Potential Use of Polypseudorotaxanes of Pegylated Polyamidoamine Dendrimer with Cyclodextrins as Novel Sustained Release Systems for DNA

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In this study, we demonstrated the potential use of polypseudorotaxanes (PPRXs) of polyethylene glycol (PEG, molecular weight: 2000)-grafted polyamidoamine dendrimer (PEG-dendrimer) with cyclodextrins (CyDs) as novel sustained release systems for plasmid DNA (pDNA). PEG-dendrimer/pDNA complex formed PPRXs with α-CyD and γ-CyD solutions, but not with β-CyD solution. In the PEG-dendrimer/CyDs PPRXs systems, 17.9 mol of α-CyD and 8.8 mol of γ-CyD were involved in the PPRXs formation with one PEG chain by α-CyD and γ-CyD, respectively. In addition, the CyDs PPRX formation provided the sustained release of pDNA from PEG-dendrimer complex with pDNA at least 72 h *in vitro*. In addition, the release of pDNA from CyDs PPRX retarded as the dissolution medium volume decreased. These results suggest that the PEG-dendrimer/CyD PPRX systems can work as a sustained DNA release system, and the PPRX formation with CyDs may be useful as a sustained drug delivery technique for other pegylated polymers.

Key words   cyclodextrin; polypseudorotaxane; pegylated dendrimer; DNA; sustained release; controlled release

Gene therapy can provide a most promising way to overcome both genetic and refractory diseases. Wide variety of vectors to deliver therapeutic nucleic acids such as plasmid DNA (pDNA) or oligonucleotides into the desired target cells have been investigated.[1] There are two categories of gene therapy vectors, *i.e.*, viral vectors and nonviral vectors. Recently, the potentials of nonviral vectors have been noticed due to easy preparation of vector/pDNA complexes, low cytotoxicity, and lack of immunogenicity. Starburst polyamidoamine (PAMAM) dendrimer (dendrimer) employed in this study is a spherical, highly ordered, dendritic polymer with positively charged primary amino groups on the surface at physiological pH[2,3] and has the widespread use as nonviral vectors. Recently, to obtain more effective gene expression, a controlled release of bioactive pDNA has been studied by encapsulating pDNA into biodegradable matrices such as gelatin,[4] atelocollagen,[5] polylactic-polyglycolic acid (PLGA).[6—8] However, these techniques have some drawbacks, *e.g.*, 1) a complicated way to encapsulate nucleic acids, 2) use of organic solvent, 3) low encapsulation efficiency, *etc.* Therefore, the development of a novel controlled release system for bioactive nucleic acids has been expected.

Recently, supramolecular assemblies have attracted a great attention, due to its intriguing topologies and its 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. CyDs have been widely applied to DDS due to their good bioadaptability.[9,10] CyDs are cyclic oligosaccharides composed of 6 (α-CyD), 7 (β-CyD), and 8 (γ-CyD) glycopyranose units that can form inclusion complexes with various organic and inorganic compounds. Harada *et al.* firstly 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 of the polymer chain when PPRXs

dissolved in water. This complexation shows the size dependency, *i.e.* the small cavity of α-CyD forms the PPRX with one PEG chain, while the middle cavity of β-CyD with polypropylene glycol, and large cavity of γ-CyD 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 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 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 DNA or oligonucleotides. In the present study, we designed and evaluated CyDs PPRXs of the pegylated dendrimer/pDNA complexes as a novel sustained release system for pDNA.

Experimental

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=3256) was purchased from Aldrich Chemical (Tokyo, Japan). Methoxypolyethylene glycol succinimidyl carboxymethyl ester (PEG-COO-NHS, SUN-BRIGHT® ME-020AS, molecular weight=2000) was obtained from NOF (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.

Preparation of PEG-Dendrimer   PEG-COO-NHS (50 mg, 2.5×10^{-5} mol) and dendrimer (G2) were dissolved in 1 ml of dimethyl sulfoxide (DMSO) and stirred for 24 h at room temperature. Then, the reactant was dialyzed using a dialysis membrane, Spectra/pore (MWCO=3500), in water for 1 week. After dialysis, the sample was concentrated and freeze dried to obtain PEG-dendrimer. The average degree of substitution of PEG (DSP) was determined by ¹H-NMR, but a peak derived from DMSO was not detected by ¹H-NMR (data not shown).

Preparation of PEG-Dendrimer/pDNA/CyD PPRXs   The solutions containing the pDNA complexes with PEG-dendrimer (G2, DSP4, 2.53×10^{-9}
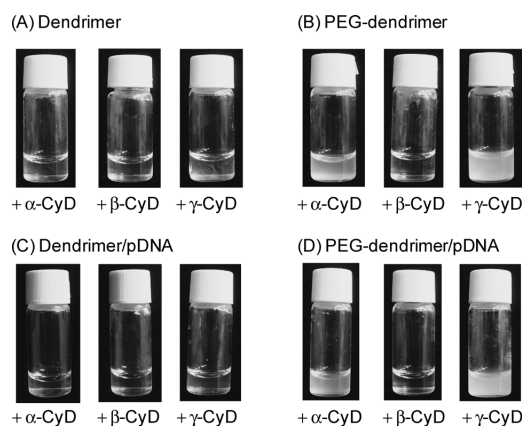
Fig. 1. Photographs of PEG-Dendrimer/CyD PPRX with or without pDNA

The solutions containing the pDNA complexes with PEG-dendrimer were incubated for 15 min at room temperature. Thereafter, solutions containing dendrimer/pDNA and PEG-dendrimer/pDNA 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 dendrimer/pDNA or PEG-dendrimer/pDNA was 2/1.

mol, 28.42 μg) were incubated for 15 min at room temperature. Thereafter, solutions containing dendrimer (G2)/pDNA or PEG-dendrimer (G2, DSP4)/pDNA were 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 4 °C. The amount of pDNA was 5 μg. The charge ratio of dendrimer/pDNA and PEG-dendrimer (G2, DSP4)/pDNA was 2/1, because the complete complexation of dendrimer/pDNA and PEG-dendrimer (G2, DSP4)/pDNA was observed at a charge ratio of 2/1 by electrophoresis (data not shown).

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$K\alpha$ 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.

Physicochemical Properties of PEG-Dendrimer/pDNA/CyD PPRXs The solutions containing pDNA complexes with PEG-dendrimer were added to Tris–HCl buffer (10 mM, pH 7.4). The ζ-potentials and particle sizes of the polyplexes with PEG-dendrimer with CyDs were determined by a Zetasizer Nano (Malvern Instruments, Worcestershire, U.K.).

Release of pDNA from PEG-Dendrimer/pDNA/CyD PPRXs The release rate of the PEG-dendrimer/pDNA was measured by the modified dispersed-amount method,[16] i.e. 300—700 μl of phosphate-buffered saline (PBS, pH 7.4) was added in the PPRXs suspensions at room temperature, and stirred at 100 rpm. The amount of pDNA was 30 μg. At appropriate intervals, an aliquot of the dissolution medium was withdrawn, centrifuged at 3000 rpm for 3 min. The concentration of pDNA in the PEG-dendrimer/pDNA complex released from PPRXs in dissolution medium was determined by measuring the absorbance at 260 nm (sample) and 280 nm (reference) using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, Delaware, U.S.A.).

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 PPRXs of PEG-Dendrimer with CyDs The PEG moiety at the primary amino group of dendrimer (G2) was introduced, using PEG-COO-NHS in DMSO. After dialysis, the PEG-dendrimer was obtained with about 24.3% product yield. The average degree of substitution of PEG (DSP) was determined by ^{1}H-NMR as about 4. PPRXs of the PEG-dendrimer with CyDs were prepared by mixing aqueous solutions containing them. Figure 1 shows the solution after mixing the dendrimer or PEG-dendrimer in the absence and presence of pDNA with α-CyD, β-CyD or γ-CyD solution and standing for 12 h at 4 °C. The addition of α-CyD or

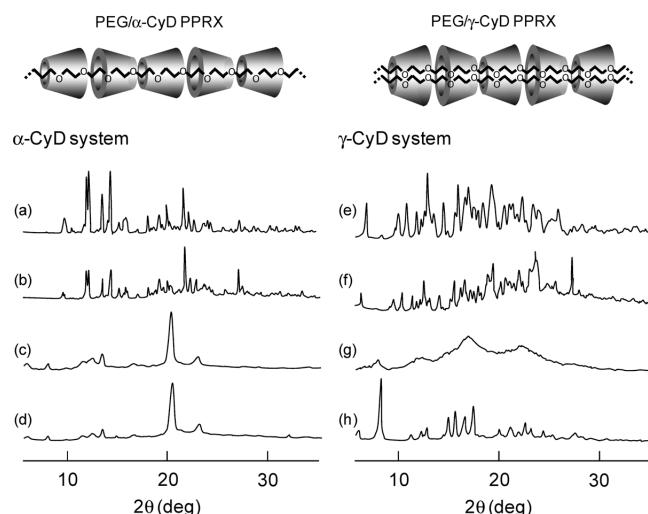

Fig. 2. Powder X-Ray Diffraction Patterns of PEG-Dendrimer/CyD PPRX

(a) α-CyD alone, (b) PEG-dendrimer/α-CyD physical mixture, (c) PEG-dendrimer/α-CyD PPRX, (d) PEG/α-CyD PPRX, (e) γ-CyD alone, (f) PEG-dendrimer/γ-CyD physical mixture, (g) PEG-dendrimer/γ-CyD PPRX, (h) PEG/γ-CyD PPRX.

γ-CyD solution to PEG-dendrimer solution provided precipitates, but not that of β-CyD solution, indicating that α-CyD and γ-CyD form PPRXs with PEG-dendrimer, like PPRX formation of PEG with these CyDs reported by Harada et al.[11,12] Importantly, we confirmed that the complexes of PEG-dendrimer with pDNA could also form PPRXs. That is, the stoichiometry of the PPRXs was accurately determined by measuring integral values of the protons derived from amino groups of PEG-dendrimer and the ethylene protons of the PEG-dendrimer in ^{1}H-NMR spectra after dissolving the solid PPRXs in DMSO. As a result, 17.9 and 8.8 mol of α-CyD and γ-CyD are involved in the PPRXs formation with one PEG chain by α-CyD or γ-CyD, respectively. Similar results were observed in the PEG-dendrimer/pDNA/CyDs PPRXs systems.

Next, we confirmed the PPRXs formation by measuring the powder X-ray diffraction patterns of the α-CyD and γ-CyD PPRXs with PEG-dendrimer, in comparison with those of PEG (Fig. 2). The diffraction pattern of the PEG-dendrimer/α-CyD PPRX (Fig. 2c) was different from that of α-CyD alone (Fig. 2a) and physical mixture (Fig. 2b), but the same as that of PEG/α-CyD PPRX (Fig. 2d).[19] These results suggest that PEG-dendrimer forms PPRXs with α-CyD in the solid state. Meanwhile, the diffraction pattern of the PEG-dendrimer/γ-CyD PPRX (Fig. 2g) was broad, and was different from that of γ-CyD alone (Fig. 2e) or physical mixture (Fig. 2f), but provided a similar pattern with that of PEG/γ-CyD PPRX only slightly (Fig. 2h). Thus, it is assumed that γ-CyD forms PPRXs with PEG-dendrimer having more complicated structures, e.g. γ-CyD formed PPRXs with 1) two PEG chains derived from one PEG-dendrimer molecule, 2) two PEG chains derived from different PEG-dendrimer molecule, and 3) bended one PEG chain (equivalent to two PEG chains were threaded into γ-CyD). These lines of evidence make it tempting to speculate the structures of α-CyD and γ-CyD PPRXs with PEG-dendrimer (Fig. 3). Thereafter, further studies should be required to reveal the detailed PPRX structures of PEG-dendrimer/CyD systems.
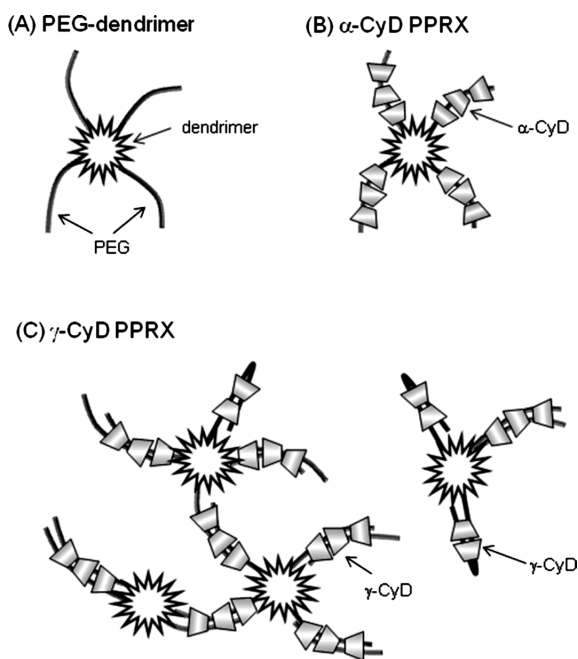
(A) PEG-dendrimer

(B) α-CyD PPRX

(C) γ-CyD PPRX

Fig. 3. Proposed Structures of PEG-Dendrimer and PEG-Dendrimer/CyD PPRXs

Table 1. Particle Sizes and ζ-Potentials of PEG-dendrimer/CyD PPRXs and PEG-Dendrimer/pDNA/CyD PPRX in Tris–HCl Buffer (pH 7.4)

System	Mean diameter (μm)	ζ-Potential (mV)
PEG-dendrimer	0.40±0.02	0.27±0.29
PEG-dendrimer/α-CyD PPRX	4.51±0.11*,†	5.67±0.04*,†
PEG-dendrimer/γ-CyD PPRX	2.91±0.08*,†	2.19±0.02*,†
PEG-dendrimer/pDNA	0.19±0.00	0.43±0.54
PEG-dendrimer/pDNA/α-CyD PPRX	4.56±0.18*,†	1.09±0.42
PEG-dendrimer/pDNA/γ-CyD PPRX	3.73±0.14*,†	0.25±0.33

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-dendrimer/pDNA was 2/1. Each value represents the mean±S.E.M. of 3—4 experiments. *$p < 0.05$ versus PEG-dendrimer. †$p < 0.05$ versus PEG-dendrimer/pDNA.

Physicochemical Properties of PEG-Dendrimer/pDNA/CyD PPRXs To clarify physicochemical properties of the PEG-dendrimer/pDNA/CyD PPRXs, we determined the particle sizes and ζ-potential values of the PEG-dendrimer/pDNA/CyD PPRXs at a charge ratio of 2 (PEG-dendrimer/pDNA) (Table 1). The mean diameters of the PEG-dendrimer and PEG-dendrimer/pDNA were significantly increased by the addition of α-CyD and γ-CyD, probably due to the formation of PPRXs in the solution. There was no significant difference in the mean diameters between PEG-dendrimer/CyD PPRXs and PEG-dendrimer/pDNA/CyD PPRXs. Meanwhile, the ζ-potential values of the PEG-dendrimer/pDNA/CyD PPRXs were smaller than those of PEG-dendrimer/CyD PPRXs. These results indicate that pDNA lowered the ζ-potential values and did not affect the particle size of the PEG-dendrimer/CyD PPRXs.

***In Vitro* Release of pDNA from PEG-Dendrimer/pDNA/CyD PPRXs** Figure 4 shows the release profiles of the pDNA from PEG-dendrimer/pDNA/CyDs PPRXs in PBS (pH 7.4). Both α-CyD and γ-CyD PPRXs systems showed
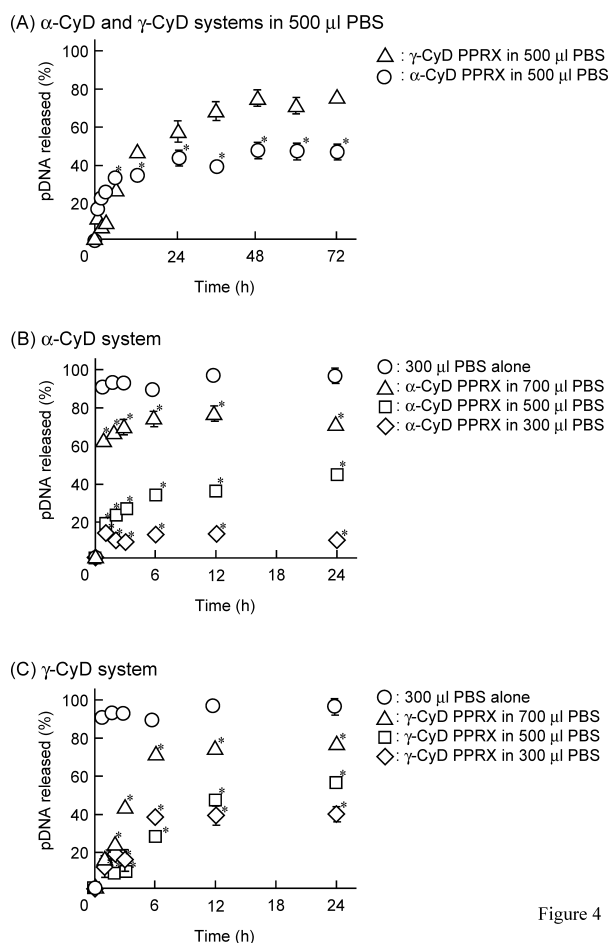

Fig. 4. *In Vitro* Release Profiles of pDNA from PEG-Dendrimer/pDNA/CyD PPRXs in Phosphate Buffer (pH 7.4)

(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-dendrimer/pDNA was 2/1. The PPRX solutions were stirred at 100 rpm. Each point represents the mean±S.E. of 5—7 experiments. *$p < 0.05$ versus γ-CyD PPRX with 500 μl PBS (pH 7.4).

sustained release profiles at least up to 72 h (Fig. 4A). The release rate of the PEG-dendrimer/pDNA/α-CyD PPRX system was lower than that of the PEG-dendrimer/pDNA/γ-CyD PPRX system. In addition, the release of pDNA from their PPRXs retarded as the volume of the dissolution medium decreased in the order of 700>500>300 μl (Figs. 4B, C). This may be due to the threading and dethreading of PPRXs in an equilibrium with free host and guest molecules.[19] This explanation is strongly supported by our previous data that pegylated insulin or pegylated lysozyme was gradually released from their PPRXs through the dethreading of PPRXs.[15—18] These results indicate that PEG-dendrimer/pDNA/CyDs PPRXs can provide 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. The PEG-dendrimer/pDNA/CyDs PPRXs are expected to use for sustained release systems of pDNA after subcutaneous, intramuscular or intratumoral administration, but not intravenous administration, due to their relatively high particle sizes (Table 1). In addition, the release rate of pDNA from PEG-dendrimer/pDNA/CyDs PPRXs could be extended by the introduction of PEG with high molecular weight onto dendrimer. It is important to know transfection efficiency of

PEG-dendrimer/pDNA complex released from PEG-dendrimer/pDNA/CyDs PPRXs. In our preliminary study, luciferase activity of 24 h after transfection of PEG-dendrimer/pDNA complex at an amount of 2.0 μg as pDNA at a charge ratio of 2/1 in Colon-26 cells was 6.6×10^5 relative light unit/mg proteins, suggesting that PEG-dendrimer/pDNA/CyDs PPRXs have the potentials for transfection efficiency, although the efficiency was not enough high, possibly due to PEG dilemma.

Further investigation on the improvement of transfection efficiency of PEG-dendrimer/pDNA complex released from PPRXs is undergoing.

Conclusions

In the present study, we demonstrated the potential use of PEG-dendrimer/pDNA/CyDs PPRXs as a novel sustained release system for pDNA. This is the first report on the application of PPRXs of a pegylated cationic polymer with CyDs for the controlled release of DNA. These results suggest that the CyD/PEG-dendrimer PPRX systems can work as a sustained DNA release system, and the PPRX formation with CyDs may be useful as a sustained drug delivery technique for other pegylated polymers.

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