
: γ-CyD

Figure 11

Table 1. Crystallographic Characteristics of PEG-α-CDE/pDNA/CyDs PPRXs

(A) PEG-α-CDE/pDNA/α-CyD				(B) PEG-α-CDE/pDNA/γ-CyD			
2θ (deg)	(hkl)	d_{obs} (Å)	d_{cal}[1)] (Å)	2θ (deg)	(hkl)	d_{obs} (Å)	d_{cal}[2)] (Å)
7.43	(200)	11.89	11.89	7.33	(200)	12.05	12.05
12.83	(220)	6.89	6.87	14.86	(400)	5.96	6.03
19.78	(420)	4.49	4.49	16.65	(420)	5.32	5.39
22.50	(600)	3.95	3.96	22.34	(600)	3.98	4.02

1) Calculated assuming a hexagonal unit cell with $a = b = 27.46$Å.
2) Calculated assuming a tetragonal unit cell with $a = b = 24.10$Å.

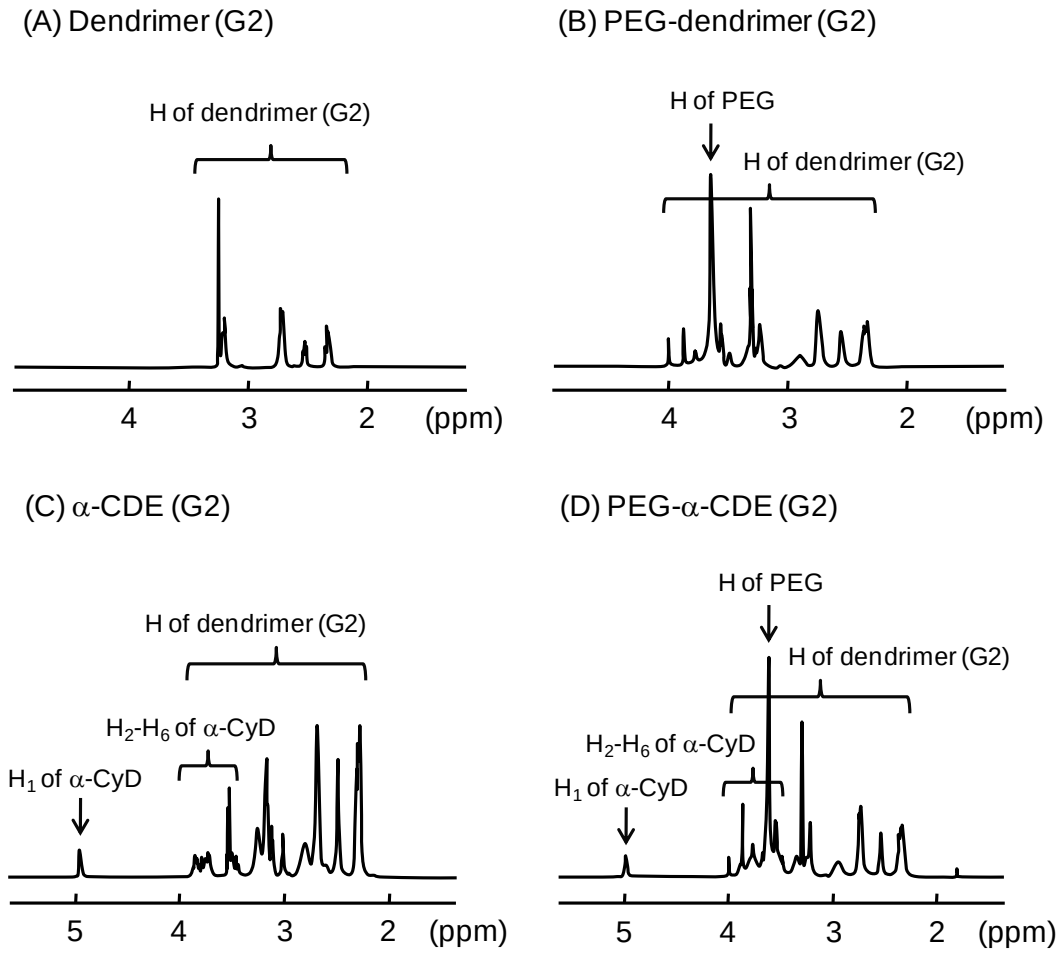
Table 1

Table 2. Particle Sizes and ζ-Potentials of PEG-α-CDE/CyD PPRXs and PEG-α-CDE/pDNA/CyD PPRXs in Tris-HCl Buffer (pH 7.4).

System	Mean diameter (μm)	ζ-potential (mV)
PEG-α-CDE	N.D.	N.D.
PEG-α-CDE/α-CyD PPRX	5.40±0.12*,†	3.72±0.42 *,†
PEG-α-CDE/γ-CyD PPRX	4.47±0.19*,†	1.96±0.27 *,†
PEG-α-CDE/pDNA	0.64±0.02	0.59±0.25
PEG-α-CDE/pDNA/α-CyD PPRX	5.66±0.36*,†	-6.09±0.33
PEG-α-CDE/pDNA/γ-CyD PPRX	4.63±0.35*,†	-7.49±1.92

Particle sizes and ζ-potentials of the samples were measured by Zetasizer Nano. The pDNA complexes with carriers were added to Tris-HCl buffer (10 mM, pH 7.4). The amount of pDNA was 5 μg. The charge ratio of PEG-α-CDE/pDNA was 2. Each value represents the mean±S.E.M. of 3-4 experiments. *p<0.05 *versus* PEG-α-CDE. †p<0.05 *versus* PEG-α-CDE/pDNA.

Table 2



Supplementary Fig. S1.
^{1}H-NMR Spectra of Various Carriers in D_2O at 25°C