

farther in the interior of the HuH-7 spheroids (Figure 7b) than that of the FITC-labeled lipoplexes after 48 h of incubation (Figure 7b). This result suggests that the small size of approximately 100 nm and the nonionic and hydrophilic PEG shell of the PEGylated polyplexes may reduce the nonspecific interaction of the micelles with the cell membrane and/or ECM, allowing their smooth penetration into the HuH-7 spheroids.<sup>[18]</sup>

#### Detection of apoptosis in the HuH-7 spheroids

To confirm whether or not the observed growth-inhibitory effect of the PEGylated polyplexes on the HuH-7 spheroids is due to the induction of cell death, a Live/Dead staining assay was carried out for the HuH-7 spheroids treated with mock, lipoplex (100 nm), and lac-PEGylated polyplex (50 nm), respectively. By means of confocal fluorescence-scanning microscopy, living and dead cells in the HuH-7 spheroids were individually detected as green fluorescence and red fluorescence, respectively. As can be seen in Figure 8, cell death at the center of the mock-treated HuH-7 spheroid was clearly observed on day 7, whereas living cells were only observed at the periphery of the spheroid. The observed cell death in the mock-treated sample was apparently due to necrosis resulting from the insufficient supply of oxygen and nutriment to the spheroid interior, deficiencies which become significant with increasing spheroid size.<sup>[11]</sup> Note that the spheroids treated with the lipoplexes showed some zones at the periphery containing dead cells on day two, yet after ten days the distribution of dead and living cells was the same as in the mock-treated samples.

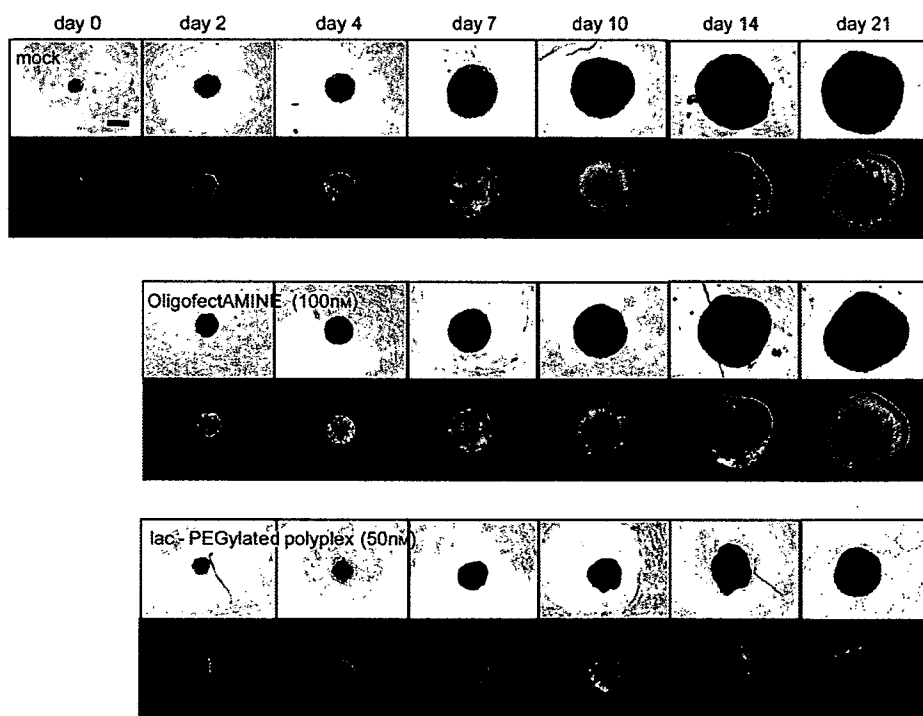
In contrast, the spheroids treated with lac-PEGylated polyplexes showed continuous cell death at the periphery even after day ten, resulting in the significant death of cells in the spheroids.

In addition to the Live/Dead staining assay, we performed the detection of activated caspase-3 in the HuH-7 spheroids to confirm the induction of apoptosis. The activation of caspase-3 is known to play a central role in the induction of the apoptosis;<sup>[19]</sup> therefore, caspase-3 is an appropriate marker for measuring apoptosis induced by RecQL1 siRNA. Activated caspase-3 was detected using Magic Red fluorescence probe (MR-(DEVD)<sub>2</sub>). In the presence of activated caspase-3, the DEVD amino-acid sequence in the MR-(DEVD)<sub>2</sub> is cleaved to generate a red fluorescence.<sup>[20]</sup> As can be seen in Figure 9, apoptotic cells (fluorescence signals) were not observed in the mock-treated spheroids. In contrast, apoptotic cells (fluorescent signals) were observed at the periphery of the spheroids treated with the lipoplex as early as day one. Nevertheless, there was no sign of apoptosis after seven days. It is worth noting that the apoptotic cells were also observed at the periphery of spheroids treated with the lac-PEGylated polyplex, and the number of apoptotic cells increased with prolonged incubation time, from day four to day ten. Note that apoptotic cells were still observed even after ten days. Other spheroids treated with the lac-PEGylated polyplex also showed a similar tendency. These results strongly suggest that the observed growth-inhibitory effect and cell death in the spheroids treated with PEGylated polyplexes are likely to be due to apoptosis induced by RecQL1 siRNA in the long term. In addition, the significant difference in the observation period for apoptotic cells between the PEGylated polyplexes and the lipoplexes may be ascribed to the lower tolerance of the lipoplexes for the culture environment than the PEGylated polyplexes. The appreciable stability under physiological conditions and the uniform size of 100 nm of the PEGylated polyplexes may contribute to their smooth penetration into the spheroids, eventually facilitating the RNAi effect through the continuous uptake into the tumor cells located in the interior and the periphery of the spheroids.

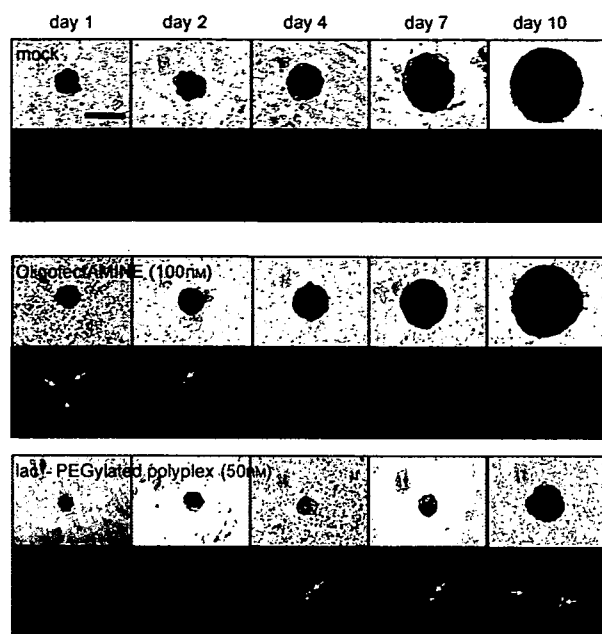
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#### Conclusions

In conclusion, we have demonstrated that MCTSs are useful for evaluating the long-term (up to 21 days) efficacy of siRNA delivery systems (therapeutic value). Note that MCTSs are a versatile *in vitro* model which can be



**Figure 8.** Live/Dead staining assay of the HuH-7 spheroids treated with mock, OligofectAMINE ([siRNA] = 100 nm) and lac-PEGylated polyplexes ([conjugate] = 50 nm) (bar = 100  $\mu$ m). Living and dead cells emit green and red fluorescences, respectively.



**Figure 9.** Detection of the activated caspase-3 as the signal of apoptosis in the HuH-7 spheroids treated with mock, OligofectAMINE ([siRNA] = 100 nM), and lac-PEGylated polyplexes ([conjugate] = 50 nM) (bar = 200 μm).

used to estimate the penetration of carriers into 3D tumor tissues and the effect of the ECM and cell-cell contacts (microenvironment) that could otherwise only be examined *in vivo* using animal models. Indeed, the lac-PEGylated polyplexes composed of the Lac-PEG-siRNA conjugate and PLL showed a remarkable growth-inhibitory effect ( $IC_{50} = 6$  nM) on the HuH-7 spheroids, inducing long-term apoptotic cell death by means of RecQL1 siRNA. Several important factors are likely to be synergistically involved in the pronounced growth-inhibitory effect of the PEGylated polyplexes, such as the improvement of the stability against enzymatic degradation, smooth penetration into the spheroid interior, and enhancement of the cellular uptake through ASGP receptor-mediated endocytosis. In sharp contrast, the lipoplexes showed almost no growth-inhibitory effect even at a siRNA concentration 100 nM in the HuH-7 spheroids, presumably due to the poor penetration into the spheroids. Furthermore, spheroids derived directly from patients' tumor tissues offer the opportunity of studying the efficacy of delivery systems in the unique tumor cell microenvironments characteristic to individual patients. Thus, MCTSs, as an *in vitro* tumor model, are expected to be useful in the assessment of the usefulness of siRNA carrier systems in tumor targeting.

## Experimental Section

**Materials:** PLLs (degree of polymerization (DP) = 40,  $M_w = 8300$ ; DP = 100,  $M_w = 20900$ ; DP = 460,  $M_w = 75900$ ) were purchased from Sigma. OligofectAMINE and LipofectAMINE were purchased from Invitrogen. 5'-Thiol-modified sense RNAs (HS-(CH<sub>2</sub>)<sub>6</sub>-CUU ACG

CUG AGU ACU UCG AdTdT-3', firefly luciferase, pGL3-control sense sequence<sup>[1]</sup> and HS-(CH<sub>2</sub>)<sub>6</sub>-GUU CAG ACC ACU UCA GCU UdTdT-3', RecQL1, sense sequence<sup>[10]</sup>) and unmodified antisense RNA (5'-UCG AAG UAC UCA GCG UAA GdTdT-3', firefly luciferase, pGL3-control antisense sequence and 5'-AAG CUG AAG UGG UCU GAA CdTdT-3', RecQL1, antisense sequence) were purchased from Dharmacon. Water was purified using a Milli-Q instrument (MILLIPORE). Plasmid DNAs (pDNA) encoding firefly luciferase (pGL3-Control, Promega; 5256 bp) and renilla luciferase (pRL-TK, Promega; 4045 bp) were amplified using EndoFree Plasmid Maxi or Mega Kits (QIAGEN). The DNA concentration was determined by reading the absorbance at 260 nm. A spheroid culture plate, Celltight Spheroid Culture Plate, was purchased from Sumitomo Bakelite. MTT assay reagents and a double staining kit (Live/Dead assay) were purchased from DOJINDO. A Magic Red Caspase Detection Kit was purchased from Immunochemistry Technologies.

**Preparation of the PEGylated polyplexes:** The PEG-siRNA conjugates were prepared as described in the previous report.<sup>[5]</sup> Specific amounts of the PEG-siRNA conjugate (50 μM) and PLL were separately added to 10 mM Tris-HCl buffer (pH 7.4) to prepare the 10 μM stock solutions. The solutions were filtered through a 0.1 μm filter to remove the dust. The PEG-siRNA conjugate in 10 mM Tris-HCl buffer (pH 7.4) was mixed with PLL stock solution in 10 mM Tris-HCl buffer (pH 7.4) at an equal unit molar ratio of phosphate groups in the PEG-siRNA conjugate to amino groups in PLL (N/P = 1), followed by the addition of 10 mM Tris-HCl buffer (pH 7.4), adding 0.3 M NaCl to adjust the ionic strength of the solution to the physiological condition (0.15 M NaCl).

**Cell culture:** HuH-7 human cancer cells, derived from a hepatocarcinoma cell line, were obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging, and Cancer, Tohoku University. The monolayer-cultured cells and multicellular spheroids were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg mL<sup>-1</sup> streptomycin at 37°C in a humidified 5% CO<sub>2</sub> atmosphere.

**MTT assay:** HuH-7 cells were plated in a 96-well plate (10<sup>4</sup> cells/well) to allow them to reach about 50% confluence after 24 h and the medium was then changed to fresh DMEM with 10% FBS (180 μL/well). To each well, appropriate amounts of sample were added in 20 μL aliquot. After 48 h of incubation, fresh medium (100 μL/well) was added, and further incubation was carried out for 48 h. The metabolic activity of each well was determined by an MTT assay. The optical absorbance was measured at 560 nm using a microplate reader and converted to the percentage relative to that for mock cells (buffer-treated cells).

**Growth inhibition of the HuH-7 spheroids:** Single-cell suspensions were obtained by the trypsinization of monolayer-cultured HuH-7 cells: 45 μL of single-cell suspensions (80 cells) were seeded in individual wells of a 96-well Celltight Spheroid Culture Plate to form the 100 μm HuH-7 spheroids. After 24 h, the PEGylated polyplexes (N/P = 1), siRNA, Lac-PEG-siRNA conjugate or OligofectAMINE/siRNA (5 μL/well) were added to the well at the prescribed concentration on day 0. Fresh DMEM with 10% FBS was added to each wells on day 2 (25 μL/well), 7 (50 μL/well), 10 (50 μL/well), and 14 (50 μL/well), compensating for the decrease in the medium volume due to the natural evaporation. The perpendicular diameters of the HuH-7 spheroids were measured by means of phase-contrast microscope (Olympus IX71) on day 0, 2, 4, 7, 10, 14, and 21. The volume of the HuH-7 spheroids was calculated using the following formula:<sup>[21]</sup>

$$4\pi a^2 b/3 = \text{Tumor Volume (mm}^3\text{)}, \quad (1)$$

where  $a$  and  $b$  are the smallest and largest radius (nm) of the HuH-7 spheroids, respectively.

**Distribution study of FITC-labeled oligodeoxynucleotide in the HuH-7 spheroids:** 5'-FITC-labeled (fluorescein isothiocyanate) oligodeoxynucleotide (ODN) having the same antisense sequence as the firefly luciferase siRNA was hybridized with the sense firefly luciferase PEG-ODN conjugate to form an FITC-labeled Lac-PEG-dsODN conjugate. The lac-PEGylated polyplex was then prepared by mixing FITC-labeled Lac-PEG-dsODN conjugate with PLL (DP = 100). FITC-labeled dsDNA/OligofectAMINE was also prepared as the control. FITC-PEGylated polyplexes or FITC-dsODN/OligofectAMINE were added to the 100  $\mu$ m HuH-7 spheroids at conjugate or siRNA concentration of 400 nM, and incubated for 48 h. After the incubation, the HuH-7 spheroids were washed three times with phosphate-buffered saline (PBS) and imaged directly in the cell culture medium using a confocal fluorescence-scanning microscope (Olympus IX71 equipped with a confocal IX2-DSU system and an appropriate filter).

**Live/Dead staining assay:** A Live/Dead staining assay was carried out using a double staining kit. The staining solution (15  $\mu$ L) containing calcein-acetoxymethyl (10  $\mu$ M) and propidium iodide (30  $\mu$ M) were added to the HuH-7 spheroids (100  $\mu$ m initial diameter) treated with the lac-PEGylated polyplexes (50 nM), OligofectAMINE/siRNA (100 nM), or mock on day 0, 2, 4, 7, 10, 14, and 21. After 2 h of incubation, the HuH-7 spheroids were washed three times with PBS and imaged directly in the cell culture medium using a confocal fluorescence-scanning microscope (Olympus IX71 equipped with a confocal IX2-DSU system and an appropriate filter).

**Detection of apoptosis:** The detection of apoptosis was carried out using a Magic Red Caspase Detection Kit. Staining solution (5  $\mu$ L) containing MR-(DEVD)<sub>2</sub> (30  $\mu$ M) were added to the HuH-7 spheroids (100  $\mu$ m initial diameter) treated with the lac-PEGylated polyplexes (50 nM), OligofectAMINE/siRNA (100 nM) or mock on day 0, 1, 2, 4, 7, 10, 14, and 21. After 2 h of incubation, the HuH-7 spheroids were washed three times with PBS and imaged directly in the cell culture medium using a confocal fluorescence-scanning microscope (Olympus IX71 equipped with a confocal IX2-DSU system and an appropriate filter).

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**Keywords:** antitumor agents • bioconjugates • drug delivery • siRNA • spheroids

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# PEG-based block cationomers possessing DNA anchoring and endosomal escaping functions to form polyplex micelles with improved stability and high transfection efficacy

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## Abstract

For the development of polyplex systems showing a high transfection efficacy without a large excess of polycations, a lysine (Lys) unit as a DNA anchoring moiety was introduced into the amino acid sequence in poly(ethylene glycol)-*b*-cationic poly(*N*-substituted asparagine) with a flanking *N*-(2-aminoethyl)-2-aminoethyl group (PEG-*b*-Asp(DET)) resulting in PEG-*b*-P[Lys/Asp(DET)], in which the Asp(DET) unit acts as a buffering moiety inducing endosomal escape with minimal cytotoxicity. PEG-*b*-P[Lys/Asp(DET)]/DNA polyplexes exhibited a narrow size distribution of ~90 nm without secondary aggregates at the stoichiometric N/P 1, suggesting the formation of PEG-shielded polyplex micelles. The introduction of Lys units into the cationomer sequence facilitated cellular uptake and a 100-fold higher level of gene expression with PEG-*b*-P[Lys/Asp(DET)]/DNA polyplex micelles prepared even at a lowered N/P 2, possibly due to the enhanced association power of the anchoring Lys units.

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**Keywords:** Gene delivery; PEG; Polyplex micelle; Block copolymer; Nonviral

## 1. Introduction

In recent years, enormous efforts have been devoted to the development of polycation-based gene delivery systems (polyplexes) due to their safety for clinical use, simplicity of preparation, and adaptability to large-scale production [1,2]. In particular, poly(ethylene glycol)-modified (PEGylated) polyplexes (polyplex micelles) formed through the electrostatic interaction between plasmid DNA (pDNA) and PEG-*b*-polycation copolymers (PEG-based block cationomers) are

promising for *in vivo* gene therapy applications. The unique core-shell architecture of PEG-based block cationomers when combined with pDNA shows particle size data <100 nm under physiological conditions [3]. Indeed, polyplex micelles from PEG-*b*-poly(L-lysine) copolymers showed a high colloidal stability in biological media, excellent biocompatibility, and prolonged circulation periods in the blood stream [4–6]. However, further improvement in the transfection efficacy of these polyplex systems is needed for translation into the clinic.

Previous studies have revealed that a high transfection efficacy was obtained from polycations with a relatively low p*K*<sub>a</sub> value, such as polyethylenimine (PEI), which is explained by their buffering effect in endosomal compartments, as described by the proton sponge hypothesis [7,8]. In this regard, we have developed PEG-*b*-poly(*N*-substituted asparagine) copolymers having the *N*-(2-aminoethyl)-2-aminoethyl group

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in the side chain (PEG-*b*-PAsp(DET)). Consequently, these polyplex micelles exhibited a remarkably high transfection activity possibly due to the high buffering capacity based on the distinctive two-step protonation behavior of the flanking ethylenediamine moiety [9]. Also, we found that PEG-*b*-PAsp(DET) copolymers showed minimal cytotoxicity, allowing the successful transfection to primary cells [9–12]. However, an excess of PEG-*b*-PAsp(DET) copolymers with high N/P ratios were required for successful polyplex transfection; consequently, we concluded that micelle solutions prepared under such conditions are likely to contain a mixed population of: (i) block cationers firmly condensing DNA, (ii) block cationers loosely associated with DNA, and (iii) free or non-DNA condensing block cationers. Hence, *in vivo* use of such PEG-*b*-PAsp(DET) polyplex micelles, particularly for systemic administration, may be limited, because loosely associated block cationers easily desorb from the polyplex micelles during blood circulation, leading to the decreased transfection efficacy at the target site. Therefore, the micelle systems showing efficient transfection at lower N/P ratios without such non-associated or loosely associated cationers should be next developed for *in vivo* gene delivery.

The present study was devoted to improving the transfection efficacy of the PEG-*b*-PAsp(DET)-based polyplex micelles at N/P ratios near unity by enhancing their association power through the introduction of Lys residues into the amino acid sequence of the block cationer. Note that Lys residues are expected to anchor the associated block cationers to the polyplex micelles. In this way, a significantly improved efficacy of transfection was achieved with the polyplex micelles with a subtle excess of block cationers even after preincubation in the medium containing serum. This result seems to be associated with the facilitated cellular internalization of the polyplex micelles with stably incorporated block cationers showing a high buffering capacity for endosomal escape.

## 2. Materials and methods

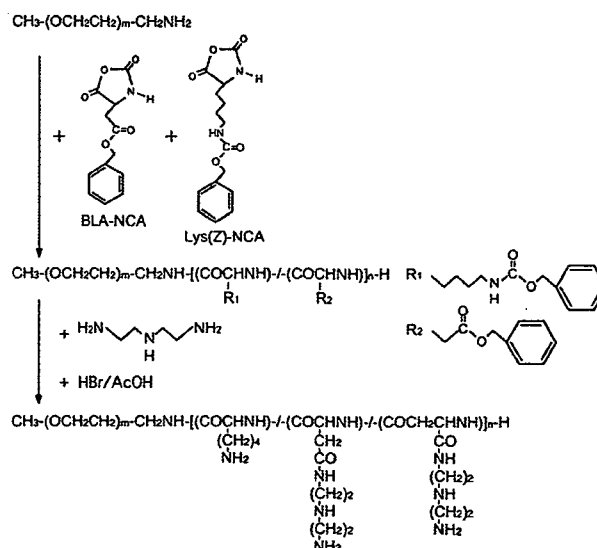
### 2.1. Materials

$\alpha$ -Methoxy- $\omega$ -amino-poly(ethylene glycol) (Mw 12,000) and  $\beta$ -benzyl-L-aspartate *N*-carboxyanhydride (BLA-NCA) were obtained from Nippon Oil and Fats Co., Ltd. (Tokyo, Japan).  $\epsilon$ -(Benzyloxycarbonyl)-L-lysine *N*-carboxyanhydride (Lys(Z)-NCA) was synthesized from  $\epsilon$ -(benzyloxycarbonyl)-L-lysine (Wako Pure Chemical Industries, Ltd., Osaka, Japan) by the Fuchs–Farthing method using bis(trichloromethyl) carbonate (triphosgene) (Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan) [13]. Diethylenetriamine (DET), *N,N*-dimethylformamide (DMF), dichloromethane, benzene, and trifluoroacetic acid were purchased from Wako Pure Chemical Industries, Ltd. Hydrogen bromide (HBr) (30% in acetic acid) was purchased from Tokyo Kasei Kogyo Co., Ltd. Branched polyethylenimine (25 kDa) (BPEI) was purchased from Sigma-Aldrich Co. (St. Louis, MO). The pDNA coding for *luciferase* with a CAG promoter (RIKEN, Japan) was amplified in competent DH5 $\alpha$  *E. coli* and purified with a QIAGEN HiSpeed Plasmid MaxiKit

(Germantown, MD). Luciferase Assay System Kit was purchased from Promega (Madison WI); Label IT Fluorescein Labeling Kit from Mirus Co. (Milwaukee, WI); and Micro BCA™ Protein Assay Reagent Kit from Pierce Co., Inc. (Rockford, IL).

### 2.2. Synthesis of PEG-*b*-P[Lys/Asp(DET)]

Block copolymers of PEG and L-lysine(Z)/ $\beta$ -benzyl-L-aspartate copolymer (P[Lys(Z)/BLA]), further referenced as PEG-*b*-P[Lys(Z)/BLA], were synthesized by ring-opening polymerization of a mixture of Lys(Z)-NCA and BLA-NCA initiated by the terminal primary amino group of  $\alpha$ -methoxy- $\omega$ -amino-PEG (Scheme 1). The typical synthetic procedure is described as follows for the PEG-*b*-P[Lys(Z)/BLA] with 47 units of Lys(Z) and 52 units of BLA. Lys(Z)-NCA (1.29 g) and BLA-NCA (1.25 g) were dissolved in a mixture of DMF and dichloromethane (6.8 mL and 31.7 mL, respectively). This NCA solution was added to the PEG (1.0 g) in dichloromethane (15 mL) in a stream of dry argon and stirred at 35 °C for 48 h for copolymerization. The polymer in the reaction medium was precipitated in a mixture of hexane and ethyl acetate (600 mL and 400 mL, respectively), and purified by filtration. The acetylation of the *N*-terminal amino group of the obtained polymer (1.5 g) was subsequently performed at 35 °C for 1 h using acetic anhydride (500  $\mu$ L) in a dichloromethane solution (22 mL). The polymer solution was precipitated into a mixture of hexane and ethyl acetate (300 mL and 200 mL, respectively), purified by filtration, and lyophilized from a benzene solution. The resulting copolymers had molecular weight distributions (Mw/Mn) of 1.1 to 1.3 as determined by GPC (data not shown). The degree of polymerization (DP) of the P[Lys(Z)/BLA] segment in the PEG-*b*-P[Lys(Z)/BLA] was determined from the peak intensity ratio of the methylene protons of PEG (OCH<sub>2</sub>-CH<sub>2</sub>,  $\delta$ =3.5 ppm) to the aryl protons of the benzyl groups in the



Scheme 1. Synthetic procedure of PEG-*b*-P[Lys/Asp(DET)].

Lys(Z) and BLA units ( $C_6H_6$ ,  $\delta=7.2\text{--}7.3$  ppm) in the  $^1H$  NMR spectra taken in dimethylsulfoxide at 80 °C. The compositions of the P[Lys(Z)/BLA] segments were determined from the peak intensity ratio of the protons of the  $\beta$  to  $\delta$  methylene groups ( $CH_2CH_2CH_2$ ,  $\delta=1.2\text{--}2.0$  ppm) in the side chain of the Lys(Z) units to the protons of the benzyl groups in the Lys(Z) and BLA units in the  $^1H$  NMR spectra under the same conditions.

The PEG-*b*-P[Lys(Z)/BLA] copolymers (300 mg) were dissolved in DMF (6 mL), after which DET (2.5 mL; 50 eq to the benzyl group of PBLA) was added to the polymer solution, and stirred for 24 h at 40 °C under a dry argon atmosphere. After 24 h, the mixture was dropped into diethylether (120 mL) with stirring, and then the white precipitate was filtered and re-dissolved in trifluoroacetic acid (4 mL). To deprotect the Z group, HBr (30% in acetic acid) was then added and stirred for 1 h, after which the solution was dropped into diethylether (100 mL) with stirring, and the resulting precipitate was purified by filtered and dried *in vacuo*. The crude product was dissolved in distilled water, dialyzed against 0.01 N HCl and distilled water, and lyophilized to obtain the final product, PEG-*b*-P[Lys/Asp(DET)] as the hydrochloride salt form. The PEG-*b*-P[Lys/Asp(DET)] copolymers with varying ratios of Lys units to Asp(DET) units were obtained by changing the initial feeding ratio of Lys(Z)-NCA to BLA-NCA upon polymerization. The introduction of a DET moiety was confirmed from the peak intensity ratio of the methylene protons of PEG ( $OCH_2CH_2$ ,  $\delta=3.5$  ppm) to the methylene protons of the introduced DET moieties ( $CH_2CH_2NHCH_2CH_2$ ,  $\delta=2.6\text{--}3.6$  ppm) in the  $^1H$  NMR spectra taken in  $D_2O$  at 25 °C.

### 2.3. Preparation of polyplex micelles from PEG-*b*-P[Lys/Asp(DET)]

The synthesized PEG-*b*-P[Lys/Asp(DET)] copolymer was dissolved in 10 mM Tris-HCl (pH 7.4) buffer at 5 mg/mL. This polymer solution was then mixed with pDNA in 10 mM Tris-HCl (pH 7.4) (50  $\mu$ g/mL) at varying N/P ratios (residual molar ratio of amino groups in Lys and Asp(DET) units to pDNA phosphate groups), followed by a 24 h incubation at ambient temperature. The final concentration of pDNA in all the samples was adjusted to 33  $\mu$ g/mL. The complexation of pDNA with the polycations was confirmed by agarose gel retardation analysis and ethidium bromide (EtBr) dye exclusion assay. In the gel retardation analysis, each sample was prepared by the dilution of the micelle solutions to the concentration of 8.3  $\mu$ g pDNA/mL. 20  $\mu$ L of each sample (166 ng pDNA) with a loading buffer was then electrophoresed at 100 V for 1 h on a 0.9 wt% agarose gel in 3.3 mM Tris-acetic acid buffer containing 1.7 mM sodium acetate. The migrated pDNA was visualized by soaking the gel in distilled water containing EtBr (0.5  $\mu$ g/mL). In the EtBr dye exclusion assay, each sample (33  $\mu$ g pDNA/mL) with varying N/P ratios was adjusted to 10  $\mu$ g pDNA/mL with 2.5  $\mu$ g EtBr/mL and 150 mM NaCl by adding 10 mM Tris-HCl (pH 7.4) buffer containing EtBr and NaCl. The solutions were incubated at ambient temperature overnight. The fluorescence intensity of the samples excited at 510 nm was measured at 590 nm and a temperature of 25 °C using a spectrofluorometer

(Jasco, FP-777). The relative fluorescence intensity was calculated as follows:

$$F_r = (F_{\text{sample}} - F_0) / (F_{100} - F_0)$$

where  $F_{\text{sample}}$  is the fluorescence intensity of the micelle samples,  $F_{100}$  is the free pDNA, and  $F_0$  is the background without pDNA.

### 2.4. Zeta-potential and dynamic light scattering (DLS) measurements

The zeta-potential of the polyplex micelles was determined from the laser-Doppler electrophoresis using the Zetasizer nanoseries (Malvern Instruments Ltd., UK) at a detection angle of 173° and a temperature of 25 °C. Each sample was prepared by simply mixing the polymer solutions with the pDNA solution at varying N<sup>+</sup>/P ratios (33  $\mu$ g pDNA/mL). The N<sup>+</sup>/P ratio was defined as the residual molar ratio of protonated amino groups in PEG-*b*-P[Lys/Asp(DET)] to phosphate groups in DNA. The fraction of protonated amino groups in P[Lys/Asp(DET)] segment was calculated assuming that 100% and 50% of the amino groups in the Lys and Asp(DET) units, respectively, were protonated at pH 7.4 and a temperature of 25 °C based on the potentiometric titration results [9]. The samples were adjusted to 14  $\mu$ g pDNA/mL by adding 10 mM Tris-HCl (pH 7.4) buffer, and then, injected into folded capillary cells (Malvern Instruments, Ltd.), followed by the measurement. From the obtained electrophoretic mobility, the zeta-potentials of each micelle were calculated by the Smoluchowski equation:  $\zeta = 4\pi\eta v / e$  in which  $\eta$  is the viscosity of the solvent,  $v$  is the electrophoretic mobility, and  $e$  is the dielectric constant of the solvent. The results are represented as the average of three experiments.

The sizes of each polyplex micelle were also measured by the DLS using the same apparatus. Micelle samples were prepared by mixing each polymer solution with pDNA solution at varying N<sup>+</sup>/P ratios (33  $\mu$ g pDNA/mL). After an overnight incubation at ambient temperature, the samples were adjusted to 14  $\mu$ g pDNA/mL by adding 10 mM Tris-HCl (pH 7.4) buffer, and then injected into low volume glass cuvettes, ZEN2112 (Malvern Instruments, Ltd.), followed by the measurement. The data obtained from the rate of decay in the photon correlation function were analyzed by the cumulant method, and the corresponding hydrodynamic diameter of micelles was then calculated by the Stokes-Einstein equation [14].

### 2.5. Stability of polyplex micelles against counter polyanion exchange reaction

The stability of the polyplex micelle was estimated from the release of pDNA from the micelle caused by the exchange reaction with poly(aspartic acid) (PAsp, DP 66) as a polyanion. Ten mM Tris-HCl buffer (pH 7.4) solutions with varying concentrations of PAsp were added to the micelle solution with the pDNA concentration of 33  $\mu$ g/mL. After overnight incubation at ambient temperature, each sample solution containing 167 ng of pDNA was electrophoresed through a 0.9 wt% agarose gel with

a running buffer of (3.3 mM Tris–acetic acid (pH 7.4)+1.7 mM sodium acetate+1 mM EDTA2Na). The pDNAs in the gel were visualized by soaking the gel into distilled water containing EtBr (0.5 mg/L).

### 2.6. Radiolabeling of pDNA for the cellular uptake study of polyplex micelles

pDNA was radioactively labeled with  $^{32}\text{P}$ -dCTP using the Nick Translation System (Invitrogen, San Diego, CA). Unincorporated nucleotides were removed using High Pure PCR Product Purification Kit (Roche Laboratories, Nutley, NJ). After the purification, the 2  $\mu\text{g}$  of labeled pDNA was mixed with 400  $\mu\text{g}$  of non-labeled pDNA. The polyplex micelle samples were prepared by mixing the radioactive pDNA solution with each polymer solution (33  $\mu\text{g}$  pDNA/mL). For cellular uptake experiments, HeLa cells were seeded on 24-well cultured plates 24 h before the experiments in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS). The cells were incubated with 30  $\mu\text{L}$  of the radioactive micelle solution (1  $\mu\text{g}$  pDNA/well) in 400  $\mu\text{L}$  of DMEM containing 10% FBS. After 24 h incubation, the cells were washed three times with Dulbecco's PBS and lysed with 400  $\mu\text{L}$  of the cell culture Promega lysis buffer. The lysates were mixed with 5 mL of scintillation cocktail, Ultima Gold (PerkinElmer, MA), and then, the radioactivity of the lysate solution was measured by a scintillation counter. The results are presented as a mean and standard error of mean obtained from four samples.

### 2.7. In vitro transfection

HeLa cells were seeded on 24-well culture plates and incubated overnight in 400  $\mu\text{L}$  of DMEM containing 10% FBS. The medium was changed to 400  $\mu\text{L}$  of fresh DMEM containing 10% FBS, and then 30  $\mu\text{L}$  of each micelle solution was applied to each well (1  $\mu\text{g}$  of pDNA/well). In the estimation of the effect of serum incubation on the transfection capacity of the micelle samples, the micelle solutions were preincubated with DMEM containing 10% FBS for 4 h and then added to the wells. After 24 h incubation, the medium was changed to 400  $\mu\text{L}$  of fresh DMEM without micelle samples, followed by an additional 24 h incubation. The cells were washed with 400  $\mu\text{L}$  of Dulbecco's PBS, and lysed by 100  $\mu\text{L}$  of the cell culture Promega lysis buffer. The luciferase activity of the lysates was evaluated from the photoluminescence intensity using Mithras LB 940 (Berthold Technologies). The obtained luciferase activity was normalized with the amount of proteins

Table 1  
A series of synthesized block cationomers

Code	Feeding unit ratio (Lys: Asp)	DP of poly (amino acid)	Unit number of Lys in poly(amino acid)	% of Lys units
L0/101	0:120	101	0	0
L24/102	25:90	102	24	24
L47/99	50:60	99	47	47
L70/98	75:30	98	70	71
L109/109	120:0	109	109	100

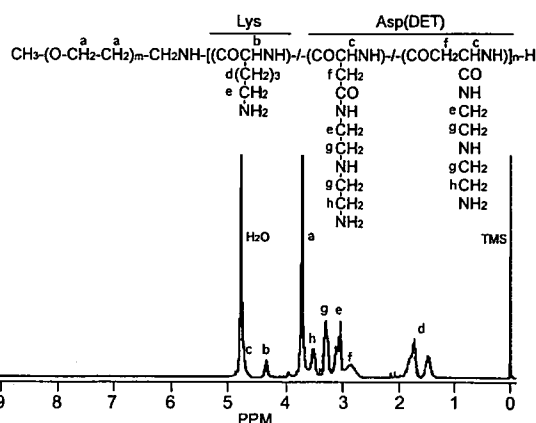


Fig. 1.  $^1\text{H}$ -NMR spectrum of the PEG-*b*-P[Lys/Asp(DET)] (L47/99) ( $\text{D}_2\text{O}$ ; 25  $^\circ\text{C}$ ; concentration, 10 mg/mL).

in the lysates determined by the Micro BCA<sup>TM</sup> Protein Assay Reagent Kit (Pierce).

### 2.8. Tolerability of polyplex micelles in serum-containing medium

The tolerability of polyplex micelles in serum-containing medium was estimated from the change in the fluorescence intensity of the fluorescein-labeled pDNA (F-pDNA) contained in the micelles. A pDNA was labeled using a Label IT Fluorescein Labeling Kit. This system promotes the covalent attachment of specific fluorescent molecules to guanine residues in nucleic acids. Each polyplex micelle sample was prepared by simply adding the polymer solution to the F-pDNA solution. After an overnight incubation at ambient temperature under dark conditions, the micelle solutions were mixed with 9 times volume of FBS solution, and then incubated at 37  $^\circ\text{C}$  for 4 h. The fluorescence emission of each sample excited at 492 nm was measured at 520 nm and a temperature of 37  $^\circ\text{C}$  using a spectrofluorometer (Jasco, FP-777). The obtained fluorescence intensities were expressed as the relative value to the fluorescence intensity of naked F-pDNA.

## 3. Results and discussion

### 3.1. Synthesis of PEG-*b*-P[Lys/Asp(DET)]

Block copolymers of PEG and P[Lys(Z)/BLA] (PEG-*b*-P[Lys(Z)/BLA]) were prepared by the ring-opening copolymerization of Lys(Z)- and BLA-NCAs as shown in Scheme 1. The poly(amino acid) segments with similar DP and varying Lys(Z)/BLA unit ratios were synthesized by changing the feeding ratio of Lys(Z)-NCA to BLA-NCA in the reaction mixture. The DPs and the unit ratios of Lys(Z)/BLA in the obtained copolymers were calculated from the peak intensity ratio in the  $^1\text{H}$  NMR spectra (data not shown). As summarized in Table 1, five types of copolymers were prepared. The DP of the poly(amino acid) segments in a series of copolymers was confirmed to be approximately 100, regardless of the composition. These copolymers were then subjected to aminolysis reaction with DET, followed

by deprotection of the Z groups. The  $^1\text{H}$  NMR spectra of the obtained cationers, as typically seen in Fig. 1, reveal the quantitative aminolysis of BLA units as well as the complete deprotection of Lys(Z) units in the poly(amino acid) segment, because the methylene protons in the DET moiety and the  $\beta$ -methylene protons in the asparagine unit had a 4:1 peak intensity ratio and the peaks of the Z group disappeared in the  $^1\text{H}$  NMR spectrum. These cationers were abbreviated as  $Lx/y$ , where  $x$  and  $y$  represent the unit number of Lys and the total DP of the poly(amino acid) segment, respectively.

### 3.2. Formation of polyplex micelles from PEG-*b*-P[Lys/Asp(DET)] cationers

The polyplex micelles were prepared by simply mixing each cationer solution with pDNA solution at varying N/P ratios. The complex formation of pDNA was confirmed by the agarose gel electrophoresis of each sample. With an increase in the N/P ratio, the amount of migrating free pDNA decreased, indicating the complex formation of pDNA with the PEG-*b*-P[Lys/Asp(DET)] cationers (Supplementary Fig. 1). The critical N/P ratio where the migration of pDNA was completely retarded differed depending on the composition of the cationers; i.e., the micelle of the block cationers with a lower ratio of Lys units required the higher N/P ratio for the complete retardation of pDNA. To quantitatively evaluate the relationship between the N/P ratio

and the condensation behavior of pDNA, the EtBr dye exclusion assay by fluorometry was completed. Fig. 2 (a) shows that the fluorescence intensity of EtBr decreased with an increase in the N/P ratio, and leveled off at a critical N/P ratio for each micelle system. These fluorescence data were then re-plotted against  $N^+/P$  ratio, the molar ratio of protonated amino groups at pH 7.4 in the block cationers to phosphate groups in pDNA, as seen in Fig. 2 (b). Note that the ratios of the protonated amino groups in the block cationer were calculated as 100% for Lys units and 50% for Asp(DET) units at pH 7.4, respectively, from the potentiometric titration results of PEG-*b*-PLys and PEG-*b*-PAsp(DET) block cationers [9,15]. Interestingly, Fig. 2 (b) reveals that the fluorescence intensity of all the micelles leveled off at the  $N^+/P$  ratio of approximately 1 regardless of the composition of the cationers, indicating that the condensation behavior of pDNA might be closely correlated with the ratio of charged groups ( $N^+/P$ ). Also, this result strongly suggests that the PAsp(DET) segment in the micelles is likely to maintain the same protonation degree ( $\alpha=0.5$  at pH 7.4) as that in the free cationer without the facilitated protonation by the complexation. As previously reported [9], this limited protonation for the proton sponge potential of the Asp(DET) units in the micelles is assumed to contribute to endosomal escape of PEG-*b*-P[Lys/Asp(DET)] polyplex micelles.

Fig. 3 (a) and (b) show the results obtained from DLS and zeta-potential measurements. The cumulant diameters of the polyplex micelles from the PEG-*b*-P[Lys/Asp(DET)]s were determined to be 70–100 nm throughout the range of the examined  $N^+/P > 1$ . As seen in the Fig. 3 (b) inset, the zeta-potentials of each polyplex micelle appear nearly neutral at an  $N^+/P$  1, indicating the formation of the charge stoichiometric micelle from pDNA and the block cationers. It should be emphasized that the polyplex micelles from PEG-*b*-P[Lys/Asp(DET)] had a narrowly distributed size of approximately 90 nm without secondary aggregates even at the charge neutralized condition ( $N^+/P$  1) as previously demonstrated for those from PEG-*b*-PLys and PEG-*b*-PAsp(DET) cationers [9,16]. It should also be noted that all polyplex micelles from the block cationers had a much lower absolute value in zeta-potentials than polyplexes prepared from PAsp(DET) homopolymer (DP 98) (Fig. 3 (b)), possibly due to the shielding effect of the PEG layer surrounding the polyplex core. Nevertheless, there was a slight increase in the zeta-potentials of the polyplex micelles from neutral to positive values in the region of  $N^+/P > 1$ . This zeta-potential increase is likely to be ascribed to the loose association of excess cationers with the polyplex micelles as previously reported for the PEG-poly(2-dimethylamino)ethyl methacrylate cationer/pDNA micelle [17]. Interestingly, the tendency of such increasing zeta-potentials with  $N^+/P$  ratios varied according to the composition of the block cationers. The zeta-potential value of the polyplex micelles from PEG-*b*-PLys leveled off at an  $N^+/P$  4 (+10 mV), while that from PEG-*b*-PAsp(DET) seemed to reach a plateau at a higher  $N^+/P$  16 (+10 mV), suggesting that the association profile of the block cationers with the polyplex micelles varied between PEG-*b*-PLys and PEG-*b*-PAsp(DET) micelles at  $N^+/P$  ratios ranging from 1 to 16. Presumably, PEG-*b*-PLys may reach the saturated association with pDNA at the lower

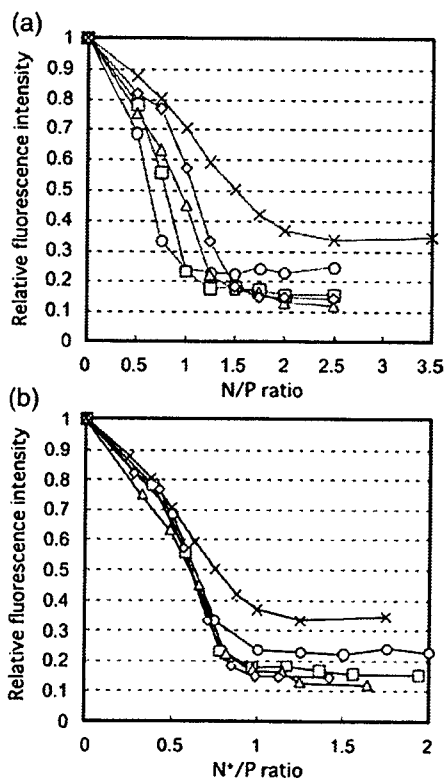


Fig. 2. EtBr dye exclusion assay on a series of polyplex micelles. Micelles included are:  $\times$ : L0/101;  $\diamond$ : L24/102;  $\triangle$ : L47/99;  $\square$ : L70/98;  $\circ$ : L109/109. (a) Relative fluorescence intensity vs. N/P ratio. (b) Relative fluorescence intensity vs.  $N^+/P$  ratio.



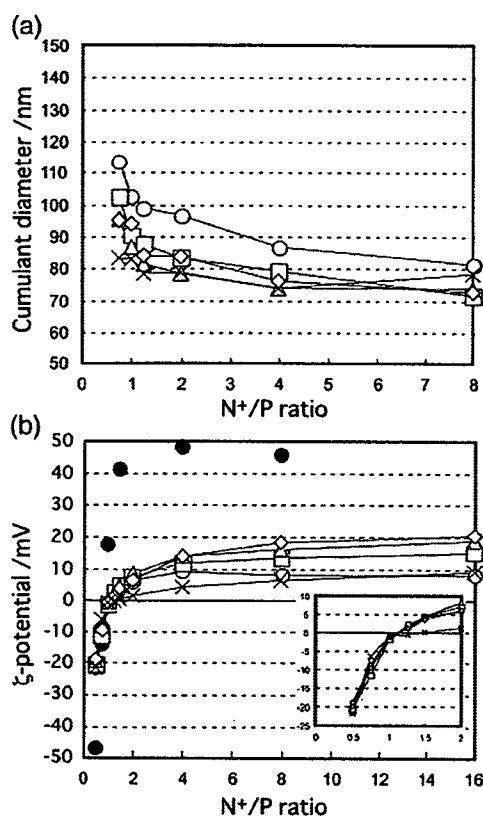


Fig. 3. (a) Size and (b) ζ-potential of a series of polyplex micelles and a PAsp (DET) polyplex. X: L0/101 micelle, ◇: L24/102 micelle, △: L47/99 micelle, □: L70/98 micelle, ○: L109/109 micelle, ●: PAsp(DET) (DP 98) polyplex.

concentration (lower N<sup>+</sup>/P) than PEG-*b*-PAsp(DET) due to the effective anchoring effect of the Lys units. Although PEG-*b*-P[Lys/Asp(DET)] micelles displayed the similar profiles of the zeta-potential to PEG-*b*-PLys micelles at lower N<sup>+</sup>/P ratios, the micelles showed further increase in the surface charge in the range of N<sup>+</sup>/P > 4 without leveling off. This result suggests that the association of PEG-*b*-P[Lys/Asp(DET)] with pDNA may not be saturated even in the range of N<sup>+</sup>/P > 4, despite introduction of Lys units as a DNA anchoring moiety. This phenomenon may be ascribed to the unique structure of PEG-*b*-P[Lys/Asp(DET)] possessing two types of cationic units with different pDNA affinity. The presence of the Lys units in the block cationer is likely to facilitate the binding of Asp (DET) units to pDNA, promoting the block cationer association to the polyplex micelles at N<sup>+</sup>/P 2 or lower; however, at high concentrations of the block cationers (high N<sup>+</sup>/P), Lys units may preferentially bind to the polyplex micelles to replace the Asp (DET) units, resulting in the continuous binding of the block cationers until the Lys units saturate the available binding sites.

### 3.3. Stability of polyplex micelles

The result of the zeta-potential measurement suggested that the affinity of PAsp(DET) to pDNA seems to be enhanced by the incorporation of Lys units. The complexing stability of the polyplex micelles prepared from PEG-*b*-P[Lys/Asp(DET)] was evaluated directly from the standpoint of the counter polyion exchange reaction. The solutions with varying concentrations of

PAsp were added to the solution of the PEG-*b*-PAsp(DET) micelle (N<sup>+</sup>/P 2) in different A/P ratios (the molar ratio of carboxyl groups of PAsp to phosphate groups of pDNA). Consequently, the pDNA was released from the PEG-*b*-PAsp (DET) micelles at A/P > 3 due to the counter polyanion exchange of pDNA by PAsp (Fig. 4 (a)). In contrast, the improved stability of the polyplex micelles from PEG-*b*-P[Lys/Asp(DET)] was

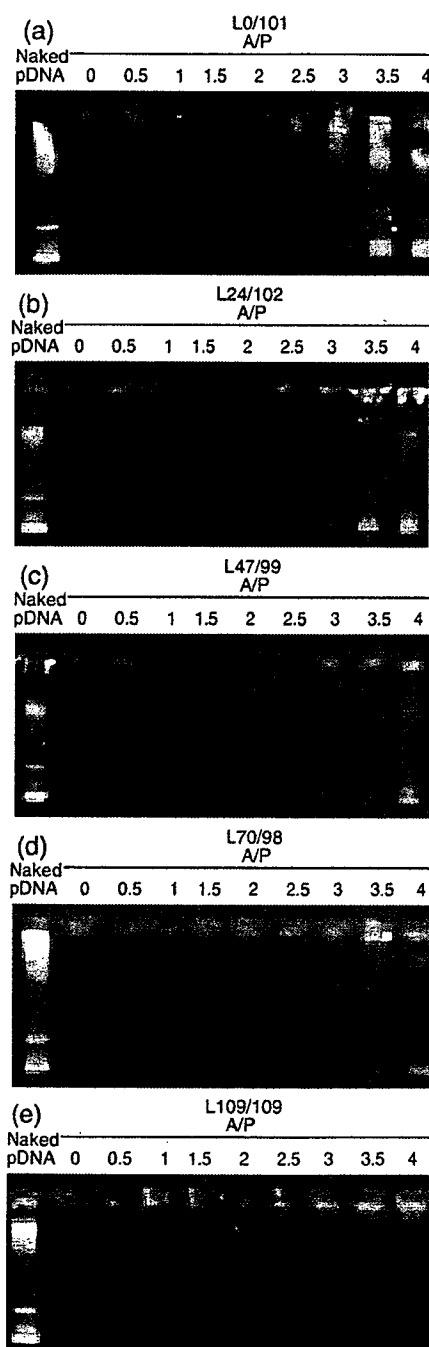


Fig. 4. Electrophoretic evaluation of polyplex micelle (N<sup>+</sup>/P 2) tolerability against an exchange reaction with polyaspartic acid (DP 66). Note: A/P stands for the molar ratio of carboxyl groups in polyaspartic acid to phosphate groups in pDNA. Micelle samples prepared at N<sup>+</sup>/P 2 were mixed with polyaspartic acid solution and electrophoresed after overnight incubation.

confirmed as shown in Fig. 4 (b)–(d): higher A/P ratios were required for the pDNA release with the increment in the percentage of Lys units in the amino acid sequence of the PEG-*b*-P[Lys/Asp(DET)]. In the case of the micelles from PEG-*b*-PLys, the pDNA release was not observed in the range of the examined A/P ratios (0–4) (Fig. 4 (e)). The similar tendency was also confirmed for the micelles prepared at  $N^+/P$  4 (Supplementary Fig. 2). These results support our hypothesis that a Lys unit has a higher association power with pDNA than the Asp(DET) unit, and consequently, the PEG-*b*-P[Lys/Asp(DET)] micelles acquired the tolerability against the dissociation by polyanions through the anchoring effect of Lys units in the block cationer.

### 3.4. Transfection with polyplex micelles from PEG-*b*-P[Lys/Asp(DET)]

Preliminary experiments on the cellular uptake of complexed pDNA revealed that the pDNA incorporated into PEG-*b*-PAsp(DET) at  $N^+/P < 4$  was marginally internalized by cultured cells as is the case with naked pDNA (Supplementary Fig. 3). We speculate that this may contribute to the significantly lower transfection ability of PEG-*b*-PAsp(DET) micelles prepared at low  $N^+/P$  ratios. Herein, we hypothesize that the complexing stability promoted by the Lys anchors may facilitate the cellular uptake of polyplex micelles prepared even at low  $N^+/P$  ratios. To confirm this hypothesis, we completed a cellular uptake study using  $^{32}\text{P}$ -radiolabeled pDNA. Fig. 5 reveals that the cellular uptake of pDNA complexed with block cationers at an  $N^+/P$  ratio of 2 increased by the introduction of the Lys unit into the amino acid sequence. Especially, the polyplex micelles from the PEG-*b*-P[Lys/Asp(DET)] with the percentage of Lys units  $> 47$  exhibited more than a 10-fold uptake of pDNA compared to PEG-*b*-PAsp(DET). This result strongly suggests that the increased association power of the polyplex micelles may contribute to their facilitated cellular uptake without a large excess of block cationers, i.e., at low  $N^+/P$  ratios. Interestingly, the cellular uptake of radioactive pDNA was maximized at the L70/98, even through its stability was deemed comparable to that of PEG-*b*-PLys (L109/109), as evidenced from the counter polyanion exchange reaction (Fig. 4

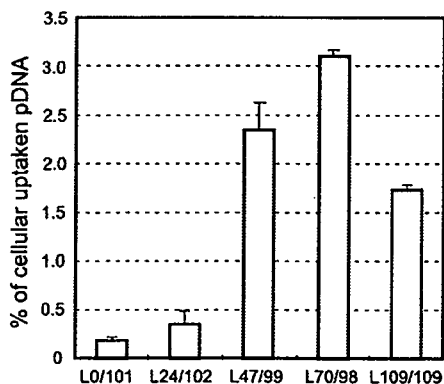


Fig. 5. The uptake of pDNA complexed with block cationers at  $N^+/P$  2 into HeLa cells.  $^{32}\text{P}$ -labeled pDNA micelles were incubated with the cells in DMEM containing 10% FBS at 37 °C for 24 h. The amount of internalized pDNA is represented as a percentage for the dosed pDNA (1  $\mu\text{g}/\text{well}$ ).

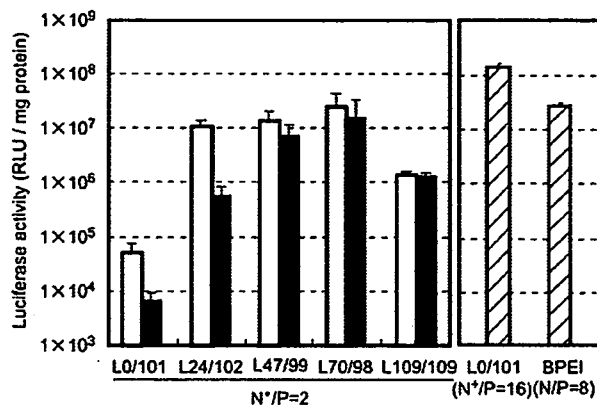


Fig. 6. *In vitro* transfection efficacy of polyplex micelles ( $N^+/P$  2) against HeLa cells. Open bars: Transfection efficacy without serum-preincubation. Closed bars: Transfection efficacy after 4 h incubation in the DMEM containing 10% FBS. Hatched bars: Transfection efficacy of the control; L0/101 micelles ( $N^+/P$  16) and BPEI (25 kDa) polyplexes ( $N/P$  8).

(d) and (e)). These data indicate that the increased cellular uptake of PEG-*b*-P[Lys/Asp(DET)] micelles compared to PEG-*b*-PLys micelles was not simply correlated to the enhanced stability. In this regard, a slightly positive zeta-potential of PEG-*b*-P[Lys/Asp(DET)] micelles compared to PEG-*b*-PAsp(DET) and PEG-*b*-PLys micelles is noteworthy, suggesting the surface charge may indeed affect the cellular uptake of the micelles with varying stabilities.

The effect of the introduction of the Lys unit as an anchoring moiety on the transfection ability of the polyplex micelles was then evaluated from the expression of a luciferase gene in the transfected cells. Although the PEG-*b*-PAsp(DET) micelles prepared at the high  $N^+/P$  16 (ca.  $N/P$  32) showed appreciably high transfection efficacy, which was one order of magnitude higher than that of BPEI polyplexes (25 kDa,  $N/P$  8), reduction in the  $N^+/P$  ratio resulted in a sharp decline of the transfection ability of the PEG-*b*-PAsp(DET) micelles probably due to the lowered cellular uptake (Fig. 6). In contrast, the transfection efficacy could be maintained to be a high value even in the range of lowered  $N^+/P$  ratios by introducing Lys units into the block cationer (Supplementary Fig. 4). Eventually, the PEG-*b*-P[Lys/Asp(DET)] micelles revealed an appreciably improved transfection efficacy at  $N^+/P$  2 compared to the polyplex micelles of PEG-*b*-PLys and PEG-*b*-PAsp(DET) (Fig. 6, open bars). These results suggest that PEG-*b*-P[Lys/Asp(DET)] micelles may provide high stability and promote endosomal escape, presumably due to the synergistic effect of Lys and Asp(DET) units. The improved stability through the anchoring effect of Lys units was also confirmed from the transfection results after the preincubation of micelles in the serum-containing medium (Fig. 6, closed bars). The micelles from PEG-*b*-P[Lys/Asp(DET)], with the higher percentage of Lys units (L47/99 and L70/98), maintained the initial transfection capacity even after the serum-preincubation, whereas transfection efficacies of PEG-*b*-PAsp(DET) and L24/102 micelles, with a low percentage of Lys residues, were significantly impaired by serum-preincubation. These results strongly suggest that the associated block cationers in the micelles from L47/99, L70/98, and L109/109 might be tolerable

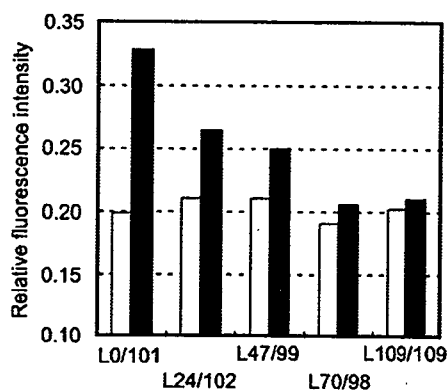


Fig. 7. Tolerability of polyplex micelles against serum incubation evaluated from the fluorescence recovery of the entrapped fluorescein-labeled pDNA due to serum-induced decondensation. Open bars: Fluorescence intensity in 10 mM Tris-HCl (pH 7.4) buffer solution without FBS. Closed bars: Fluorescence intensity after 4 h incubation in the medium containing 90% FBS.

in the transfection medium containing serum due to the strong anchoring of Lys units. This added strength of the Lys anchors is further supported by the sustained fluorescence quenching of fluorescein-labeled pDNA in micelles incubated in 90% FBS for 4 h as seen in Fig. 7. Apparently, fluorescence recovery due to the serum-inducing decondensation of pDNA was progressively inhibited with the increment in the percentage of Lys units in the PEG-*b*-P[Lys/Asp(DET)].

Although the transfection efficacy (Fig. 6) seems to be roughly correlated with the cellular uptake of the micelles (Fig. 5), careful examination reveals the tendency that the PEG-*b*-P[Lys/Asp(DET)] micelles with the lower percentage of Lys units, L24/102, achieved comparable transfection to those with the higher percentage of Lys units, 47/L98 and L70/98, even though the efficacy of pDNA uptake was fairly limited. This tendency becomes more apparent by normalizing the luciferase activity with pDNA uptake (Supplementary Fig. 5). This apparent increase in the transfection efficacy may be explained by the timely release of the loosely associated block cationers from the micelles in the endosomal compartment to exert a high buffering capacity [9] and their possible interaction with the endosomal membrane component, facilitating the endosomal escape of the complexed pDNA, followed by a smooth release of pDNA directing efficient transcription. The loosely associated nature of L24/102 in the micelle may be supported from a decreased transfection efficacy after serum-preincubation as shown in Fig. 6.

#### 4. Conclusion

Polyplex micelles from PEG-*b*-PAsp(DET) revealed a high transfection efficacy to various cell types including primary cells [9–12] presumably due to a low cytotoxicity and a strong pH-buffering capacity. Nevertheless, the weak association power of PAsp(DET) with DNA may be problematic for *in vivo* systemic administration, where the tolerability of polyplexes in proteinaceous media should be a crucial factor. Alternatively, a polylysine has an appreciably high affinity to DNA, however, the trans-

fection efficacy of polylysine polyplexes remains low, possibly due to poor endosomal escaping functions and an impaired release of pDNA from the polyplex with an over-stabilized nature. In this novel study, we sought to alter these discrepancies by placing both Asp(DET) as a buffering unit with low cytotoxicity and Lys as a strong anchoring moiety to DNA in a single polymer strand resulting in PEG-*b*-P[Lys/Asp(DET)]. Polyplexes prepared from pDNA and PEG-*b*-P[Lys/Asp(DET)] have a micellar structure with a PEG palisade, exhibiting a remarkably improved stability compared to PEG-*b*-PAsp(DET)/pDNA polyplex micelles. PEG-*b*-P[Lys/Asp(DET)] polyplex micelles were further revealed to promote cellular internalization, leading to enhanced transfection efficacy even with a subtle excess of block cationers. This enhanced transfection efficacy could be explained by the synergistic effect of Lys as an anchoring unit and Asp(DET) as a lower toxic endosomal escaping unit. This approach of placing cationic units with discriminating functions, e.g., DNA anchoring and endosomal escaping functions, into a single block cationer strand is highly promising for future construct designs for effective *in vivo* systemic applications.

#### Acknowledgement

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#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jconrel.2007.06.020.

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## Cyclic RGD Peptide-Conjugated Polyplex Micelles as a Targetable Gene Delivery System Directed to Cells Possessing $\alpha_v\beta_3$ and $\alpha_v\beta_5$ Integrins

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A cyclic RGD peptide-conjugated block copolymer, cyclo[RGDfK(CX-)]-poly(ethylene glycol)-polylysine (c(RGDfK)-PEG-PLys), was synthesized from acetal-PEG-PLys under mild acidic conditions and spontaneously associated with plasmid DNA (pDNA) to form a polyplex micelle in aqueous solution. The cyclic RGD peptide recognizes  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$  integrin receptors, which play a pivotal role in angiogenesis, vascular intima thickening, and the proliferation of malignant tumors. The c(RGDfK)-PEG-PLys/pDNA polyplex micelle showed a remarkably increased transfection efficiency (TE) compared to the PEG-PLys/pDNA polyplex micelle for the cultured HeLa cells possessing  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$  integrins. On the other hand, in the transfection against the 293T cells possessing no  $\alpha_v\beta_3$  and a few  $\alpha_v\beta_5$  integrins, the TE of the c(RGDfK)-PEG-PLys/pDNA micelle showed no increase compared to the TE of the PEG-PLys/pDNA micelle. Flow cytometric analysis revealed a higher uptake of the c(RGDfK)-PEG-PLys/pDNA micelle than the PEG-PLys/pDNA micelle against HeLa cells, consistent with the transfection results. Furthermore, a confocal laser scanning microscopic observation revealed that the pDNA in the c(RGDfK)-PEG-PLys micelle preferentially accumulated in the perinuclear region of the HeLa cells within 3 h of incubation. No such fast and directed accumulation of pDNA to the perinuclear region was observed for the micelles without c(RGDfK) ligands. These results indicate that the increase in the TE induced by the introduction of the c(RGDfK) peptide ligand was due to an increase in cellular uptake as well as facilitated intracellular trafficking of micelles toward the perinuclear region via  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$  integrin receptor-mediated endocytosis, suggesting that the cyclic RGD peptide-conjugated polyplex micelle has promising feasibility as a site-specifically targetable gene delivery system.

### INTRODUCTION

With the increase in available information on various disease genes that has resulted from progress in the Human Genome Project, gene therapy is increasingly recognized as a promising therapy for many intractable diseases. Obviously, a major key to successful gene therapy is the development of gene vectors that are particularly effective for *in vivo* use. Nevertheless, there are many restrictions in the clinical application of viral vectors, which have played a pivotal role in gene therapy up to now, because of their safety issues, including antigenicity, as well as to the difficulty of formulating them with good quality control. These limitations of viral vectors have led to the recent trend in developing nonviral vectors with safety and high productivity as alternative systems to viral vectors.

One of the important challenges in the development of nonviral vectors is the system available for systemic injection, which must be stable enough to achieve longevity in the blood

circulation but also be able to achieve high transfection efficiency (TE) in the target region with minimal toxicity. Lipoplex (1) and polyplex (2) systems based on cationic lipids and polymers, respectively, have been extensively studied as nonviral gene vectors for systemic administration. Nevertheless, there remain unresolved issues with these systems, because it is generally required that they contain excessive cationic lipids or polymers to increase solubility in an aqueous solution. Eventually, shifting their surface charge to a positive value induces nonspecific interaction with anionic components in the body such as plasma proteins and blood cells. This apparently hampers their applicability to systemic gene delivery. Polyplex micelles (3), characterized by the unique core-shell architecture of the hydrophilic shell layer surrounding their polyplex core, have the potential to acquire a so-called "stealth" property to minimize nonspecific interaction with biocomponents. Indeed, a polyplex micelle with poly(ethylene glycol) (PEG) as a hydrophilic shell layer achieved high stability in a medium containing serum compared to conventional lipoplexes and polyplexes and showed increased retention time in the bloodstream (4, 5), suggesting that a polyplex micelle may be a promising candidate for a vector that can be used in systemic gene delivery. Nevertheless, in order to increase selective uptake into the target cells, appropriate ligands are preferably introduced into the surface of the polyplex micelles. In this way, surface-installed ligands are expected to enhance the uptake rate of the

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polyplex micelles into the target cells by receptor-mediated endocytosis, which may lead to higher gene TE compared to the ligand-free polyplex micelles taken up by the cells through adsorptive or fluid-phase endocytosis (6–8).

In this study, acetal-poly(ethylene glycol)-polylysine (acetal-PEG-PLys) was synthesized as a precursor for constructing ligand-installed polyplex micelles. PLys, which is one of the first polymers used and well studied as a nonviral vector, was chosen as a cationic segment of the block copolymer. PLys-based polyplexes are known to show a relatively low TE against cultured cell lines compared to those having the endosomal escape function, such as polyethylenimine (PEI)-based polyplexes, but they have an advantage in stability even in a diluted condition, and they have shown an appreciable level of gene transfection in animal models (5, 9). A cyclic RGD peptide (c(RGDfK)), which selectively recognizes  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$  integrin receptors (10), was chosen as a ligand and introduced into the PEG terminus of aldehyde-PEG-PLys, derived from acetal-PEG-PLys, through a thiazolidine ring formation, and the c(RGDfK)-PEG-PLys block copolymer was obtained. It is generally known that the  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$  integrins are expressed on various cell types such as endothelial cells, osteoclasts, macrophages, platelets, and melanomas, and that they play a significant role in angiogenesis, vascular intima thickening, and the proliferation of malignant tumors (11). Therefore, gene delivery targeting the  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$  integrins is expected to be useful in the treatment of cancer and vascular diseases. Indeed, the c(RGDfK)-PEG-PLys/pDNA polyplex micelle thus prepared showed a remarkably increased TE compared to ligand-free polyplex micelles against the HeLa cells expressing  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$  integrins.

## EXPERIMENTAL PROCEDURES

**Materials.** 3,3-Diethoxypropanol was purchased from Aldrich Chemical Co. Ltd. (Milwaukee, WI). Ammonia solution (25%), tetrahydrofuran (THF), *N,N*-dimethylformamide (DMF), *n*-hexane, methanesulfonyl chloride (MsCl), and triethylamine (TEA) were purchased from Wako Pure Chem. Co. Ltd. (Japan). Ethylene oxide (EO) was purchased from Sumitomo Seika Chemicals Co. Ltd. (Japan). THF was distilled according to the conventional procedure as previously reported (12). DMF was dehydrated using activated molecular sieves (4A) and distilled under reduced pressure. 3,3-Diethoxypropanol and *n*-hexane were distilled over sodium wire. MsCl, TEA, and EO were dried over calcium hydride followed by distillation. CXYGRRGDS (RGDS) and cyclo[RGDfK(CX-)] (c(RGDfK)) peptides (X = 6-aminocaproic acid;  $\epsilon$ -Acp) were purchased from Peptide Institute, Inc. (Japan). The PEG-PLys block copolymer (PEG; 12 000 g/mol, polymerization degree of PLys segment; 73) was synthesized as previously reported (13). A micro BCA protein assay reagent kit was purchased from Pierce (Rockford, IL). The Luciferase assay kit was a product of Promega (Madison, WI). Plasmid pCacc+Luc coding for firefly luciferase under the control of the CAG promoter was provided by the RIKEN Gene Bank (Japan), amplified in competent DH5 $\alpha$  *Escherichia coli*, and then purified using a HiSpeed Plasmid MaxiKit purchased from QIAGEN Sciences Co., Inc. (Germany). FITC-labeled monoclonal antibodies against  $\alpha_v\beta_3$  integrin,  $\alpha_v\beta_5$  integrin, and mouse IgG were purchased from Cosmo Bio Co., Ltd. (Japan). QuantiLum recombinant luciferase was purchased from Promega (Madison, WI).

**Measurements.** Gel permeation chromatography (GPC) measurements were carried out using a TOSOH HLC-8220 equipped with TSKgel columns (G4000PWXL and G3000PWXL). The internal refractive index (RI) was used for detection of the polymer. DMF with 10 mM LiCl was used as an eluent at a flow rate of 0.8 mL min<sup>-1</sup> at 40 °C. IR was measured with an

IR Report-100 spectrometer (JASCO, Tokyo, Japan). <sup>1</sup>H NMR spectra were obtained with a JEOL EX300 spectrometer (JEOL, Tokyo, Japan). Chemical shifts are reported in ppm relative to the residual protonated solvent resonance.

**Synthesis of RGDS-PEG-PLys 5a and c(RGDfK)-PEG-PLys 5b (Scheme 1).** *Acetal-PEG-NH<sub>2</sub> 2.* A THF solution of 3,3-diethoxypropanol (0.16 mL, 1 mmol) and potassium naphthalene (2.7 mL, 0.90 mmol) were mixed in THF (75 mL) to form potassium 3,3-diethoxypropanolate (PDP) as previously reported (14). After the mixture was stirred for 10 min, liquid EO (13.5 mL, 270 mmol) chilled below 0 °C was added to the solution with additional stirring at room temperature (r.t.) for 2 days. The reactant polymer was isolated by precipitation into diethyl ether and lyophilized from benzene to obtain acetal-PEG-OH (12.65 g, quant). Acetal-PEG-OH (6.01 g, 0.50 mmol) was then dissolved in THF (40 mL), followed by the addition of TEA (0.32 mL, 2.25 mmol). Subsequently, the solution was slowly added dropwise into MsCl (0.12 mL, 1.5 mmol) in THF (20 mL) and stirred at 0 °C for 2 h. The solution was poured into diethyl ether to precipitate acetal-PEG-OMs **1**. The recovered acetal-PEG-OMs **1** was dissolved in 25% ammonia solution (500 mL) and stirred at r.t. for 4 days. The volume of the solution was concentrated to 50 mL by evaporation and dialyzed sequentially against 0.125% ammonia solution and distilled water, followed by lyophilization to obtain acetal-PEG-NH<sub>2</sub> **2** (5.40 g, 90%).

*Acetal-PEG-PLys(TFA) 3.* *N<sup>ε</sup>*-Trifluoroacetyl-L-lysine *N*-carboxyanhydride (Lys(TFA)-NCA) (3.59 g, 13.39 mmol, 80 equiv to **2**) prepared according to the protocol described in the literature (15) in DMF (40 mL) was added to acetal-PEG-NH<sub>2</sub> **2** (2.01 g, 167.5  $\mu$ mol) in DMF (20 mL) and stirred at 40 °C for 2 days. The polymerization was monitored by IR. The reactant polymer was precipitated into AcOEt/hexane (4:6) and lyophilized in dioxane to obtain acetal-PEG-PLys(TFA) **3** (4.08 g, 73%).

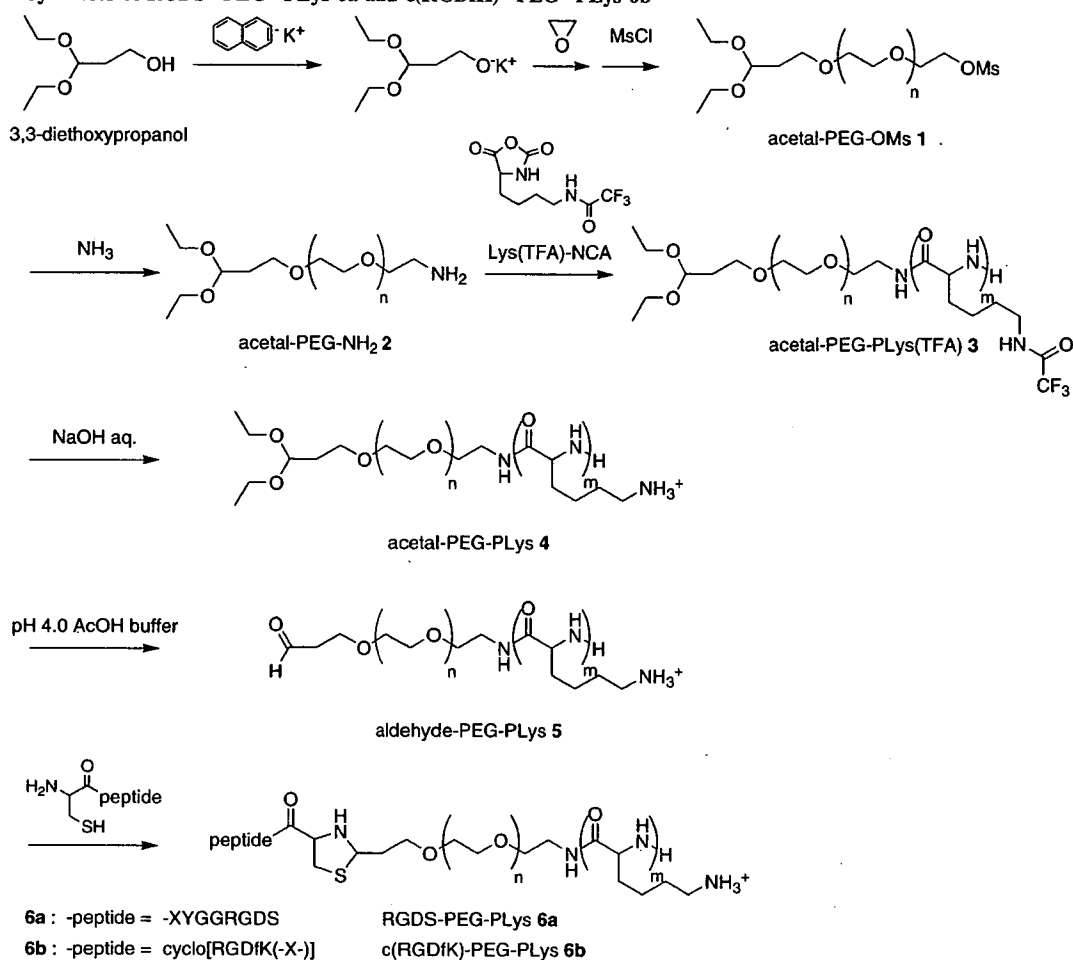
*Acetal-PEG-PLys 4.* NaOH solution (1 N, 4 mL) was added to acetal-PEG-PLys(TFA) **3** (500 mg, 17.77  $\mu$ mol) in MeOH (40 mL) and stirred at 40 °C for 24 h. The reacted polymer was purified by dialysis sequentially against 10 mM PBS solution (pH 7.0) and distilled water and lyophilized to obtain acetal-PEG-PLys **4** (498 mg, quant).

*RGDS-PEG-PLys 6a and c(RGDfK)-PEG-PLys 6b.* Conjugation of the peptide into the PEG terminus of PEG-PLys was performed through thiazolidine ring formation using RGDS or c(RGDfK) peptide. RGDS-PEG-PLys **6a** was synthesized according to the typical procedure, as follows: Acetal-PEG-PLys **4** (39.1 mg, 1.64  $\mu$ mol) was added to RGDS (15.2 mg, 16.4  $\mu$ mol) in 0.2 M AcOH buffer (pH 4.0, 8 mL) and stirred at room temperature for 5 days. The reacted polymer was purified by dialysis sequentially against 10 mM PBS solution (pH 7.0) and distilled water and lyophilized to obtain RGDS-PEG-PLys **6a** (35.4 mg, 86%). Conjugation of the c(RGDfK) to acetal-PEG-PLys **4** was carried out in a similar way, e.g., c(RGDfK) (7.4 mg, 9.03  $\mu$ mol) and acetal-PEG-PLys **4** (30.0 mg, 1.26  $\mu$ mol) were used to obtain c(RGDfK)-PEG-PLys **6b** (26.6 mg, 87%).

**Preparation of Polyplex Micelles.** Each block copolymer and pDNA was dissolved separately in 10 mM Tris-HCl buffer (pH 7.4). The polymer solution in varying concentrations was added to a twice-excess volume of pDNA solution to form polyplex micelles with different compositions. The final pDNA concentration was adjusted to 33.3 or 50  $\mu$ g/mL. The resulting solution was kept at r.t. overnight. The N/P ratio was defined as the residual molar ratio of the amino groups of PLys units to the phosphate groups of pDNA.

**Ethidium Bromide Exclusion Assay.** Polyplex micelle solutions (50  $\mu$ g pDNA/mL) prepared at various N/P ratios were

Scheme 1. Syntheses of RGDS-PEG-PLys 6a and c(RGDfK)-PEG-PLys 6b



adjusted to contain 10  $\mu\text{g}$  pDNA/mL with 2.5  $\mu\text{g}$  ethidium bromide (EtBr)/mL and 150 mM NaCl by adding 10 mM Tris-HCl solution (pH 7.4) containing EtBr and NaCl. The solutions were kept at r.t. overnight. The fluorescence intensity of the sample solutions at 590 nm (excitation wavelength: 365 nm) was measured at 25  $^{\circ}\text{C}$  using a spectrofluorometer (ND-3300, NanoDrop, Wilmington, DE). The fluorescence intensity of naked pDNA was set at 100% and measured against a background of EtBr without pDNA.

**Dynamic Light Scattering Measurement.** The size of the polyplex micelles was evaluated by dynamic light scattering (DLS) using Nano ZS (ZEN3600, Malvern Instruments, Ltd., UK). A He-Ne ion laser (633 nm) was used as the incident beam. Polyplex micelle solutions (50  $\mu\text{g}$  pDNA/mL) with an N/P = 2 were adjusted to a concentration of 10  $\mu\text{g}$  pDNA/mL. The data obtained at a detection angle of 173  $^{\circ}$  at 25  $^{\circ}\text{C}$  were analyzed by a cumulant method to obtain the hydrodynamic diameters and polydispersity indices ( $\mu\text{T}^2$ ) of the micelles. The results reported are expressed as the mean values ( $\pm\text{SEM}$ ) of three experiments.

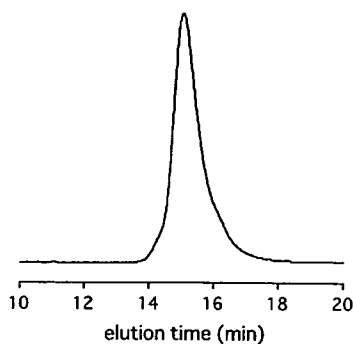
**$\zeta$ -Potential Measurement.** The  $\zeta$ -potential of polyplex micelles was evaluated by the laser-doppler electrophoresis method using Nano ZS with a He-Ne ion laser (633 nm). Sample solutions similar to those used for the DLS measurements were prepared. The  $\zeta$ -potential measurements were carried out at 25  $^{\circ}\text{C}$ . A scattering angle of 17 $^{\circ}$  was used in these measurements. The results are expressed as the mean values ( $\pm\text{SEM}$ ) of three experiments.

**Detection of  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$  Integrin Receptors.** 293T cells and HeLa cells were detached by pipetting and by harvesting

with trypsin, respectively. Both types of cells were washed twice with PBS. The  $1 \times 10^6$  cells and FITC-labeled antibody against  $\alpha_v\beta_3$  or  $\alpha_v\beta_5$  integrin (2  $\mu\text{g}$ ) were resuspended in 100  $\mu\text{L}$  of Dulbecco's Modified Eagle Medium (DMEM) (containing 10% serum) and incubated on ice for 1 h in the dark. The cells were washed three times with cold medium and, after being resuspended in PBS, were analyzed using a flow cytometer (EPICS XL, Beckman Coulter, Inc.). The cytometric data were analyzed using EXPO32 software (Beckman Coulter, Inc., Fullerton, CA).

**Transfection.** HeLa and 293T cells were respectively seeded on 24-well culture plates (10 000 cells/well) and incubated overnight in 500  $\mu\text{L}$  of DMEM containing 10% serum. After the medium was replaced with fresh medium, 20  $\mu\text{L}$  of polyplex solution (50  $\mu\text{g}$  pDNA/mL, N/P = 2) was applied to each well. The amount of pDNA was adjusted to 1  $\mu\text{g}$  per well. After incubation for various lengths of time, the medium was replaced with 500  $\mu\text{L}$  of fresh medium, followed by reincubation. The reincubation time was adjusted so the total incubation time would be 48 h. The luciferase gene expression was then evaluated based on the intensity of the photoluminescence using the Luciferase assay kit and a Luminometer (Lumat LB9507, BERTHOLD, Germany). The amount of protein in each well was concomitantly determined using a Micro BCA protein assay reagent kit. The relative light units were converted into the absolute amount of luciferase (ng) using a standard curve calibrated with recombinant luciferase (QuantiLum, Promega). One nanogram of luciferase corresponded to  $9.1 \times 10^7$  RLU in our experiments; this was defined as the conversion factor.

**Analysis of Cellular Uptake of Polyplex Micelles.** pDNA was labeled with fluorescein as previously reported (4). Briefly,



**Figure 1.** Gel permeation chromatogram of acetal-PEG-PLys(TFA) **3** (instrument TOSOH HLC-8220, detector RI, columns TSKgel G4000PWXL and G3000PWXL, eluent DMF with 10 mM LiCl, flow rate 0.8 mL min<sup>-1</sup>, temperature 40 °C).

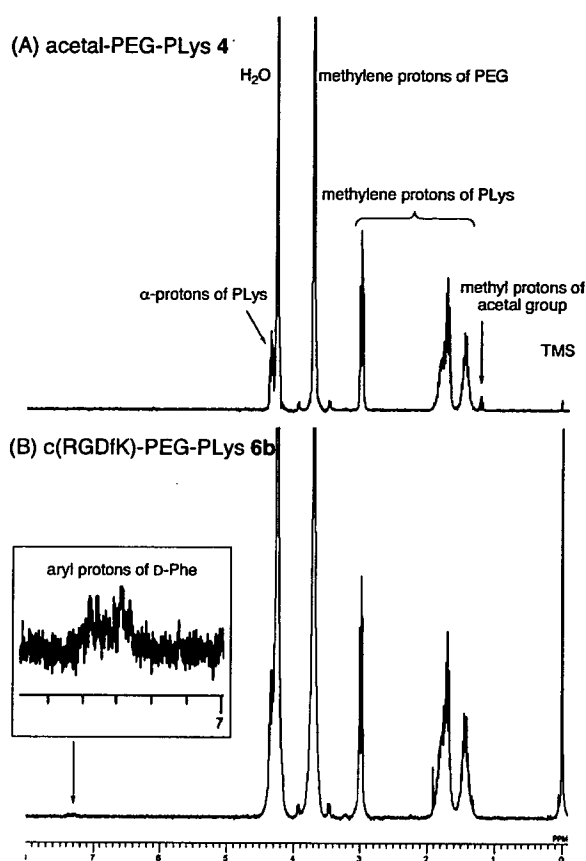
pDNA was labeled using a Label IT Nucleic Acid Labeling Kit (Mirus, Madison, WI). HeLa cells were seeded on six-well culture plates (100 000 cells/well) and incubated overnight in 1 mL of DMEM containing 10% serum. After the medium was replaced with fresh medium, 90  $\mu$ L of polyplex solution (33.3  $\mu$ g fluorescein-labeled pDNA/mL, N/P = 2) was applied to each well. The amount of fluorescein-labeled pDNA was adjusted to 3  $\mu$ g per well. After various periods of incubation, the medium was removed and the cells were washed twice with PBS. After detachment by trypsin, the cells were resuspended in PBS and analyzed using the flow cytometer.

**Confocal Laser Scanning Microscope (CLSM) Observation.** pDNA was labeled with Cy5 in the same manner as fluorescein using the Label IT Nucleic Acid Labeling Kit. HeLa cells (30 000 cells) were seeded on a 35-mm glass base dish (Iwaki, Japan) and incubated overnight in 1 mL of DMEM containing 10% serum. After the medium was replaced with fresh medium, 90  $\mu$ L of polyplex solution containing 3  $\mu$ g Cy5-labeled pDNA (N/P = 2) was applied to a glass dish. After 3 h incubation, the medium was removed and the cells were washed twice with PBS. The intracellular distributions of the polyplex micelles were observed by CLSM following acidic late endosome and lysosome staining with LysoTracker Green (Molecular Probes, Eugene, OR) and nuclear staining with Hoechst 33342 (Dojindo Laboratories, Japan). The CLSM observation was performed using LSM 510 (Carl Zeiss, Germany) with a 63 $\times$  objective (C-Apochromat, Carl Zeiss, Germany) at excitation wavelengths of 488 nm (Ar laser), 633 nm (He-Ne laser), and 710 nm (Mai Tai laser) for fluorescein, Cy5, and Hoechst 33342, respectively.

## RESULTS

### Synthesis of Peptide-Conjugated PEG-PLys (Scheme 1).

The synthesis of acetal-PEG-OMs **1** has been previously reported (14). Briefly, PDP from 3,3-diethoxypropanol and potassium naphthalene initiated the anionic polymerization of EO to form acetal-PEG-OH ( $M_n$  11 825,  $M_w$  12 089,  $M_w/M_n$  1.02), followed by the mesylation of alcohol using MsCl to obtain acetal-PEG-OMs **1**. Acetal-PEG-NH<sub>2</sub> **2** was then synthesized by amination of acetal-PEG-OMs **1** using ammonia solution and was confirmed to be unimodal with a narrow molecular weight distribution ( $M_w/M_n$  1.03). Polymerization of Lys(TFA)-NCA using acetal-PEG-NH<sub>2</sub> **2** as an initiator led to the formation of acetal-PEG-PLys(TFA) **3**. A GPC of the obtained acetal-PEG-PLys(TFA) **3** showed a single peak with a narrow molecular weight distribution ( $M_w/M_n$  1.12) (Figure 1). From the peak intensity ratio of the methylene protons of PEG ( $\text{OCH}_2\text{CH}_2$ ,  $\delta = 3.6$  ppm) and the methylene protons of Lys(TFA) ( $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}$ ,  $\delta = 1.4, 1.5, 1.9,$  and  $3.2$  ppm) measured by <sup>1</sup>H NMR, the polymerization degree (DP) of Lys-

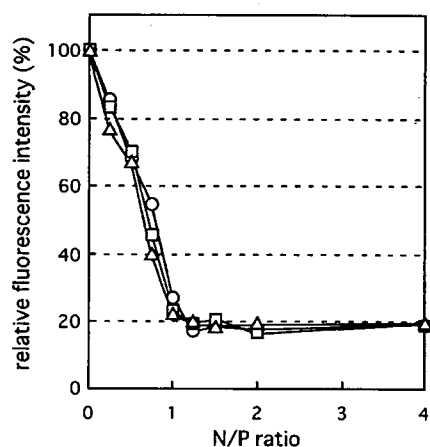


**Figure 2.** <sup>1</sup>H NMR spectra of acetal-PEG-PLys **4** (A) and c(RGDfK)-PEG-PLys **6b** (B) in D<sub>2</sub>O at 80 °C.

(TFA) was calculated to be 70 (data not shown). Acetal-PEG-PLys **4** was then quantitatively obtained by the deprotection of acetal-PEG-PLys(TFA) **3** under basic conditions. From the <sup>1</sup>H NMR spectrum, the acetal group ( $\text{CH}_3\text{CH}_2\text{O}$ ,  $\delta = 1.2$  ppm) was confirmed to be almost intact without converting to an aldehyde group (Figure 2). The DP of Lys was calculated to be 72 from the peak intensity ratio of the methylene protons of PEG ( $\text{OCH}_2\text{CH}_2$ ,  $\delta = 3.6$  ppm) and the methylene protons of PLys ( $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}_3$ ,  $\delta = 1.4, 1.7,$  and  $3.0$  ppm) (Figure 2). The DP of acetal-PEG-PLys(TFA) **3** and acetal-PEG-PLys **4** were almost identical, indicating that main chain cleavage under the deprotection reaction was negligible.

Conjugation of peptide ligands into the PEG terminus of acetal-PEG-PLys **4** was achieved through the formation of a thiazolidine ring. The acetal group was deprotected under acidic conditions to an aldehyde group, giving aldehyde-PEG-PLys **5**. The aldehyde group is known to react with cysteine to form a stable thiazolidine ring (16). In this study, CXYGGRGDS (RGDS) and cyclo[RGDfK(CX-)] (c(RGDfK)), having an N-terminal cysteine residue (C: L-Cys), were reacted with acetal-PEG-PLys **4** under mild acidic conditions (pH 4.0) to prepare the peptide-conjugated PEG-PLys through thiazolidine formation. Note that Schiff base formation due to the reaction of the aldehyde and primary amino groups of the PLys segment was prohibited under the acidic conditions because of the complete protonation of the primary amino groups. The methyl protons of the acetal group ( $\delta = 1.2$  ppm) completely disappeared with the appearance of protons assigned to the aromatic rings of L-tyrosine (Y: L-Tyr) ( $\delta = 6.9$  and  $7.1$  ppm) in the RGDS and D-phenylalanine (f: D-Phe) ( $\delta = 7.3$  and  $7.4$  ppm) in the c(RGDfK) (Figure 2). Based on the peak intensity ratios of the aromatic ring protons of peptide ligands and the methylene





**Figure 3.** Ethidium bromide exclusion assays of PEG-PLys (open circle), RGDS-PEG-PLys (open square), and c(RGDfK)-PEG-PLys (open triangle). Fluorescence measurements were carried out as described in the Experimental Procedures.

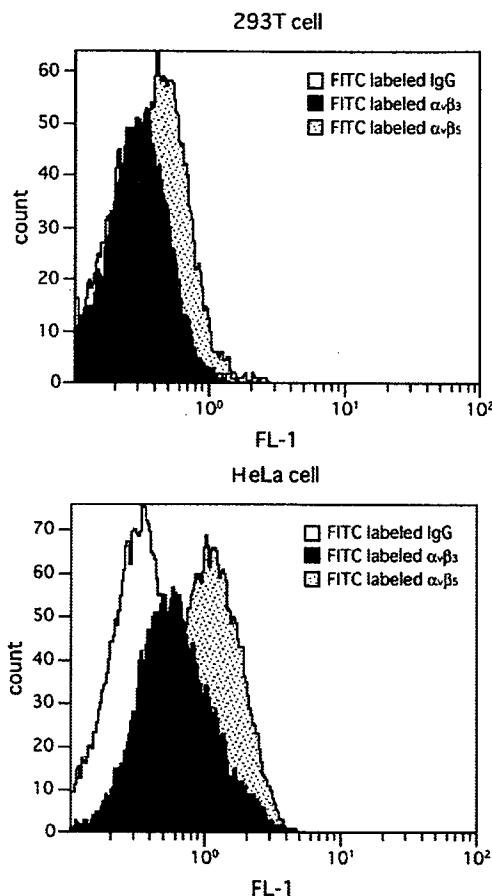
**Table 1.** Size and  $\zeta$ -Potentials of Polyplex Micelles

sample	cumulant diameter (nm)/ polydispersity index ( $\mu/\Gamma^2$ )	$\zeta$ -potential (mV)
PEG-PLys	124 $\pm$ 0.27/0.158 $\pm$ 0.014	-0.19 $\pm$ 0.195
RGDS-PEG-PLys	124 $\pm$ 0.88/0.177 $\pm$ 0.012	1.12 $\pm$ 0.349
c(RGDfK)-PEG-PLys	125 $\pm$ 1.00/0.166 $\pm$ 0.006	0.68 $\pm$ 0.238

protons of PEG ( $\text{OCH}_2\text{CH}_2$ ,  $\delta = 3.6$  ppm), the introduction rates of peptide ligands were determined to be 100% in the RGDS-PEG-PLys **6a** and 66% in the c(RGDfK)-PEG-PLys **6b**.

**Formation of Polyplex Micelles.** Ethidium bromide (EtBr) is known to form an intercalating complex with double helical polynucleotides and subsequently show a striking enhancement of its fluorescence intensity (17). This enhancement is quenched by the formation of a complex between DNA and cationic components, because cationic components prevent EtBr from intercalating into the double-strand DNA. Thus, EtBr exclusion assay is frequently utilized to estimate the degree of pDNA condensation in the complex with a cationer (18, 19). As shown in Figure 3, as the N/P ratio increased, the fluorescence intensity in all the polyplex micelles decreased correspondingly and leveled off at around N/P = 1.25. This result indicates that the degree of pDNA condensation was not influenced by the introduction of peptide ligands. Table 1 summarizes the cumulant diameters and  $\zeta$ -potentials of the polyplex micelles at N/P = 2. The cumulant diameters of all the micelles were approximately 125 nm with a moderate polydispersity index between 0.15 and 0.18. There was no change in the particle size because of the introduction of peptide ligands. Also, the  $\zeta$ -potentials of all the polyplex micelles were approximately 0 mV. As previously reported (5, 18), the PEG palisade surrounding the complex shields the charge of the micelles to maintain a very small absolute value in the  $\zeta$ -potential even in the region of N/P > 1. All of the results of the EtBr assay, DLS analysis, and  $\zeta$ -potential measurement suggest that the characteristics of the three types of polyplex micelles (PEG-PLys, RGDS-PEG-PLys, and c(RGDfK)-PEG-PLys) were quite similar regardless of the introduction of the peptide ligands.

**Detection of  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$  Integrin Receptors.** To evaluate the expression of integrin receptors on the cell surface, flow cytometric analysis of 293T and HeLa cells was carried out using FITC-labeled antibodies (Figure 4). Flow cytometric analysis revealed that the 293T cells expressed almost no  $\alpha_v\beta_3$  integrin and a slight amount of  $\alpha_v\beta_5$  integrin, while the HeLa cells expressed a considerably higher amount of  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$  integrins than the 293T cells. These results implied that HeLa

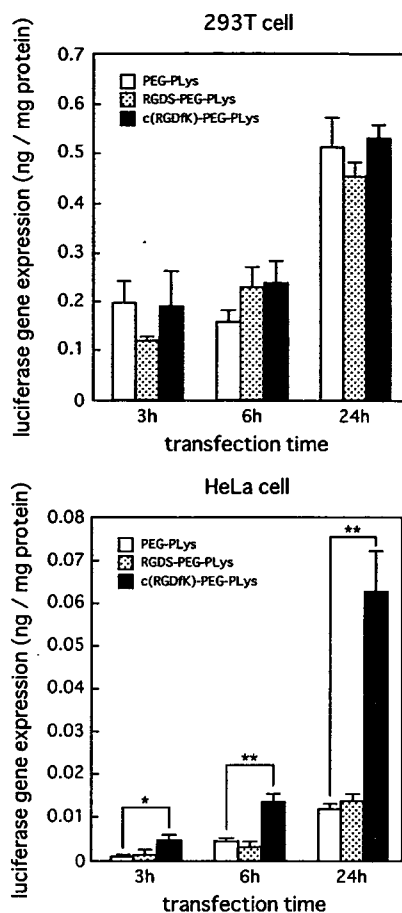


**Figure 4.** Flow cytometric analysis of integrin expression on 293T cells (left) and HeLa cells (right).

cells might show a higher uptake of the cyclic RGD peptide-conjugated polyplex micelles than 293T cells through integrin-mediated endocytosis.

**Transfection.** *In vitro* TE of polyplex micelles was evaluated for 293T cells and HeLa cells at varying transfection times (Figure 5). PEG-PLys/pDNA micelles without the peptide ligand and RGDS-PEG-PLys/pDNA micelles with a linear RGD peptide ligand were used as controls. RGD peptide is the adhesion motif of extracellular matrix proteins for the various types of integrins (20, 21). The linear RGD peptide showed a binding affinity for  $\alpha_v\beta_3$  integrin approximately 1/1000 of that of the cyclic RGD peptide (22). In the transfection experiment using 293T cells, there were no differences in the TE of the three types of micelles at any of the transfection times. This finding is consistent with the observation that 293T cells showed low integrin expression (Figure 4). On the other hand, in the transfection experiment using HeLa cells, c(RGDfK)-PEG-PLys micelles achieved a significantly higher TE than PEG-PLys and RGDS-PEG-PLys micelles. It is reasonable to assume that HeLa cells might efficiently recognize c(RGDfK) ligands on the micelle through  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$  integrins expressed on their surface. Nevertheless, the TE of linear RGD peptide-conjugated micelles toward HeLa cells was comparable to that of the micelles without any ligand, suggesting that the binding affinity of the linear RGD peptide for the integrins might be insufficient to increase the TE.

**Analysis of Cellular Uptake of Polyplex Micelles.** The cellular uptake of the micelles into the HeLa cells was evaluated by a flow cytometer using fluorescein-labeled pDNA-incorporated micelles with varying incubation times (Figure 6). The amount of the uptake was always higher for c(RGDfK)-PEG-



**Figure 5.** Effect of cell lines and transfection time on gene expression. 293T cells (left) and HeLa cells (right) were transfected with PEG-PLys, RGDS-PEG-PLys, and c(RGDfK)-PEG-PLys micelles prepared at N/P = 2 in a medium containing 10% serum. The data are the mean  $\pm$  SEM,  $n = 4$ .  $P^* < 0.05$  and  $P^{**} < 0.01$ .

PLys micelles than for PEG-PLys micelles at each incubation time, which suggests that the specific interaction of the cyclic RGD peptide with integrin receptors on the HeLa cells contributed to the uptake amount. In 24 h incubation, the amount of c(RGDfK)-PEG-PLys micelles that were taken up was 1.6 times the amount of PEG-PLys micelles taken up (Figure 6B). Nevertheless, c(RGDfK)-PEG-PLys micelles revealed an even higher TE of approximately 5 times that of the PEG-PLys micelles (Figure 5). This implied that other factors besides the enhanced uptake might also play a role in the enhanced TE observed for HeLa cells challenged with c(RGDfK)-PEG-PLys micelles.

**Intracellular Distribution of Polyplex Micelles.** The intracellular distribution of polyplex micelles was investigated by CLSM using Cy5-labeled pDNA-incorporated micelles with 3 h incubation, and typical images from this investigation are shown in Figure 7. A large fraction of the Cy5-labeled pDNA was still distributed near the cell membrane for the cells challenged with PEG-PLys polyplex micelles (Figure 7A). Alternatively, an appreciable accumulation of the Cy5-labeled pDNA in the perinuclear regions was observed for the cells challenged with c(RGDfK)-PEG-PLys polyplex micelles (Figure 7B). Further detailed observation using LysoTracker, which labels acidic late endosome and lysosome, revealed that the majority of c(RGDfK)-PEG-PLys polyplex micelles were localized in the acidic compartments as indicated by the yellow color in the merged fluorescence image of the Cy5-labeled pDNA (red) and LysoTracker (green), yet a fraction that did

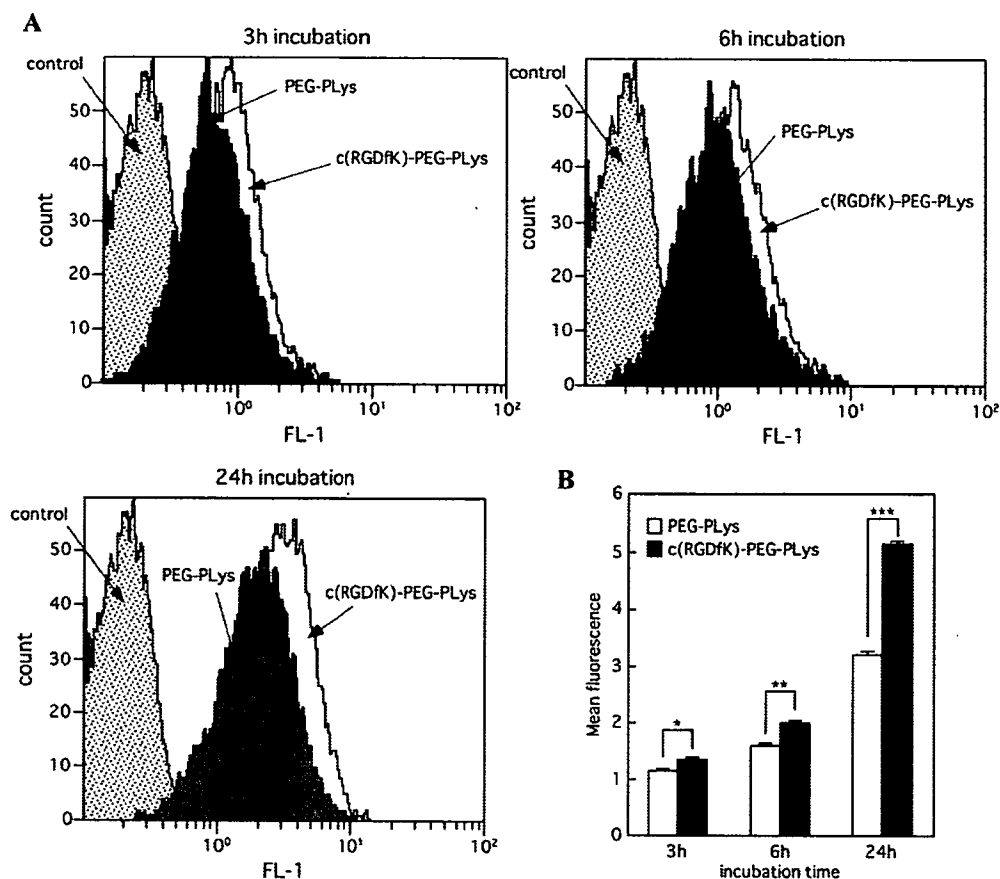
not co-localize with LysoTracker was also observed, suggesting that at least some of the polyplex micelles in the perinuclear regions may be located in a site other than the lysosomes.

## DISCUSSION

There are many barriers to achieve effective transfection using nonviral gene vectors. Stability in the bloodstream and specific cellular uptake by the target tissues and organs are major obstacles, especially for *in vivo* gene delivery. Polyplex micelles with PEG as a hydrophilic shell layer have been shown to achieve an increased retention time in the bloodstream (5), and they are promising candidates for vectors that can be used for *in vivo* gene delivery. Also, the introduction of targetable ligands onto polyplex micelles has been reported, and some of the ligand-installed polyplex micelles have been demonstrated to be effective both in *in vivo* as well as *in vitro* transfection (6–8). The ligands on polyplex micelles may facilitate their internalization into the target cells, thus increasing the TE. A number of studies have aimed to find suitable ligands for this purpose (23), and cyclic RGD peptides have been highlighted as one of the promising candidates (7). Cyclic RGD peptides are well-known to selectively recognize  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$  integrins, which are overexpressed in angiogenic endothelial cells in tumors. Therefore, a targetable gene delivery system equipped with a cyclic RGD peptide as the ligand may be useful for antiangiogenic therapy for tumors (24, 25).

In this study, to construct polyplex micelles showing integrin-mediated gene transfection, the novel synthetic route of a block copolymer with a c(RGDfK) peptide ligand was exploited as summarized in Scheme 1. The acetal group may be converted to an aldehyde group under acidic conditions. Thus, the TFA group was selected to protect the primary amino group of the lysine units, because it can be removed under basic conditions without any conversion of acetal groups to aldehyde groups. The introduction of peptide ligands was then carried out under mild acidic conditions with good yields in a one-pot reaction involving the conversion of acetal groups to aldehyde groups and the subsequent conjugation of the ligand through the formation of a thiazolidine ring as seen in Scheme 1. In this way, various peptide ligands having a cysteine end group (Cys-peptide) may be readily introduced into the block copolymer. Moreover, this type of conjugation reaction between aldehyde and Cys-peptide occurs selectively even in the presence of primary amines, because the formation of a Schiff base between a primary amine and an aldehyde is reversible and thus sensitive to pH, while the thiazolidine ring formation between the N-terminal cysteine and aldehyde is an irreversible reaction. This reaction is available for the introduction of various ligands into the block copolymer, not only peptides but also other ligand molecules possessing an N-terminal cysteine.

Indeed, as confirmed by the NMR spectra shown in Figure 2, the c(RGDfK) peptide, which can peculiarly recognize  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$  integrins, was successfully introduced into the PEG terminus of the PEG-PLys block copolymer as a targetable ligand molecule. The c(RGDfK)-PEG-PLys/pDNA micelle achieved a higher TE compared to nontargetable PEG-PLys and RGDS-PEG-PLys micelles against HeLa cells, which express an appreciable amount of  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$  integrins. In contrast, there was no difference in TE among these three types of polyplex micelles against 293T cells, which show a limited expression of  $\alpha_v\beta_5$  integrin, but no expression of  $\alpha_v\beta_3$  integrin. This result is consistent with a previous report which found that the  $\alpha_v\beta_3$  integrin has an approximately 10-times higher binding affinity for the cyclic RGD peptide than the  $\alpha_v\beta_5$  integrin (26), which suggests that the  $\alpha_v\beta_3$  integrin may be involved in an increased TE against HeLa cells by means of the c(RGDfK)-PEG-PLys/pDNA micelle. Flow cytometric analysis (Figure

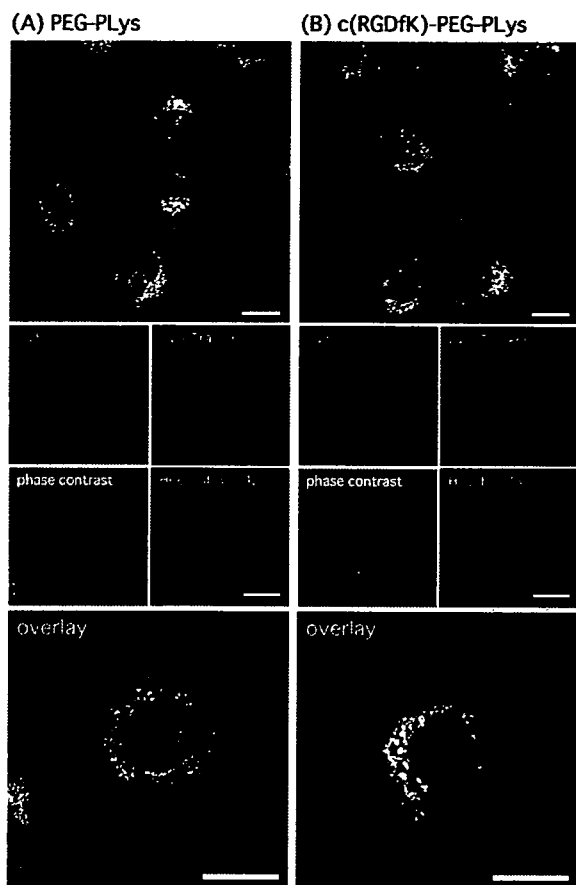


**Figure 6.** Cellular uptake of polyplex micelles. PEG-PLys and c(RGDfK)-PEG-PLys micelles loaded with fluorescein-labeled pDNA were applied to HeLa cells with varying incubation times. (A) Flow cytometric histogram profiles of time-dependent change in fluorescence intensity. (B) Mean fluorescence intensity at each incubation time. The data are the mean  $\pm$  SEM,  $n = 3$ .  $P^* < 0.05$ ,  $P^{**} < 0.01$ , and  $P^{***} < 0.001$ .

6) also suggests that the enhanced uptake of the c(RGDfK)-PEG-PLys/pDNA micelle might contribute to an increased TE against HeLa cells. Interestingly, the difference in the uptake ratio against the HeLa cells between the c(RGDfK) micelle and the ligand-free micelle became more significant with increased incubation time as seen in Figure 6. Integrin receptors are known to recycle to the plasma membrane through an endocytic cycle every 15–40 min (27). Consequently, this fast recycle of integrins might contribute to the facilitated uptake of the c(RGDfK) micelle into the HeLa cells in comparison with the ligand-free micelle. Nevertheless, as can be seen from Figures 5 and 6B, the uptake ratio is not simply correlated in a time-dependent manner with the TE. The ligand effect appeared to be more significant in the TE than in the uptake ratio, particularly after a prolonged time period such as 24 h. This result led us to assume that the c(RGDfK) ligand may also affect intracellular trafficking of the polyplex micelles. Indeed, a CLSM observation of HeLa cells challenged with polyplex micelles revealed a substantial difference in intracellular distribution between the PEG-PLys and c(RGDfK)-PEG-PLys micelles (Figure 7). In the cells challenged with the PEG-PLys polyplex micelle, a majority of pDNA was still distributed near the cell membrane, whereas in the same time frame of 3 h the cells with the c(RGDfK)-PEG-PLys polyplex micelle showed an appreciable accumulation of pDNA in the perinuclear region. It is noteworthy that a definite fraction of pDNA (colored red) in the perinuclear region did not co-localize with LysoTracker (colored green), suggesting that this fraction may be located in a compartment other than the acidic late endosome and lysosome. The  $\alpha_v\beta_3$  integrin receptors have been reported to pass rapidly through the early endosomes, arriving at the

perinuclear compartments approximately 30 min after internalization (28). Recent reports have shown an active transport pathway for a nonviral gene delivery system (29, 30), in which microtubule-associated motor proteins have contributed to a rapid perinuclear accumulation of the polyplex within minutes after transfection. Also, recent studies have revealed that polyplexes in the perinuclear region but not in the acidic compartments significantly contribute to the effective transfection (31, 32). These phenomena are consistent with our observation of a partial accumulation of the c(RGDfK)-PEG-PLys micelle in the perinuclear sites but outside of the acidic compartments, suggesting that the enhancement of the TE of the polyplex micelle by the introduction of cyclic RGD peptide ligands might be due not only to the enhanced uptake but also to the change in the intracellular trafficking route directed to the nucleus. It is likely that the micelle fraction distributed in nonacidic compartments near the nucleus may efficiently internalize into the nucleus when the cells are in the dividing phase, thus contributing to the increase in the transfection. Although the effective transport of the PLys-based polyplex micelles from the isolated compartment into the cytoplasm remains an issue because of inefficiency in the PLys function of facilitating the endosome-escaping step, the conjugation of c(RGDfK) ligands onto the polyplex micelle surface has great potential to improve the TE through modulated intracellular trafficking.

In conclusion, a successful synthetic route involving block copolymers conjugated with peptidyl ligands through thiazolidine ring formation was developed in this study. Cyclic RGD peptide-conjugated polyplex micelles showed an increased TE against HeLa cells possessing  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$  integrins because



**Figure 7.** Intracellular distribution of pDNA challenged by the polyplex micelles. PEG-PLys and c(RGDfK)-PEG-PLys micelles loaded with Cy5-labeled pDNA (red) were incubated with HeLa cells for 3 h. The CLSM observation was performed using a 63 $\times$  objective. The cell nuclei were stained with Hoechst 33342 (blue), and the acidic late endosome and lysosome were stained with LysoTracker Green (green). The scale bar represents 20  $\mu$ m.

of enhanced uptake and a possible change in the route of intracellular trafficking. These results indicate that the introduction of various peptide ligands including c(RGDfK) peptide into the block copolymers is feasible and could eventually allow the construction of targetable polyplex micelles that are useful for site-specific gene therapy through a systemic route.

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