



## Pharmaceutical Nanotechnology

Possible enhancing mechanisms for gene transfer activity of glucuronylglucosyl- $\beta$ -cyclodextrin/dendrimer conjugateTakayuki Anno<sup>a</sup>, Taishi Higashi<sup>a</sup>, Keiichi Motoyama<sup>a</sup>, Fumitoshi Hirayama<sup>b</sup>, Kaneto Uekama<sup>b</sup>, Hidetoshi Arima<sup>a,\*</sup><sup>a</sup> Graduate School of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Kumamoto 862-0973, Japan<sup>b</sup> Faculty of Pharmaceutical Sciences, Sojo University, 4-22-1 Ikeda, Kumamoto 860-0082, Japan

## ARTICLE INFO

## Article history:

Received 19 October 2011

Received in revised form

18 December 2011

Accepted 17 January 2012

Available online 25 January 2012

## Keywords:

PAMAM starburst dendrimer

Glucuronylglucosyl- $\beta$ -cyclodextrin

Conjugate

Cyclodextrins

Non-viral vector

Enhancing mechanism

## ABSTRACT

We previously reported that glucuronylglucosyl- $\beta$ -cyclodextrin (GUG- $\beta$ -CyD) conjugate with polyamidoamine starburst dendrimer (GUG- $\beta$ -CDE conjugate) with the average degree of substitution (DS) of cyclodextrin (CyD) of 1.8 (GUG- $\beta$ -CDE conjugate (DS 1.8)), showed remarkably higher gene transfer activity than  $\alpha$ -CyD/dendrimer conjugate ( $\alpha$ -CDE conjugate (DS 1.2)) and  $\beta$ -CyD/dendrimer conjugate ( $\beta$ -CDE conjugate (DS 1.3)) *in vitro* and *in vivo*. In this study, to clarify the enhancing mechanism for high gene transfer activity of GUG- $\beta$ -CDE conjugate (DS 1.8), we investigated the physicochemical properties, cellular uptake, endosomal escape and nuclear translocation of the plasmid DNA (pDNA) complexes as well as pDNA release from the complexes. The particle size,  $\zeta$ -potential and cellular uptake of GUG- $\beta$ -CDE conjugate (DS 1.8)/pDNA complex were mostly comparable to those of  $\alpha$ -CDE conjugate (DS 1.2) and  $\beta$ -CDE conjugate (DS 1.3). Meanwhile, GUG- $\beta$ -CDE conjugate (DS 1.8)/pDNA complex was likely to have high endosomal escaping ability and nuclear localization ability in A549 and RAW264.7 cells. In addition, the pDNA condensation and decondensation abilities of GUG- $\beta$ -CDE conjugate (DS 1.8) were lower and higher than that of  $\alpha$ -CDE conjugate (DS 1.2) or  $\beta$ -CDE conjugate (DS 1.3), respectively. These results suggest that high gene transfer activity of GUG- $\beta$ -CDE conjugate (DS 1.8) could be, at least in part, attributed to high endosomal escaping ability, nuclear localization ability and suitable pDNA release from its complex.

© 2012 Elsevier B.V. All rights reserved.

## 1. Introduction

Polyamidoamine starburst dendrimer (dendrimer, Fig. 1) is a spherical, highly ordered, dendritic polymer with positively charged primary amino groups on the surface at physiological pH (Tomalia et al., 1990). Although their usefulness as nonviral vectors has been reported (Bielinska et al., 1996; Qin et al., 1998), dendrimers with lower generations (generations 1–3, G1–G3) do not have efficient gene transfer activity, whereas dendrimers with higher generations exhibit cytotoxicity (Kukowska-Latallo et al., 1996).

Recently, cyclodextrins (CyDs) have been applied to gene transfer and oligonucleotide delivery (Abdou et al., 1997; Croyle et al., 1998; Freeman and Niven, 1996; Zhao et al., 1995). Huang et al. (2006) reported that two polymers of low molecular weight polyethylenimine (PEI) cross-linked by 2-hydroxypropyl- $\beta$ -CyD (HP- $\beta$ -CyD) or 2-hydroxypropyl- $\gamma$ -CyD (HP- $\gamma$ -CyD) showed lower

cytotoxicity and higher transfection efficiency for the delivery of pDNA, compared with those of PEI (25 kDa). Davis et al. reported the development of a transferrin-modified, CyD polymer-based gene delivery system (Bellocq et al., 2003). The delivery system is comprised of a nanoparticle (formed by condensation of a CyD polycation with nucleic acid) that is surface-modified to display polyethylene glycol (PEG) for increasing stability in biological fluids and transferrin for targeting of cancer cells that express transferrin receptor.

We previously reported that of various dendrimer (G3) conjugates with  $\alpha$ -CyD ( $\alpha$ -CDEs),  $\alpha$ -CDE conjugate (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 (Arima et al., 2001; Kihara et al., 2002, 2003). Recently, we reported the potential use of  $\alpha$ -CDEs bearing galactose (Gal- $\alpha$ -CDE conjugate), mannose (Man- $\alpha$ -CDE conjugate) or lactose (Lac- $\alpha$ -CDE conjugate) with the various DS of these sugar moieties as gene delivery carriers (Arima et al., 2006, 2010; Wada et al., 2005a, b). More recently, we constructed the novel dendrimer conjugate with 6-O- $\alpha$ -(4-O- $\alpha$ -D-glucuronyl)-D-glucosyl- $\beta$ -CyD (GUG- $\beta$ -CyD), and the resulting conjugate (GUG- $\beta$ -CDE conjugate (DS 1.8), Fig. 1) showed

\* Corresponding author. Tel.: +81 96 371 4160; fax: +81 96 371 4420.

E-mail address: [arimah@spo.kumamoto-u.ac.jp](mailto:arimah@spo.kumamoto-u.ac.jp) (H. Arima).

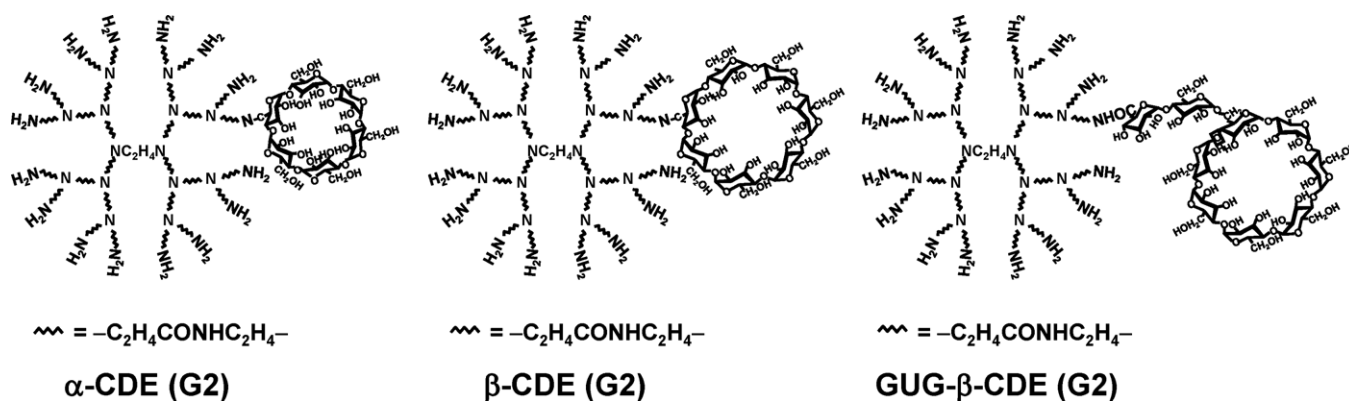


Fig. 1. Chemical structures of  $\alpha$ -CDE conjugate,  $\beta$ -CDE conjugate and GUG- $\beta$ -CDE conjugate.

markedly higher gene transfer efficiency than  $\alpha$ -CDE conjugate (DS 1.2) and dendrimer conjugate with  $\beta$ -CyD ( $\beta$ -CDE conjugate (DS 1.3) *in vitro* and *in vivo* (Anno et al., 2011; Anno et al., 2012). However, the detailed mechanism by which GUG- $\beta$ -CDE conjugate (DS 1.8) markedly increased gene transfer activity, compared to  $\alpha$ -CDE (DS 1.2) conjugate and  $\beta$ -CDE conjugate (DS 1.3) remains unclear. In general, cationic polymer-mediated transfection should overcome four major barriers: (1) cellular uptake of pDNA to cell surface, (2) endosomal escape, (3) entry of pDNA into the nucleus, and (4) release of pDNA from the complex in cells (De Smedt et al., 2000; Elouahabi and Ruyschaert, 2005). In the present study, to reveal the enhancing mechanism for superior gene transfer activity of GUG- $\beta$ -CDE conjugate (DS 1.8) to that of  $\alpha$ -CDE conjugate (DS 1.2) and  $\beta$ -CDE conjugate (DS 1.3), we investigated the physicochemical properties, cellular uptake, endosomal escape and nuclear translocation of pDNA complexes with GUG- $\beta$ -CDE conjugate (DS 1.8) as well as pDNA release from the complexes. Herein, all of the dendrimer used in this study was dendrimer (G2).

## 2. Materials and methods

### 2.1. Materials

$\alpha$ -CyD and  $\beta$ -CyD were donated by Nihon Shokuhin Kako (Tokyo, Japan) and recrystallized from water. GUG- $\beta$ -CyD was donated by Ensui Sugar Refining (Tokyo, Japan). Methyl- $\beta$ -CyD (M- $\beta$ -CyD) was obtained from Tokyo Kasei (Tokyo, Japan). Chlorpromazine hydrochloride (CPZ) and heparin were purchased from Sigma Aldrich (St Louis, MO). Nystatin was purchased from Wako Pure Chemical Industries (Osaka, Japan). Dendrimer (ethylenediamine core, generation 2, the terminal amino groups = 16, molecular weight = 3256) was purchased from Aldrich Chemical (Tokyo, Japan). Fetal calf serum (FCS) was purchased from Nichirei (Tokyo, Japan). Dulbecco's modified Eagle's medium (DMEM) and modified Eagle's medium (MEM) were purchased from Nissui Pharmaceuticals (Tokyo, Japan), respectively. 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/ $\mu$ g endotoxin). ULYSIS Alexa Fluor<sup>®</sup> 488 (Alexa) Nucleic Acid Labeling Kit and LysoTracker<sup>®</sup> were purchased from Molecular Probes (Tokyo, Japan). Tetramethylrhodamine isothiocyanate (isomer 5, TRITC) and bovine serum albumin (BSA) were obtained from Funakoshi (Tokyo, Japan) and Roche Diagnostics (Tokyo, Japan), respectively. Other chemicals and solvents were of analytical reagent grade.

### 2.2. Methods

#### 2.2.1. Preparation of GUG- $\beta$ -CDE conjugate (DS 1.8)

$\alpha$ -CDE conjugate (DS 1.2) and  $\beta$ -CDE conjugate (DS 1.3) were prepared as previously reported (Arima et al., 2001). GUG- $\beta$ -CDE conjugate (DS 1.8) was prepared by condensation between carboxyl group of GUG- $\beta$ -CyD and primary amino group of dendrimer (Anno et al., 2011). Briefly, 1.5 mL of DMSO including GUG- $\beta$ -CyD (61.4  $\mu$ mol), dendrimer (30.7  $\mu$ mol) and 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM, 73.7  $\mu$ mol) were incubated at 45 °C for 12 h. Next, the reaction solution was dialyzed using a membrane filter (Spectra/Por<sup>®</sup> membrane MWCO = 3500) to remove unreacted GUG- $\beta$ -CyD. The filtrate was added dropwise to ethanol and the resulting precipitates were recovered. The solid product was obtained by lyophilizing the precipitates.

#### 2.2.2. Cell culture

A549 cells, a human lung adenocarcinoma epithelial cell line, and RAW264.7 cells, a mouse leukemic monocyte macrophage cell line, were obtained from Riken Bioresource Center (Tsukuba, Japan). A549 cells were grown in MEM containing 1  $\times$  10<sup>5</sup> mU/mL of penicillin and 0.1 mg/mL of streptomycin supplemented with 10% FCS at 37 °C in a humidified 5% CO<sub>2</sub> and 95% air atmosphere. RAW264.7 cells were grown in RPMI-1640 containing 2  $\times$  10<sup>5</sup> mU/mL of penicillin and 0.2 mg/mL of streptomycin supplemented with 10% FCS at 37 °C in a humidified 5% CO<sub>2</sub> and 95% air atmosphere.

#### 2.2.3. *In vitro* gene transfer

*In vitro* transfection of pDNA complexes with various carriers was performed utilizing the *Renilla* luciferase expression of pDNA in A549 cells and RAW264.7 cells. The pDNA (2.0  $\mu$ g) was mixed with carriers at various charge ratios (carrier/pDNA). The pDNA complexes with carriers were then allowed to stand for 15 min at room temperature. The cells (2  $\times$  10<sup>5</sup> cells per 24 well plate) were seeded 6 h before transfection, and then washed twice with serum-free medium. Two hundred  $\mu$ L of serum-free medium containing pDNA or the complexes with carriers and 200  $\mu$ L of medium were added to each well, and then incubated at 37 °C for 3 h. After washing cells with serum-free medium twice, 200  $\mu$ L of medium containing 20% FCS (final concentration of FCS was 10%) were added to each dish, and then incubated at 37 °C for 21 h. After transfection, the 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). Total protein content

in the supernatant was determined by the Bio-Rad protein assay kit (Bio-Rad Laboratories, Tokyo, Japan).

#### 2.2.4. Particle size and $\zeta$ -potential

The solution containing various carriers at a charge ratio of 100 (carrier/pDNA) was added to Tris-HCl buffer (10 mM, pH 7.4) containing 5.0  $\mu$ g of pDNA. The solution was then incubated for 15 min. The particle sizes and  $\zeta$ -potentials of pDNA complexes with various carriers 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.

#### 2.2.5. Cellular association

Cellular association of pDNA complex was determined by a flow cytometry. Two micrograms of Alexa-labeled pDNA (Alexa-pDNA) were mixed with various carriers at a charge ratio of 100 (carrier/pDNA). After transfection with the Alexa-pDNA complexes with carriers for 1 h in A549 cells and RAW264.7 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  $1 \times 10^4$  cells on a FACS Calibur flow cytometer using CellQuest software (Becton-Dickinson, Mountain View, CA).

#### 2.2.6. Effects of endocytosis inhibitors on *in vitro* gene transfer

The pDNA (2.0  $\mu$ g) complexes with carriers at a charge ratio of 100 (carrier/pDNA) were prepared as above. After the cells ( $2 \times 10^5$  cells per 24 well plate) were seeded 5.5 h, the cells were pretreated with endocytosis inhibitors for 30 min, and then transfected with pDNA complexes at 37 °C for 3 h. After washing cells with serum-free medium twice, 400  $\mu$ L of medium containing 10% FCS were added to each dish, and then incubated at 37 °C for 21 h. After transfection, the luciferase activity was measured as above. The basal level of luciferase activity without the inhibitor was taken as 100%.

#### 2.2.7. Intracellular distribution

To observe the intracellular distribution of pDNA complexes with carriers, the cells ( $2 \times 10^5$  cells/dish) were incubated with pDNA complexes with TRITC-carriers for 6 h. In the case of the study using LysoTracker<sup>®</sup>, the cells were treated with LysoTracker<sup>®</sup> for 1 h after transfection with Alexa-pDNA complexes with carriers. After incubation, the cells were rinsed with DMEM twice and were observed with a fluorescence microscope BZ-9000 (KEYENCE, Osaka, Japan). The amount of pDNA was 2.0  $\mu$ g. The charge ratio was 100 (carrier/pDNA).

#### 2.2.8. pH titrations

The carriers were dissolved in 5 mL of purified water, and adjusted to a pH of approximately 11.5 with 1 N NaOH. The aliquots of 1 N HCl (5  $\mu$ L) were added (a total of 100  $\mu$ L), and pH of the solution was measured with a pH meter F-21 (HORIBA, Kyoto, Japan). Titrations were performed in duplicate.

#### 2.2.9. Liposome disruptive ability

Liposomes were prepared from egg phosphatidylcholine and cholesterol by the Bangham's method (Bangham et al., 1965) with an aqueous phase of 0.1 mM calcein which was dissolved by the addition of 3.75 equiv. of sodium hydroxide. The liposomal suspension was prepared using 10 mM phosphate buffer (pH 12) to keep the undissociated form of dendrimer,  $\alpha$ -CDE conjugate,  $\beta$ -CDE conjugate and GUG- $\beta$ -CDE conjugate. Ten microliters of 10 mM phosphate buffer (pH 12) containing carrier were added to 10  $\mu$ L of the liposomal suspension, and then the resulting suspension was incubated for 30 min at room temperature. Eighty microliters of phosphate buffer (pH 12) were further added to the suspension. The

fluorescence intensity of the suspension was finally measured with a fluorescence photometer (Hitachi F-4500, Tokyo, Japan) at 25 °C ( $\lambda_{\text{ex}}$  495 nm,  $\lambda_{\text{em}}$  515 nm). The value for 100% leakage was further obtained by the addition of 1  $\mu$ L of Triton X-100 to the suspension.

#### 2.2.10. pDNA condensed assay

The carriers were dissolved in HBSS (pH 7.4) containing pDNA (0.5  $\mu$ g) and Picogreen<sup>®</sup> dsDNA reagent (1  $\mu$ g) at the various charge ratios. The solutions were incubated at 25 °C for 15 min, and then the fluorescence ( $\lambda_{\text{ex}}$  495 nm,  $\lambda_{\text{em}}$  525 nm) was measured by a fluorescence photometer Hitachi F-4500 (Tokyo, Japan). Samples containing pDNA and Picogreen<sup>®</sup> dsDNA reagent were used to calibrate the apparatus to 100% fluorescence against a background of Picogreen<sup>®</sup> dsDNA reagent (1  $\mu$ g).

#### 2.2.11. Heparin competition assay

The carriers were added to HBSS (pH 7.4) containing pDNA (0.5  $\mu$ g) and Picogreen<sup>®</sup> dsDNA reagent (1  $\mu$ g) at a charge ratio of 100. After the solutions were incubated at 25 °C for 15 min, heparin solution (20 or 40  $\mu$ g/mL) was added to the solution, and then the fluorescence ( $\lambda_{\text{ex}}$  495 nm,  $\lambda_{\text{em}}$  525 nm) was measured by fluorescence spectrometer Hitachi F-4500 (Tokyo, Japan).

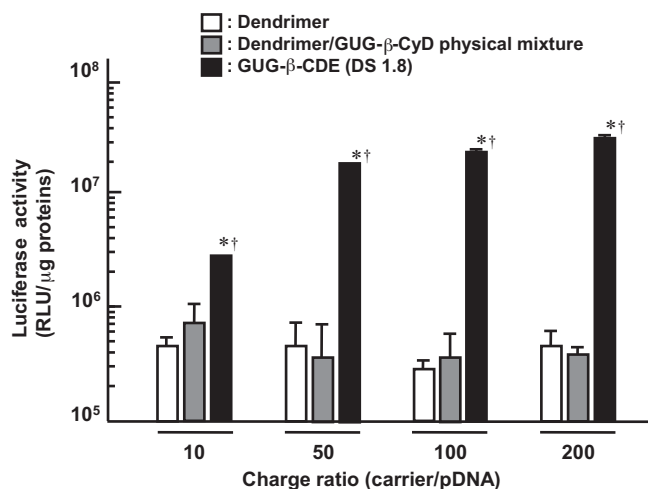
#### 2.2.12. Data analysis

Data are given as the mean  $\pm$  S.E. 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. Gene transfer activity of physical mixture of dendrimer/GUG- $\beta$ -CyD

Previously, we reported that GUG- $\beta$ -CDE conjugate (DS 1.8) provided higher gene transfer activity than those with the DS values of 1.2, 2.5 and 4.5, and the enhancing effect of GUG- $\beta$ -CDE conjugate (DS 1.8) on gene transfer activity was greater than that of  $\alpha$ -CDE conjugate (DS 1.2) or  $\beta$ -CDE conjugate (DS 1.3) at a charge ratio of 20 or 100 in A549 cells (Anno et al., 2011). Meanwhile, it is well known that CyDs extract membrane components such as phospholipids and cholesterol from plasma membranes of various cells and enhance membrane permeability of hydrophilic compounds (Irie and Uekama, 1997, 1999; Marttin et al., 1998). Additionally, Roessler et al. (2001) reported that when anionic  $\beta$ -CyD was added to the complex of PAMAM dendrimer (G5) with pDNA, transfection efficiency was increased by lowering the particle size and polydispersity of the complex in the solution. Therefore, in order to evaluate the effect of the free GUG- $\beta$ -CyD, not a conjugate, on transfection efficiency of the pDNA complex with dendrimer, we examined gene transfer activity of the physical mixture of dendrimer and GUG- $\beta$ -CyD in A549 cells (Fig. 2). As shown in Fig. 2, gene transfer activity of physical mixture of dendrimer/GUG- $\beta$ -CyD was comparable to that of dendrimer alone, indicating that the addition of GUG- $\beta$ -CyD to dendrimer/pDNA complex does not affect gene transfer activity of dendrimer. Generally, CyDs are acknowledged not to entry cells due to hydrophilicity and high molecular weight (Uekama et al., 1998). Taken together, these results suggest that GUG- $\beta$ -CyD attached to the dendrimer molecule plays an important role for the high gene transfer activity of GUG- $\beta$ -CDE conjugate (DS 1.8) in cells, probably not cell surface, because the physical mixture of GUG- $\beta$ -CyD, a membrane-impermeable compound, and dendrimer could not enhance luciferase activity after transfection in A549 cells.



**Fig. 2.** Transfection efficiencies of pDNA complexes with dendrimer/GUG-β-CyD physical mixture and GUG-β-CDE conjugate (DS 1.8) at various charge ratios in A549 cells. Transfection was performed with culture medium without FCS for 3 h. The luciferase activity in cell lysates was determined 21 h after incubation. Culture medium was supplemented with 10% FCS. The molar ratio of dendrimer/GUG-β-CyD was 1:1. Each value represents the mean ± S.E. of 4 experiments. \* $p < 0.05$ , compared with dendrimer. † $p < 0.05$ , compared with dendrimer/GUG-β-CyD physical mixture.

### 3.2. Physicochemical properties of the pDNA complex with GUG-β-CDE conjugate (DS 1.8)

The physicochemical properties of the polyplex, such as net charge and particle size markedly affect the gene transfer activity. Therefore, to reveal the physicochemical properties of the pDNA complexes with GUG-β-CDE conjugate (DS 1.8), we compared its particle size and  $\zeta$ -potential with the pDNA complexes with  $\alpha$ -CDE conjugate (DS 1.2) and  $\beta$ -CDE conjugate (DS 1.3) at a charge ratio of 100. Here, we chose a charge ratio of 100, because all of the carriers showed the highest gene transfer activity at this charge ratio *in vitro*. As shown in Table 1, the particle size of the pDNA complex with GUG-β-CDE conjugate (DS 1.8) was about 190 nm, and the value was not significantly different from that of  $\alpha$ -CDE conjugate (DS 1.2) or  $\beta$ -CDE conjugate (DS 1.3). Meanwhile, the  $\zeta$ -potential value of the pDNA complex with GUG-β-CDE conjugate (DS 1.8) was also equivalent to those of the other carries. These results suggest that higher gene transfer activity of GUG-β-CDE (DS 1.8) than  $\alpha$ -CDE (DS 1.2) and  $\beta$ -CDE (DS 1.3) cannot be explained by the particle sizes and  $\zeta$ -potential values of the pDNA complexes, although the size, surface charge and compactness of the pDNA/vector complexes as well as their association and dissociation kinetics of the pDNA/vector complexes are important for gene delivery activity.

### 3.3. Cellular association of the pDNA complex with GUG-β-CDE conjugate (DS 1.8)

Early steps in the transfection process involve binding of the carriers to cell surface and its internalization inside the cell cytosol (Elouahabi and Ruyschaert, 2005). Therefore, we examined cellular association of Alexa-pDNA complexes with various carriers 1 h

after transfection in A549 cells and RAW264.7 cells (Fig. 3). The cellular association of Alexa-pDNA in the GUG-β-CDE (DS 1.8) system was almost comparable to that in the  $\alpha$ -CDE (DS 1.2) or the  $\beta$ -CDE (DS 1.8) system in A549 cells and RAW264.7 cells. These results suggest that higher gene transfer activity of GUG-β-CDE (DS 1.8) than that of  $\alpha$ -CDE (DS 1.2) and  $\beta$ -CDE (DS 1.3) cannot be explained by the cellular association of the pDNA complexes, although efficient binding of the carriers to cell surface and its internalization to the cell cytosol is the prerequisite for high gene transfer activity.

### 3.4. Effects of endocytosis inhibitors on gene transfer activity of GUG-β-CDE conjugate (DS 1.8)

Endocytosis has been proposed as the main pathways of internalization for polyplexes, although the exact nature of endocytic vesicles and the influence of serum and extracellular matrix components on the type of endocytosis involved are still, however, a matter of debate (Elouahabi and Ruyschaert, 2005). Additionally, the importance of the uptake step in the final transfection efficiency has been demonstrated using different lipoplexes, polyplexes, and cell lines (Elouahabi and Ruyschaert, 2005). In our preliminary study, we clarified that pDNA complex with  $\alpha$ -CDE conjugate was mainly entered cells *via* clathrin- or raft-dependent endocytosis pathways (unpublished data). Therefore, to reveal the cellular uptake pathway of pDNA complex with GUG-β-CDE conjugate (DS 1.8), we examined the effects of endocytosis inhibitors on gene transfer activity of GUG-β-CDE conjugate (DS 1.8) in A549 cells. We used CPZ, nystatin and M-β-CyD as clathrin-, raft-, and clathrin/raft-dependent endocytosis inhibitors, respectively. Herein, we confirmed that these inhibitors did not cause cytotoxicity under the present experimental conditions (data not shown). Additionally, we could not perform the experiments regarding the effects of the concentrations of endocytosis inhibitors on transfection efficiency of the pDNA complexes with carriers, because we used the maximum tolerated dose. As shown in Fig. 4, in A549 cells, the gene transfer activity of GUG-β-CDE conjugate (DS 1.8) was decreased to approximately 60%, 70% and 60% by the addition of CPZ, nystatin and M-β-CyD, respectively, in A549 cells. Additionally, the activities were almost comparable to those of  $\alpha$ -CDE conjugate (DS 1.2). Likewise, the gene transfer activity of GUG-β-CDE conjugate (DS 1.8) was decreased by the addition of these inhibitors in RAW264.7 cells, and the extent of decrease in the activity was equivalent to those of  $\alpha$ -CDE conjugate (DS 1.2). Meanwhile, amiloride did not decrease gene transfer activity of the carriers. These results suggest that the endocytosis pathway of pDNA complex with GUG-β-CDE conjugate (DS 1.8) may contribute to clathrin- and raft-dependent endocytosis, not macropinocytosis, as that of the pDNA complex with  $\alpha$ -CDE conjugate. Collectively, endocytosis pathway may not be associated with high gene transfer activity of GUG-β-CDE conjugate (DS 1.8).

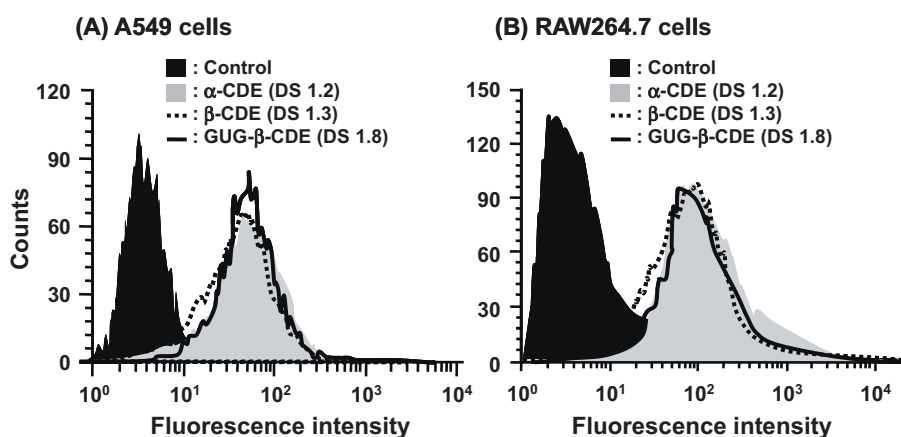
### 3.5. pH buffering capacity of GUG-β-CDE conjugate (DS 1.8)

In an effort to increase the efficiency of the endosomal escape step, cationic polymers with intrinsic endosomolytic activity, including dendrimers (Tang et al., 1996) and PEI (Kichler et al.,

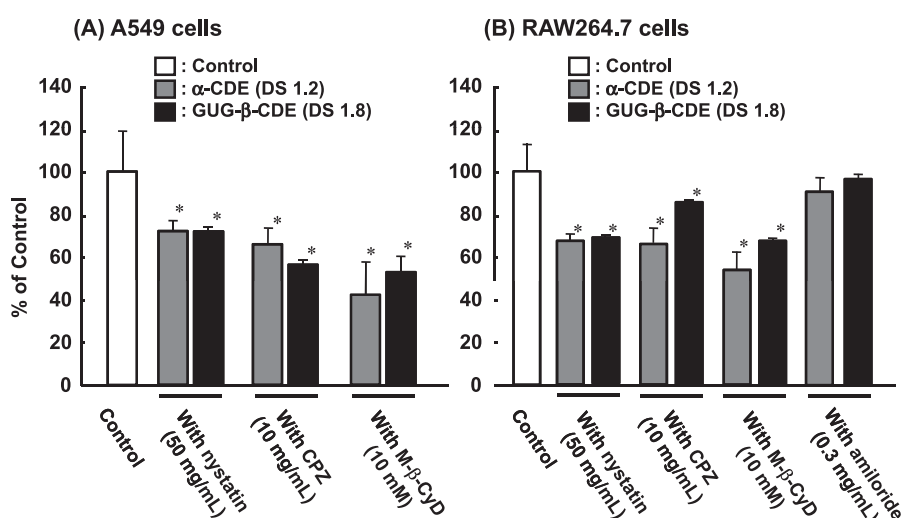
**Table 1**  
Particle sizes and  $\zeta$ -potentials of pDNA complexes with  $\alpha$ -CDE conjugate (DS 1.2),  $\beta$ -CDE conjugate (DS 1.3) and GUG-β-CDE conjugate (DS 1.8).

Carrier	Charge ratio (carrier/pDNA)	Mean diameter (nm)	Polydispersity index	$\zeta$ -Potential (mV)
$\alpha$ -CDE (DS 1.2)	100	187.3 ± 2.9	0.26 ± 0.02	27.2 ± 1.8
$\beta$ -CDE (DS 1.3)	100	178.1 ± 8.7	0.22 ± 0.01	28.1 ± 1.1
GUG-β-CDE (DS 1.8)	100	186.7 ± 1.7	0.23 ± 0.01	22.1 ± 0.6

The particle sizes and the  $\zeta$ -potentials were measured by Zetasizer Nano. The pDNA complexes with carriers were added to Tris-HCl buffer (10 mM, pH 7.4). The concentration of pDNA was 5  $\mu$ g/mL. Each value represents the mean ± S.E. of 3 experiments.



**Fig. 3.** Cellular association of Alexa-pDNA complexes with various carriers into A549 cells (A) and RAW264.7 cells (B). The fluorescence intensities of Alexa-pDNA in cells 1 h after incubation at 37 °C were determined by a flow cytometer. The amount of Alexa-pDNA was 2  $\mu$ g. The charge ratio of carriers/pDNA was 100.

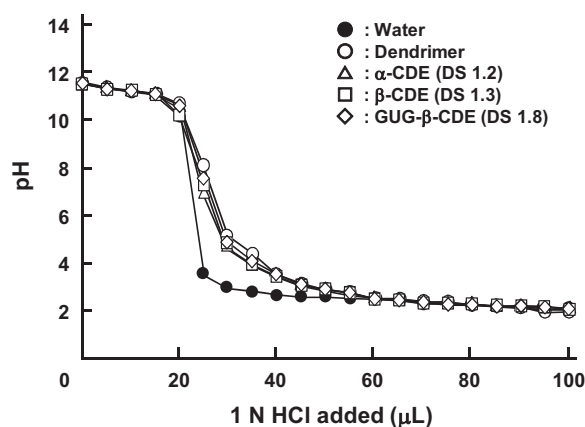


**Fig. 4.** Transfection efficiencies of pDNA complexes with GUG- $\beta$ -CDE conjugate (DS 1.8) and  $\alpha$ -CDE conjugate (DS 1.2) in the absence and presence of endocytosis inhibitors in A549 cells (A) and RAW264.7 cells (B). Cells were pretreated with the inhibitors for 30 min, and then transfected with GUG- $\beta$ -CDE conjugate (DS 1.8)/pDNA complex for 24 h. The percent of luciferase activity without inhibitors is 100%. The amount of pDNA was 2.0  $\mu$ g. The charge ratio was 100. Each value represents the mean  $\pm$  S.E. of 4 experiments. \* $p$  < 0.05, compared with control.

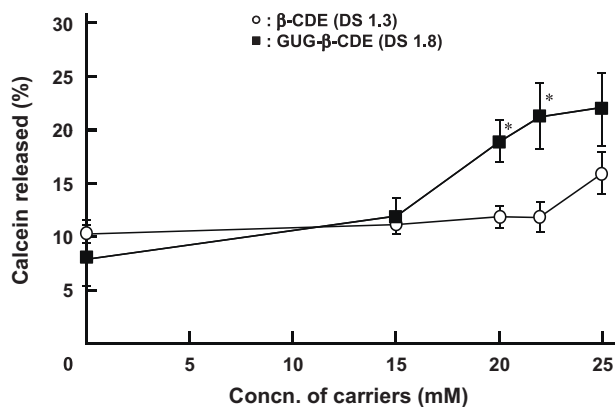
2001), have been developed. Dendrimer has a large number of secondary and tertiary amines, so exhibits buffering capacity. This buffering capacity as known to be the proton sponge effect is essential for swelling of endocytic vesicles, escape into the cytoplasm and overall gene delivery efficiency (Boussif et al., 1995). Therefore, to investigate buffering capacity of GUG- $\beta$ -CDE conjugate (DS 1.8), we titrated a solution containing GUG- $\beta$ -CDE conjugate (DS 1.8) from pH 11.5 to 2.0 with the addition of HCl. As shown in Fig. 5, the buffering capacity of GUG- $\beta$ -CDE conjugate (DS 1.8) was not different from those of the other carriers. These results suggest that an introduction of GUG- $\beta$ -CyD to the dendrimer molecule does not affect the buffering capacity of dendrimer, i.e. GUG- $\beta$ -CDE conjugate (DS 1.8) may enough maintain the proton sponge effect of dendrimer because of low DS value.

### 3.6. Liposome disruptive ability of GUG- $\beta$ -CDE conjugate (DS 1.8)

CyDs are known to induce hemolysis and to enhance the permeation of water soluble drugs through biological membranes of epithelial cells via the release of membrane components such as phospholipids and cholesterol as described above (Irie and Uekama,



**Fig. 5.** Titration curves of solutions containing various carriers (0.1 mM) with 1 N HCl. Carriers were dissolved in 5 mL of water. The solutions were firstly adjusted to pH 11.5 with 1 N NaOH. Next, 5  $\mu$ L aliquots of 1 N HCl were added to solutions, and the pH was recorded. Each point represents the mean  $\pm$  S.E. of 3 experiments.



**Fig. 6.** Membrane-disruptive activity of  $\beta$ -CDE conjugate (DS 1.3) and GUG- $\beta$ -CDE conjugate (DS 1.8) to liposomes. The solutions containing carriers at various concentrations were added to the suspension containing liposomes encapsulated calcein and mixed for 30 min at room temperature. The fluorescence intensity of liposomal suspension was measured with a fluorescence photometer. Each value represents the mean  $\pm$  S.E. of 3–9 experiments. \* $p < 0.05$ , compared with  $\beta$ -CDE conjugate (DS 1.3).

1997, 1999; Marttin et al., 1998). In addition, we reported the possibility that the enhancing mechanism by which  $\alpha$ -CDEs augment the release of pDNA from endosomes to cytoplasm after cellular uptakes (Arima et al., 2001; Kihara et al., 2002, 2003). To confirm the membrane disruptive ability of GUG- $\beta$ -CDE conjugate (DS 1.8), we examined the effects of GUG- $\beta$ -CDE conjugate (DS 1.8) on the release of calcein from liposomes (Fig. 6). GUG- $\beta$ -CDE conjugate (DS 1.8) significantly released calcein from liposomes compared to  $\beta$ -CDE conjugate (DS 1.3), suggesting that GUG- $\beta$ -CDE conjugate (DS 1.8) possesses higher membrane-disruptive ability than  $\beta$ -CDE conjugate (DS 1.3). As shown in Fig. 2, the physical mixture of GUG- $\beta$ -CyD and dendrimer did not increase gene transfer activity, compared to dendrimer. Therefore, GUG- $\beta$ -CDE conjugate (DS 1.8) may strongly interact with endosomal membrane, leading to disruption of endosomal membranes after transfection, compared to other carriers. Since GUG- $\beta$ -CDE conjugate (DS 1.8) actually has the GUG moiety as a spacer between  $\beta$ -CyD and dendrimer molecule, this spacer may enhance the interaction of this carrier with endosomal membranes in cells.

### 3.7. Intracellular distribution of pDNA complex with GUG- $\beta$ -CDE conjugate (DS 1.8)

To examine intracellular behavior of pDNA complexes, we observed the fluorescence in A549 cells after transfection of pDNA complexes with TRITC-carriers using a fluorescence microscope (Fig. 7). Herein, we observed the fluorescence 6 h after transfection, because the  $\alpha$ -CDE conjugate/pDNA complex was found to enter nucleus as of 6 h after transfection in our preliminary study. In all of the systems, the fluorescence derived from TRITC-carriers was observed in cytoplasm, suggesting the effective endosomal escape of the pDNA complexes after cellular uptake. However, the difference of the fluorescence intensity in cytoplasm among these carriers was not determined, because a quantitative evaluation was not carried out under the present experimental conditions. Therefore, to clarify the subtle difference in the intracellular behaviors between these pDNA complexes, the elaborate studies by a quantitative and diachronic analysis should be necessary.

Next, we further examined whether high gene transfer activity of GUG- $\beta$ -CDE conjugate (DS 1.8) is attributed to its endosomal escape by using LysoTracker<sup>®</sup>, which is a marker for late endosomes/lysosomes with red fluorescence in A549 cells (Fig. 8). In the images obtained, Alexa-pDNA and LysoTracker<sup>®</sup> were shown

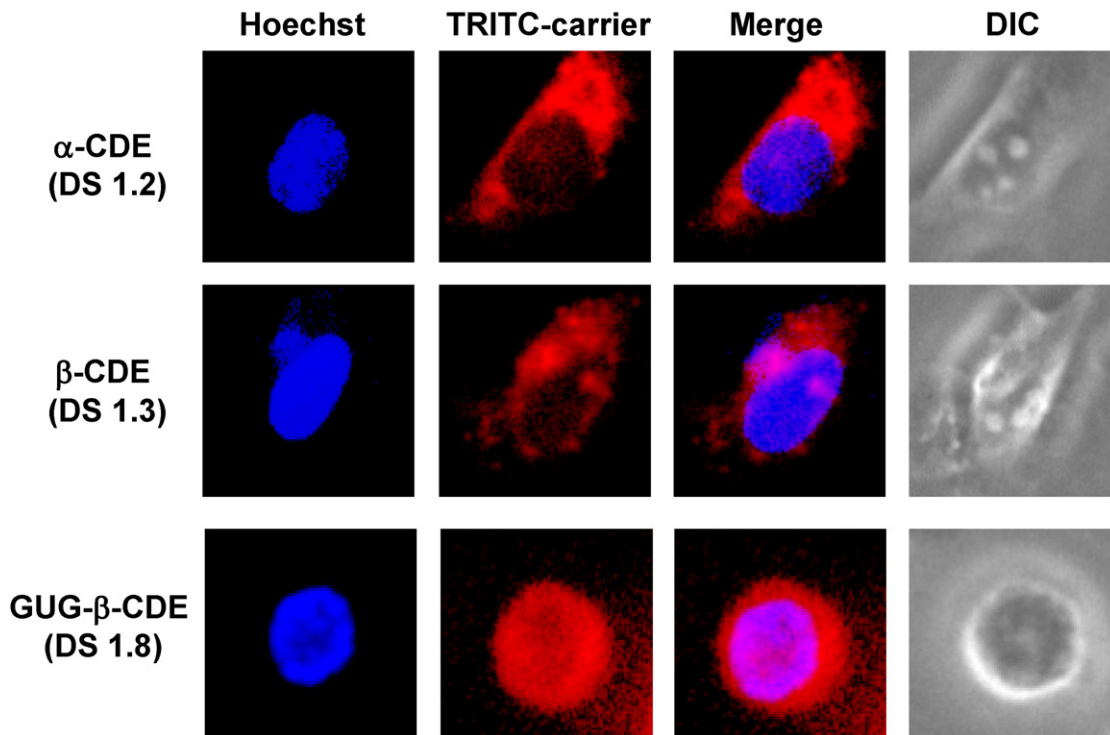
in green and red, respectively, and the merge of green and red pixels was observed in yellow. As shown in Fig. 8, the fluorescence derived from Alexa-pDNA in the three carrier systems was almost comparable in A549 cells, but the yellow pixels were decreased in the order of the  $\beta$ -CDE conjugate (DS 1.3) system > the  $\alpha$ -CDE conjugate (DS 1.2) system > the GUG- $\beta$ -CDE conjugate (DS 1.8) system. These results suggest the superior endosomal escaping ability of pDNA/GUG- $\beta$ -CDE conjugate (DS 1.8) complex to that of the pDNA/ $\beta$ -CDE conjugate (DS 1.3) and the pDNA/ $\alpha$ -CDE conjugate (DS 1.2) complexes. We previously reported that gene transfer activity decreased in the order of GUG- $\beta$ -CDE conjugate (DS 1.8) >  $\alpha$ -CDE conjugate (DS 1.2) >  $\beta$ -CDE conjugate (DS 1.3) (Anno et al., 2011), corresponding to the order of the results shown in Fig. 8. Collectively, high gene transfer activity of GUG- $\beta$ -CDE conjugate (DS 1.8) may be, at least in part, attributed to its high endosomal escape ability.

Once released into the cytoplasm, the pDNA transfected has to move into the nucleus in order to be transcribed. Several studies have established, indeed, that among cells showing cytoplasmic pDNA delivery, only those with evidence of nuclear pDNA localization resulted in efficient transgene expression. As shown in Fig. 7, the fluorescence of TRITC-GUG- $\beta$ -CDE conjugate (DS 1.8) after transfection of its pDNA complex was strongly observed in the nucleus, compared with those of other carriers. The mechanism for the nuclear localization of GUG- $\beta$ -CDE conjugate (DS 1.8) is so far not clear. Here, some lectins are known to exist in the nuclear membrane, and they can recognize sugars such as glucose or galactose (Monsigny et al., 2004). In addition, the carbohydrate-recognition domain (CRD) of the lectins possibly recognizes 2-, 4-hydroxyl group of sugars (Feinberg et al., 2000), so we hypothesized that GUG- $\beta$ -CDE conjugate having 2-, 4-hydroxyl groups in the spacer moiety may be recognized by the nuclear lectins. These results suggest that the nuclear localization ability of pDNA complex with GUG- $\beta$ -CDE conjugate (DS 1.8) may contribute to its high gene transfer activity, although thereafter we should perform the experiments using a confocal microscopy to determine the distribution and colocalization of TRITC-GUG- $\beta$ -CDE conjugate and Alexa-pDNA in detail and reveal its nuclear localization mechanism.

### 3.8. pDNA compaction and decompaction ability of GUG- $\beta$ -CDE conjugate (DS 1.8)

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 (Schwartz et al., 1999; Thomas and Klivanov, 2002). To examine the effects of various carriers on pDNA condensation, fluorescence intensity of Picogreen<sup>®</sup> dsDNA reagent was determined (Fig. 9). The relative fluorescence intensity was decreased to 20% in the dendrimer,  $\alpha$ -CDE conjugate (DS 1.2) and  $\beta$ -CDE conjugate (DS 1.3) systems at a charge ratio of 100. Meanwhile, the relative fluorescence intensity was increased in the GUG- $\beta$ -CDE conjugate (DS 1.8) system, compared with other carrier systems, suggesting that the compaction ability of GUG- $\beta$ -CDE conjugate (DS 1.8) to pDNA was lower than that of dendrimer,  $\alpha$ -CDE conjugate (DS 1.2) and  $\beta$ -CDE conjugate (DS 1.3). This low compaction ability of GUG- $\beta$ -CDE conjugate (DS 1.8) may be due to a steric hindrance by introduction of GUG- $\beta$ -CyD to the dendrimer molecule. Here, the release of pDNA from complexes of cationic polymer, such as PEI, in cellular nucleus is known to be crucial for higher gene transfer activity (Godbey et al., 1999). Therefore, it is likely that low compaction ability of GUG- $\beta$ -CDE conjugate (DS 1.8) may accelerate the release of pDNA from the complex in cells.

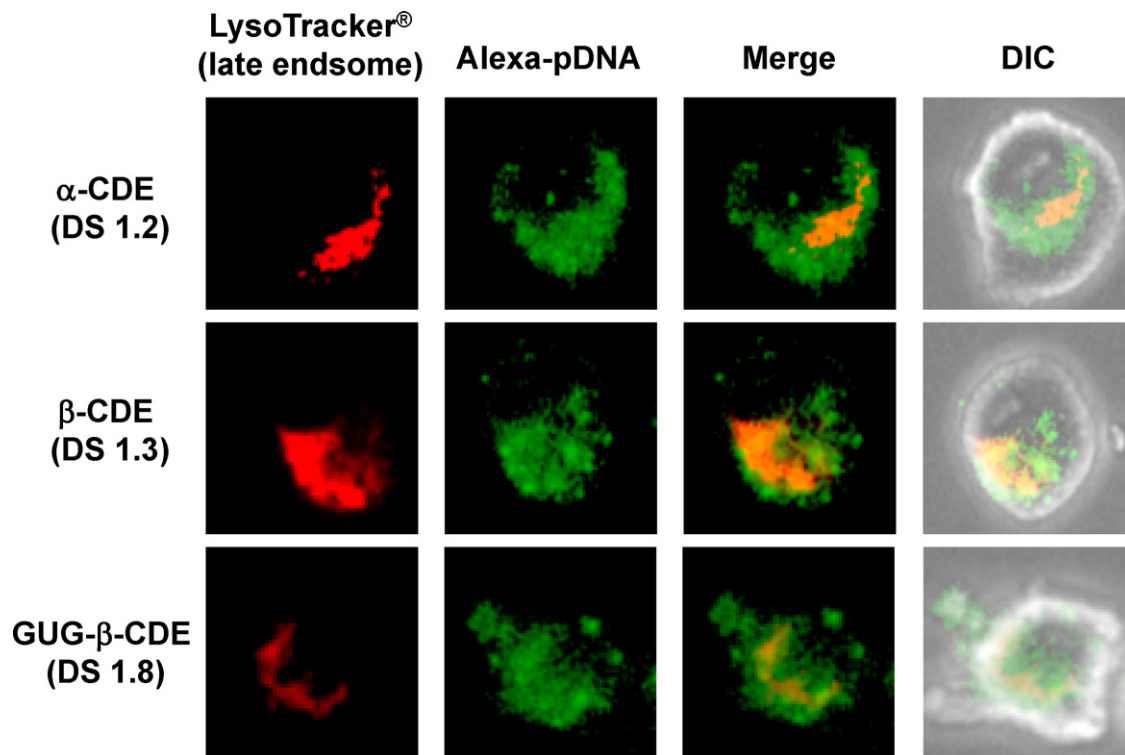
To next examine the decompaction ability of pDNA from polyplexes, fluorescence intensity of Picogreen<sup>®</sup> dsDNA reagent was determined in the presence of heparin sulfate, a competitor of



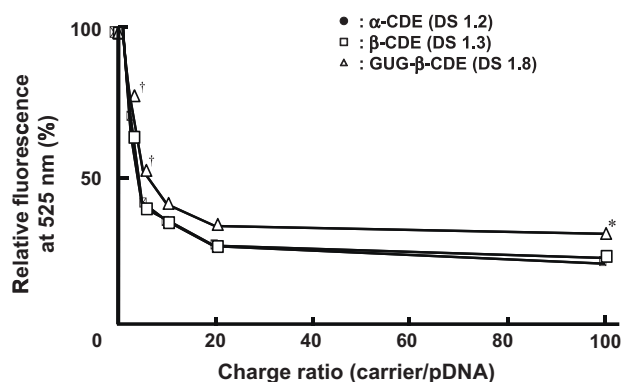
**Fig. 7.** Intracellular distribution of pDNA complexes with various TRITC-carriers in A549 cells. The cells were transfected with TRITC-carriers/pDNA complexes for 6 h. After washing twice with DMEM, the cells were observed with a fluorescence microscope. The amount of pDNA was 2.0  $\mu$ g. The charge ratio of carriers/pDNA was 100.

pDNA (Fig. 10). In the  $\alpha$ -CDE conjugate (DS 1.2) and  $\beta$ -CDE conjugate (DS 1.3) systems, the relative fluorescence intensities were mostly equivalent to that in the dendrimer system. Meanwhile, the relative fluorescence intensity was increased in the GUG- $\beta$ -CDE

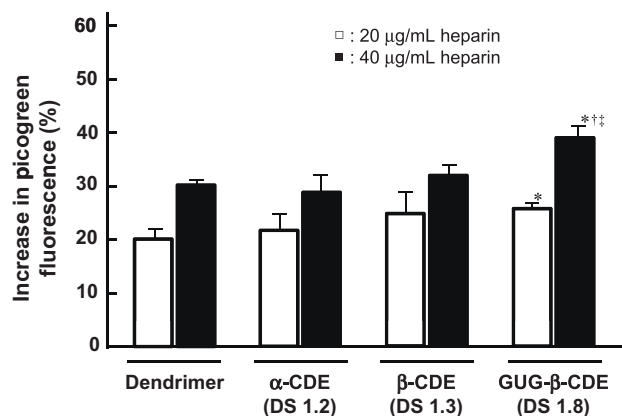
conjugate (DS 1.8) system, compared with the other carrier systems. Obviously, these results seem to be consistent with those shown in Fig. 9, which suggests that the decompaction ability of GUG- $\beta$ -CDE conjugate (DS 1.8) to pDNA was higher than that of



**Fig. 8.** Intracellular distribution of Alexa-pDNA complexes with various carriers in A549 cells. Cells were transfected with carriers/Alexa-pDNA complexes for 6 h. After cells were treated with LysoTracker<sup>®</sup> for 1 h, cells were washed with PBS twice, and then observed with a fluorescence microscope. The amount of Alexa-pDNA was 2.0  $\mu$ g. The charge ratio was 100.



**Fig. 9.** Condensed states of pDNA complexes with various carriers. pDNA (0.5  $\mu$ g), Picogreen<sup>®</sup> dsDNA reagent and carriers were added to HBSS (pH 7.4) at various charge ratios. The solutions were incubated for 15 min at 25 °C, and then the fluorescence ( $\lambda_{ex}$  = 495 nm,  $\lambda_{em}$  = 525 nm) was measured by a fluorescence photometer. Each value represents the mean  $\pm$  S.E. of 3 experiments. \* $p$  < 0.05, compared with  $\alpha$ -CDE conjugate (DS 1.2). † $p$  < 0.05, compared with  $\beta$ -CDE conjugate (DS 1.3).



**Fig. 10.** Competitive effect of heparin on pDNA dissociation from pDNA complexes with various carriers. Picogreen<sup>®</sup> dsDNA reagent was added to HBSS (pH 7.4) containing pDNA. After the solutions were incubated at 25 °C for 5 min, various carriers were added to the solution at a charge ratio of 100. After the solutions were incubated at 25 °C for 15 min, heparin solution (20 or 40  $\mu$ g/mL) was added to the solution, and then a fluorescence ( $\lambda_{ex}$  = 495 nm,  $\lambda_{em}$  = 525 nm) was measured by a fluorophotometer. Each value represents the mean  $\pm$  S.E. of 3 experiments. \* $p$  < 0.05, compared with dendrimer. † $p$  < 0.05, compared with  $\alpha$ -CDE conjugate (DS 1.2). ‡ $p$  < 0.05, compared with  $\beta$ -CDE conjugate (DS 1.3).

dendrimer,  $\alpha$ -CDE conjugate (DS 1.2) and  $\beta$ -CDE conjugate (DS 1.3). Taken together, the high decompaction ability of GUG- $\beta$ -CDE conjugate (DS 1.8) may result in appropriate release of pDNA from the polyplex in cells, and then may provide the high gene transfer activity.

#### 4. Conclusions

GUG- $\beta$ -CDE conjugate (DS 1.8) provided excellent gene transfer activity because of its efficient endosomal escaping ability, high nuclear localization and/or appropriate release of pDNA from the polyplex. Additionally, we demonstrated that pDNA complex with GUG- $\beta$ -CDE conjugate (DS 1.8) provides negligible cytotoxicity after transfection in A549 cells and RAW264.7 cells (Anno et al., 2011). Although further investigation is necessary to clarify the enhancing mechanisms of GUG- $\beta$ -CDE conjugate (DS 1.8) on gene transfer efficiency, these data may be useful for design of GUG- $\beta$ -CyD conjugates with other non-viral vectors.

#### Acknowledgment

We thank Ensuiko Sugar Refining for supply of GUG- $\beta$ -CyD.

#### References

- Abdou, S., Collomb, J., Sallas, F., Marsura, A., Finance, C., 1997. beta-Cyclodextrin derivatives as carriers to enhance the antiviral activity of an antisense oligonucleotide directed toward a coronavirus intergenic consensus sequence. *Arch. Virol.* 142, 1585–1602.
- Anno, T., Higashi, T., Motoyama, K., Hirayama, F., Uekama, K., Arima, H., 2012. Potential use of glucuronylglucosyl- $\beta$ -cyclodextrin/dendrimer conjugate (G2) as a DNA carrier in vitro and in vivo. *J. Drug Target.*, in press.
- Anno, T., Motoyama, K., Higashi, T., Hirayama, F., Uekama, K., Arima, H., 2011. Preparation and evaluation of polyamidoamine dendrimer (G2)/branched- $\beta$ -cyclodextrin conjugate as a novel gene transfer carrier. *J. Incl. Phenom. Macrocycl. Chem.* 70, 339–344.
- Arima, H., Chihara, Y., Arizono, M., Yamashita, S., Wada, K., Hirayama, F., Uekama, K., 2006. Enhancement of gene transfer activity mediated by mannosylated dendrimer/ $\alpha$ -cyclodextrin conjugate (generation 3, G3). *J. Control. Release* 116, 64–74.
- Arima, H., Kihara, F., Hirayama, F., Uekama, K., 2001. Enhancement of gene expression by polyamidoamine dendrimer conjugates with  $\alpha$ -,  $\beta$ -, and  $\gamma$ -cyclodextrins. *Bioconjug. Chem.* 12, 476–484.
- Arima, H., Yamashita, S., Mori, Y., Hayashi, Y., Motoyama, K., Hattori, K., Takeuchi, T., Jono, H., Ando, Y., Hirayama, F., Uekama, K., 2010. In vitro and in vivo gene delivery mediated by lactosylated dendrimer/ $\alpha$ -cyclodextrin conjugates (G2) into hepatocytes. *J. Control. Release* 146, 106–117.
- Bangham, A.D., Standish, M.M., Watkins, J.C., 1965. Diffusion of univalent ions across the lamellae of swollen phospholipids. *J. Mol. Biol.* 13, 238–252.
- Belloq, N.C., Pun, S.H., Jensen, G.S., Davis, M.E., 2003. Transferrin-containing, cyclodextrin polymer-based particles for tumor-targeted gene delivery. *Bioconjug. Chem.* 14, 1122–1132.
- Bielinska, A., Kukowska-Latallo, J.F., Johnson, J., Tomalia, D.A., Baker Jr., J.R., 1996. Regulation of in vitro gene expression using antisense oligonucleotides or antisense expression plasmids transfected using starburst PAMAM dendrimers. *Nucleic Acids Res.* 24, 2176–2182.
- Boussif, O., Lezoualc'h, F., Zanta, M.A., Mergny, M.D., Scherman, D., Demeneix, B., Behr, J.P., 1995. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. *Proc. Natl. Acad. Sci. U.S.A.* 92, 7297–7301.
- Croyle, M.A., Roessler, B.J., Hsu, C.P., Sun, R., Amidon, G.L., 1998. Beta cyclodextrins enhance adenoviral-mediated gene delivery to the intestine. *Pharm. Res.* 15, 1348–1355.
- De Smedt, S.C., Demeester, J., Hennink, W.E., 2000. Cationic polymer based gene delivery systems. *Pharm. Res.* 17, 113–126.
- Elouahabi, A., Ruyschaert, J.M., 2005. Formation and intracellular trafficking of lipoplexes and polyplexes. *Mol. Ther.* 11, 336–347.
- Feinberg, H., Park-Snyder, S., Kolatkar, A.R., Heise, C.T., Taylor, M.E., Weis, W.I., 2000. Structure of a C-type carbohydrate recognition domain from the macrophage mannose receptor. *J. Biol. Chem.* 275, 21539–21548.
- Freeman, D.J., Niven, R.W., 1996. The influence of sodium glycocholate and other additives on the in vivo transfection of plasmid DNA in the lungs. *Pharm. Res.* 13, 202–209.
- Godbey, W.T., Wu, K.K., Mikos, A.G., 1999. Tracking the intracellular path of poly(ethylenimine)/DNA complexes for gene delivery. *Proc. Natl. Acad. Sci. U.S.A.* 96, 5177–5181.
- Huang, H., Tang, G., Wang, Q., Li, D., Shen, F., Zhou, J., Yu, H., 2006. Two novel non-viral gene delivery vectors: low molecular weight polyethylenimine cross-linked by (2-hydroxypropyl)- $\beta$ -cyclodextrin or (2-hydroxypropyl)- $\gamma$ -cyclodextrin. *Chem. Commun. (Camb.)* 14, 2382–2384.
- Irie, T., Uekama, K., 1997. Pharmaceutical applications of cyclodextrins. III. Toxicological issues and safety evaluation. *J. Pharm. Sci.* 86, 147–162.
- Irie, T., Uekama, K., 1999. Cyclodextrins in peptide and protein delivery. *Adv. Drug Deliv. Rev.* 36, 101–123.
- Kichler, A., Leborgne, C., Coeytaux, E., Danos, O., 2001. Polyethylenimine-mediated gene delivery: a mechanistic study. *J. Gene Med.* 3, 135–144.
- Kihara, F., Arima, H., Tsutsumi, T., Hirayama, F., Uekama, K., 2002. Effects of structure of polyamidoamine dendrimer on gene transfer efficiency of the dendrimer conjugate with  $\alpha$ -cyclodextrin. *Bioconjug. Chem.* 13, 1211–1219.
- Kihara, F., Arima, H., Tsutsumi, T., Hirayama, F., Uekama, K., 2003. In vitro and in vivo gene transfer by an optimized  $\alpha$ -cyclodextrin conjugate with polyamidoamine dendrimer. *Bioconjug. Chem.* 14, 342–350.
- Kukowska-Latallo, J.F., Bielinska, A.U., Johnson, J., Spindler, R., Tomalia, D.A., Baker Jr., J.R., 1996. Efficient transfer of genetic material into mammalian cells using Starburst polyamidoamine dendrimers. *Proc. Natl. Acad. Sci. U.S.A.* 93, 4897–4902.
- Martini, E., Verfoef, J.C., Merkus, F.W., 1998. Efficacy, safety and mechanism of cyclodextrins as absorption enhancers in nasal delivery of peptide and protein drugs. *J. Drug Target.* 6, 17–36.
- Monsigny, M., Rondanino, C., Duverger, E., Fajac, I., Roche, A.C., 2004. Glyco-dependent import of glycoproteins, glycoconjugates and glycosylated plasmids. *Biochim. Biophys. Acta* 1673, 94–103.



- Qin, L., Pahud, D.R., Ding, Y., Bielinska, A.U., Kukowska-Latallo, J.F., Baker Jr., J.R., Bromberg, J.S., 1998. Efficient transfer of genes into murine cardiac grafts by Starburst polyamidoamine dendrimers. *Hum. Gene Ther.* 9, 553–560.
- Roessler, B.J., Bielinska, A.U., Janczak, K., Lee, I., Baker Jr., J.R., 2001. Substituted  $\beta$ -cyclodextrins interact with PAMAM dendrimer-DNA complexes and modify transfection efficiency. *Biochem. Biophys. Res. Commun.* 283, 124–129.
- Schwartz, B., Ivanov, M.A., Pitard, B., Escriou, V., Rangara, R., Byk, G., Wils, P., Crouzet, J., Scherman, D., 1999. Synthetic DNA-compacting peptides derived from human sequence enhance cationic lipid-mediated gene transfer in vitro and in vivo. *Gene Ther.* 6, 282–292.
- Tang, M.X., Redemann, C.T., Szoka Jr., F.C., 1996. In vitro gene delivery by degraded polyamidoamine dendrimers. *Bioconjug. Chem.* 7, 703–714.
- Thomas, M., Klibanov, A.M., 2002. Enhancing polyethylenimine's delivery of plasmid DNA into mammalian cells. *Proc. Natl. Acad. Sci. U.S.A.* 99, 14640–14645.
- Tomalia, D.A., Naylor, A.M., Goddard, W.A., 1990. Starburst dendrimers: molecular-level control of size, shape, surface chemistry, topology, and flexibility from atoms to macroscopic matter. *Angew. Chem. Int. Ed.* 29, 138–175.
- Uekama, K., Hirayama, F., Irie, T., 1998. Cyclodextrin drug carrier systems. *Chem. Rev.* 98, 2045–2076.
- Wada, K., Arima, H., Tsutsumi, T., Chihara, Y., Hattori, K., Hirayama, F., Uekama, K., 2005a. Improvement of gene delivery mediated by mannosylated dendrimer/ $\alpha$ -cyclodextrin conjugates. *J. Control. Release* 104, 397–413.
- Wada, K., Arima, H., Tsutsumi, T., Hirayama, F., Uekama, K., 2005b. Enhancing effects of galactosylated dendrimer/ $\alpha$ -cyclodextrin conjugates on gene transfer efficiency. *Biol. Pharm. Bull.* 28, 500–505.
- Zhao, Q., Tamsamani, J., Agrawal, S., 1995. Use of cyclodextrin and its derivatives as carriers for oligonucleotide delivery. *Antisense Res. Dev.* 5, 185–192.