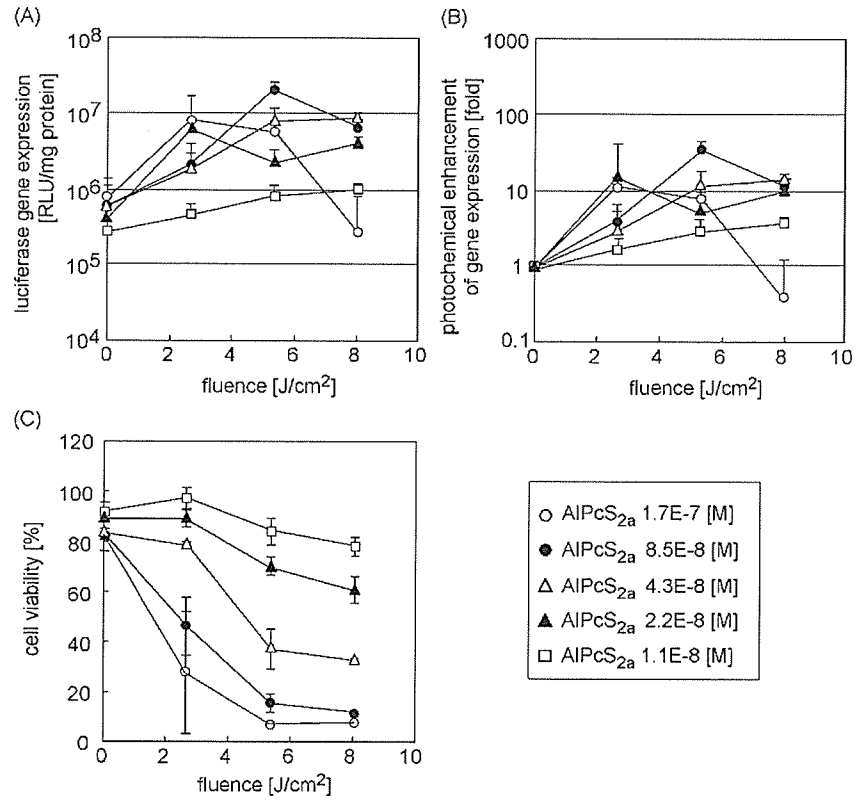

Figure 8. The effects on the $AlPcS_{2a}$ concentration and fluence on the transfection efficiency (A), photochemical enhancement of the gene expression (B) and photocytotoxicity (C) in the PCI-mediated transfection using $AlPcS_{2a}$. The light irradiation was performed 6 h after incubation with the pDNA-incorporated micelles and $AlPcS_{2a}$, followed by 48 h post-incubation in a fresh medium.

In this regard, we have recently developed DPc as a new photosensitizer for the PCI (Nishiyama et al. 2005). It has been demonstrated that the anionic DPc might electrostatically interact with the surface of the cationic polyplex to form the ternary complex enveloped with DPc, thus giving the same internalization and subcellular localization between DPc and the polyplex. Meanwhile, DPc exhibited a pH-dependent membrane binding ability, allowing the selective photodamaging of the endosomal membrane. Consequently, the ternary complex showed more than 100-fold photochemical enhancement of the gene expression with reduced photocytotoxicity on the cultured cells and *in vivo* light-selective gene transfer to the conjunctival tissues in rat eyes (Nishiyama et al. 2005). Thus, it was suggested that the design of the ternary complex might be effective for the PCI-mediated gene delivery; however, the ternary complex system is unlikely to be used for the systemic delivery. It is known that highly negatively-charged macromolecules can be excluded from the blood circulation due to the hepatic uptake by scavenger receptors of the non-parenchymal cells (Takakura and Hashida 1996).

In this study, the combinational formulation of the pDNA- and DPc-incorporated micelles was used in the PCI-mediated transfection. Polymeric micelles, of which the drug-loaded core is covered with a dense and hydrophilic PEG palisade, are characterized by their small size (less than 100 nm), excellent biocompatibility and stability in biological media (Kataoka et al. 1993, 2001; Nishiyama and Kataoka, 2006). We have so far demonstrated that polymeric micelles incorporating antitumor drugs can stably circulate in the bloodstream and effectively accumulate in solid tumors (Kwon et al. 1994; Nishiyama et al. 2003; Bae et al. 2005). Accordingly, in this study, we have used polymeric micelles as nanocarriers for the pDNA and DPc delivery, expecting their future application in systemic administration. In particular, this paper focused on demonstrating the feasibility of our strategy using the combinational formulation of the pDNA- and DPc-incorporated micelles in the PCI-mediated transfection *in vitro*. In this strategy, both polymeric micelles are assumed to be taken up by the cell through the endocytic pathway and show the same subcellular localization, allowing selective photochemical damaging of the endosomal membrane and effective cytoplasmic delivery of the pDNA-incorporated micelles upon photoirradiation, which might be a key to the successful PCI-mediated transfection. Indeed, the PCI using the DPc-incorporated micelles achieved more than 200-fold photochemical enhancement of the gene transfection of the polyplex micelles. Importantly, the PCI using the combinational micellar formulation achieved a wide range of safe DPc

Table I. Photochemical enhancement of the gene expression and cell viability in the PCI-mediated transfection using each photosensitizer (fluence: 5.4 J/cm^2).

DPc-loaded micelle	DPc conc. (μM)	0.64	0.32	0.16	0.08
	(a) Photochemical enhancement (fold)	72.6 ± 30	211 ± 150*	123 ± 58***	55.7 ± 25
	(b) Cell viability (%)	50.7 ± 1.9	73.8 ± 6.4**	81.2 ± 4.8	86.1 ± 11
DPc	DPc conc. (μM)	0.64	0.32	0.16	0.08
	(a) Photochemical enhancement (fold)	47.1 ± 20	117 ± 16**	64.3 ± 13***	34.2 ± 4.5
	(b) Cell viability (%)	15.7 ± 2.0	18.0 ± 2.3**	73.8 ± 5.8	92.0 ± 4.0
$AlPcS_{2a}$	$AlPcS_{2a}$ conc. (μM)	0.17	0.085	0.043	0.022
	(a) Photochemical enhancement (fold)	8.09 ± 8.9	35.5 ± 8.9*, **	12.9 ± 6.0***	5.63 ± 2.8
	(b) Cell viability (%)	7.15 ± 1.0	15.9 ± 3.7**	37.8 ± 7.8	71.4 ± 3.8

*$P > 0.05$; **$P < 0.01$; and ***$P < 0.05$ ($n = 4$, unpaired t-test).

concentrations and light doses, in which remarkable enhancement of the transfection was achieved without a significant decrease in the cell viability. Such expanded ranges of safe DPc concentrations and light doses should be favorable for the *in vivo* PCI-mediated gene delivery.

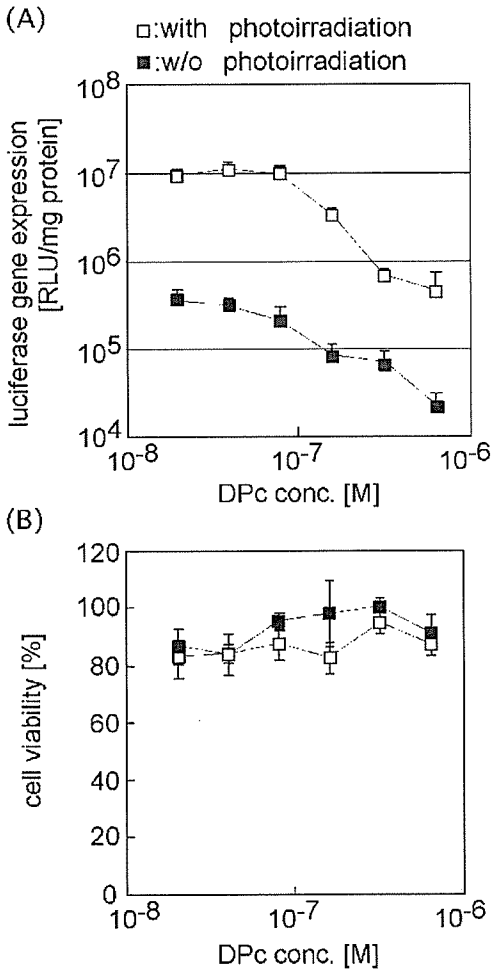

Figure 9. The transfection efficiency (A) and photocytotoxicity (B) in the PCI-mediated transfection using the DPc-incorporated micelles. The light irradiation (fluence: 5.4 J/cm^2) was performed after prolonged incubation (i.e. 24 h) with the pDNA- and DPc-incorporated micelles, followed by 48 h post-incubation in a fresh medium.

In conclusion, polymeric micelles might be a useful nanocarrier, which has been demonstrated to show prolonged blood circulation and thereby accumulate in the target tissues after intravenous administration, motivating us to study the PCI-mediated transfection using the combinational formulation of the pDNA- and DPc-incorporated micelles. Indeed, the PCI using the combinational micellar formulation achieved a remarkable photochemical enhancement of the transgene expression while maintaining an approximately 80% cell viability over a wide range of DPc concentrations and light doses. Thus, the usefulness of our strategy for *in vitro* trasnfection was successfully demonstrated. This system can be potentially useful for the gene therapy of solid tumors and ophthalmic diseases such as age-related macular regeneration (AMD) (Ideta et al. 2005). In addition to therapeutic genes, small interfering RNA (siRNA), which is known as the most powerful tool for sequence-specific silencing of the target genes (Elbashir et al. 2001), might also be delivered in a light-inducible manner by this strategy. The target-selective delivery of the therapeutic genes and siRNA

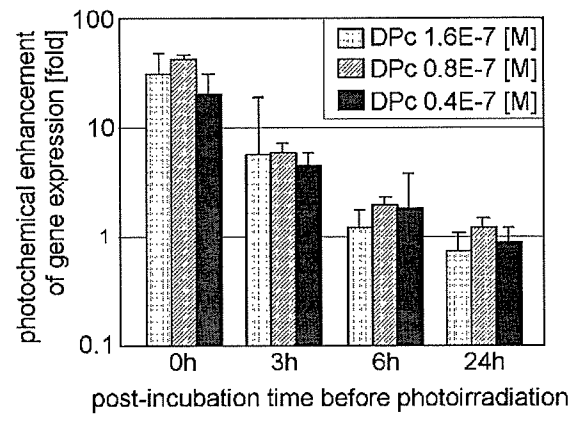

Figure 10. The effect of post-incubation time in the micelle-free medium before photoirradidation on the photochemical enhancement of the transfection of the polyplex micelles in the PCI-mediated transfection using the DPc-incorporated micelles. The cells were photoirradiated (5.4 J/cm^2) at defined post-incubation time after 6 h incubation with the pDNA- and DPc-incorporated micelles and medium replacement with a fresh one, followed by 48 h post-incubation.

by micellar nanocarriers followed by their light-induced activation at the target site might ensure the safety and effectiveness of *in vivo* gene and siRNA therapy. At present, this strategy could be applied only to the tissues, to which a visible light is accessible; however, recent advances in the laser technology such as a two-photon excitation laser (Oh et al. 1997) will solve the problems of limited tissue penetration of the light in the near future. We are now investigating *in vivo* PCI-mediated gene delivery using polymeric micelles via systemic administration and the results will be reported in a forthcoming paper.

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PEGylated gene nanocarriers based on block catiomers bearing ethylenediamine repeating units directed to remarkable enhancement of photochemical transfection

Arnida [a], Nobuhiro Nishiyama [b,c,*], Naoki Kanayama [a,d], Woo-Dong Jang [e], Yuichi Yamasaki [a,c,d], Kazunori Kataoka [a,b,c,d,*]

[a] *Department of Materials Engineering, Graduate School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan*
[b] *Center for Disease Biology and Integrative Medicine, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan*
[c] *Center for NanoBio Integration, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan*
[d] *Core Research for Evolutional Science and Technology (CREST) from the Japan Science and Technology Agency (JST), Japan*
[e] *Department of Chemisty, College of Science, Yonsei University, 134 Sinchondong, Seodaemun-gu, Seoul 120-749, Korea*

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Abstract

The therapeutic usefulness of macromolecular drugs such as plasmid DNA is often limited by the inefficient transfer of macromolecules to the cytosol. Photochemical internalization (PCI) technology, in which the endosomal escape of DNA or its complex is assisted by co-incubated photosensitizers that photodamage endosome membrane, offers a solution for this problem. A series of poly(ethylene glycol) (PEG)-based block polycatiomers with increasing number of ethylenediamine repeating unit at side chain of polycatiomers were complexed with pDNA to form the PEGylated polyplexes as a biocompatible gene carrier. Dendrimeric phthalocyanine (DPc)-incorporated micelle was used to assist the gene transfer of these polyplexes in a light-inducible manner. As a result, the light-inducible transfection activity was significantly enhanced as the number of amino group at the side chain of PEG-*b*-polycatiomer increased. The polyplex from PEG-*b*-polycatiomer having the longest ethylenediamine structure achieved approximately 1000-fold enhancement of transfection upon photoirradiation. This result supports the underlying hypothesis that photochemical transfection and proton sponge effect of polycations can work synergistically to enhance the transfection efficiency. With careful balance between photochemical transfection enhancement and cytotoxicity, PEG-*b*-polycatiomers used in this study might be a potential candidate for *in vivo* PCI-mediated gene transfer.

Keywords: Gene delivery; Polyplex; Polymeric micelle; Photochemical internalization; Photosensitizer

1. Introduction

Gene therapy is a promising approach to conquer intractable diseases for which there is little hope of finding conventional cure. But it still poses several hurdles that need to be overcome before it could enter a clinical phase. Gene therapy mostly depends on the development of the methods for efficient and specific delivery of the gene into the target cells [1–3]. Using viruses as a vector is limited by the safety factor and difficulties in production. By contrast, synthetic gene carriers are versatile and safe, however, its transfection ability might be substantially lower than viral vector [4]. A great deal of challenges in developing non-viral vectors, especially polycatiomer based gene carriers are ongoing in the world.

In non-viral or polycatiomer-mediated gene delivery, endosomal membrane could be a major biological barrier that should be overcome to deliver DNA or its complex to cytosol. In this regard, polyethylenimine (PEI) has been successfully used for the transfection of various types of cells. The early escape of the PEI/DNA complexes from the endosome, arising from "proton

* Corresponding authors. Kataoka is to be contacted at Department of Materials Engineering, Graduate School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan. Nishiyama, Center for Disease Biology and Integrative Medicine, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan. Tel.: +81 3 5841 7138; fax: +81 3 5841 7139.
E-mail addresses: nishiyama@bmw.t.u-tokyo.ac.jp (N. Nishiyama), kataoka@bmw.t.u-tokyo.ac.jp (K. Kataoka).

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sponge effect" was postulated to be the cause of high transfection efficiency [5–7]. However, the polyplexes based on PEI might usually contain an excess of free polymer that is not complexed with DNA. Ultrafiltration or size exclusion chromatography (SEC) can remove free PEI from the polyplexes; however, the purified polyplexes displayed lower transfection efficiency at low DNA concentration [8].

On the other hand, Berg et al. have introduced a novel technology called "photochemical internalization (PCI)", which allows the endosomal escape of the polyplexes in a light-inducible manner. This technology is based upon light activation of photosensitizer specifically locating at the membrane of endocytic vesicles and photochemically disrupting the membrane to release the content from endosome to cytoplasm [9–12]. This method enables the site-specific gene expression in a light-sensitive manner. Indeed, this strategy allowed the light-induced transfection; however, the enhancement of gene expression was accompanied by the photocytotoxicity [11]. The photodamage to sensitive organelles other than endosomal membrane, e.g., the plasma and mitochondrial membranes might be responsible for such photocytotoxicity [13]. Hence, increasing the selectivity of the photodamage to the endosome/lysosome is assumed to lead to the photochemical enhancement of transfection with reduced cytotoxicity. Recently, a ternary complex enveloped with anionic dendrimer phthalocyanine photosensitizer has been developed to achieve the PCI-mediated gene delivery [14]. The ternary complexes showed an expanded range of safe light dose where the photochemical enhancement of the transfection was achieved with a minimal photocytotoxicity, resulting in the success of this system for PCI-mediated gene delivery *in vivo* by local injection [14]. However, this ternary complex system is unlikely to be used for the systemic delivery due to its highly negative charges, which might be recognizable by the scavenger receptor [15]. Hence, it might be required to develop the light-responsive gene carrier applicable for the systemic delivery.

Alternatively, we have developed a biocompatible gene carrier, polyplex micelle based on the micellar assembly of the polyion complex (PIC) with block copolymer consisting poly (ethylene glycol) (PEG) and polycation segments. Their excellent properties for *in vitro* and *in vivo* application have been confirmed such as increased nuclease resistance and high stability under physiological conditions [16,17]. However, the polyplex micelles formed from PEG-*block*-poly(L-lysine) (PEG-*b*-PLL) copolymers possess significantly low transfection ability, which might be due to their inefficient transport from the endosome/lysosome to the cytosol [18]. In this regard, the feasibility of the use of the combination of polymeric micelles incorporating pDNA and dendrimer phthalocyanine (DPc) photosensitizer for PCI-mediated gene delivery has been carried out using PEG-*b*-PLL as carriers. The usefulness of this system for transfection enhancement *in vitro* was successfully demonstrated [19]. This system might be useful for *in vivo* application after systemic delivery. The schematic illustration of this strategy is shown in Fig. 1.

Motivated by this success, we tried to find another vector for pDNA other than PEG-*b*-PLL that might show higher photochemical transfection efficiency. In addition, we also intend to elucidate the structure–photochemical transfection efficiency

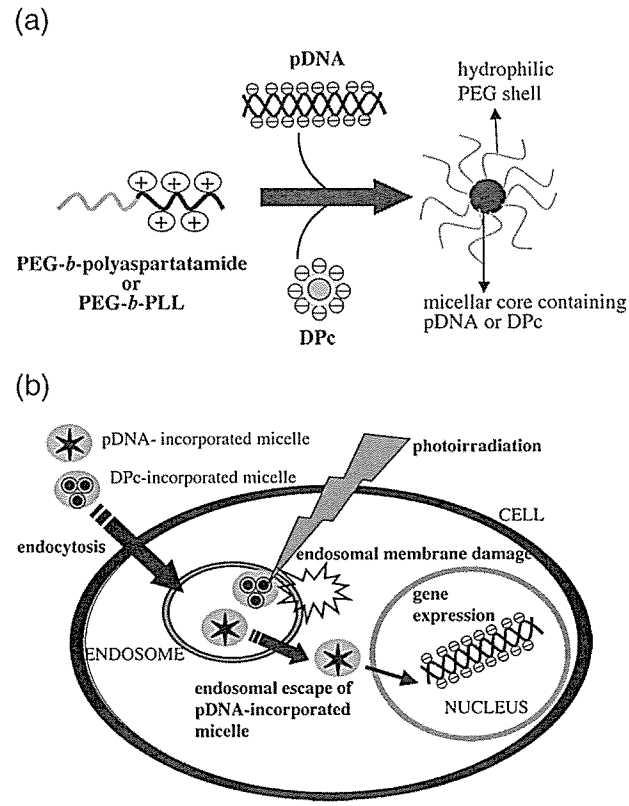

Fig. 1. Schematic illustration of (a) formation of pDNA- and DPc-incorporated micelles through electrostatic interaction between PEG-polycatiomer and pDNA or negatively charged DPc; (b) intracellular trafficking of the pDNA- and DPc-incorporated micelles in the PCI-mediated transfection. Both polymeric micelles are assumed to be taken up by the cell through the endocytic pathway. The localization of the DPc-incorporated micelles in the endosome may allow the selective photodamaging to the endosomal membrane upon photoirradiation, thereby inducing the cytoplasmic delivery of the pDNA-incorporated micelles.

relationship. In the present study, a series of PEG-*b*-polycatiomers bearing a different number of ethylenediamine repeating units at the side chain were used. Recently, we have successfully synthesized PEG-*b*-polyaspartatamide copolymers carrying the ethylenediamine unit at the side chain, which showed an appreciable buffering capacity under endosomal acidic conditions [20]. We hypothesize that the use of buffering polycations may assist the photochemical disruption of the endosomal membrane, thereby accelerating the cytoplasmic delivery of the polyplex micelles upon photoirradiation. The combination of polyplex micelles and DPc micelles was used for PCI-mediated gene transfer in this study.

2. Experimental section

2.1. Materials

N-[*tert*-Butoxycarbonyl (*Z*)]-L-lysine and bis(trichloromethyl)carbonate (triphosgene), for the synthesis of PEG-*b*-PLL diblock catiomers, were purchased from Sigma Aldrich Co., Inc. (St. Louis, MO) and Tokyo Kasei Co., Ltd. (Tokyo, Japan), respectively. β-Benzyl-L-aspartate-*N*-carboxy anhydride (BLA-NCA) and α-methoxy-ω-aminopoly(ethylene glycol)

(MeO-PEG-NH_2, Mw=12 kg/mol) were obtained from Nippon Oil and Fats, Co., Ltd. (Tokyo, Japan). Amine reagents used in aminolysis reaction were purchased from Tokyo Kasei Kogyo, Co., Ltd. (Tokyo, Japan). Chemicals for dendrimer synthesis were purchased from Tokyo Kasei Kogyo and Sigma Aldrich, Co. Inc (St. Louis, MO). *n*-Pentanol and 1,8-diazabicyclo-(5,4,0)-undec-7-ene (DBU) were purchased from Tokyo Kasei Kogyo, and used without further purification. All solvents for the polymer synthesis were distilled before use.

2.1.1. Synthesis of polymers

PEG-*block*-poly(β-benzyl-L-aspartate) (PEG-*b*-PBLA) was prepared according to the previously reported method [20]. PEG-*b*-polyaspartatamide cationers carrying ethylenediamine repeating units at the side chain were prepared through the quantitative aminolysis reaction of PEG-*b*-PBLA in dry DMF at 40 °C for 24 h in the presence of 50-fold molar of ethylenediamine (EDA), diethylenetriamine (DET), triethylenetetramine (TET) and tetraethylenepentamine (TEP) according to the previously reported method [20]. The details of the synthetic procedures and the confirmation of the chemical structures of the synthesized PEG-*b*-polycation copolymers are shown in Figs. S1, S2, S3, S4, S5 and Table S1 in Supporting information.

Synthesis of ionic dendrimer phthalocyanine (DPc) was performed according to the method reported by Ng's group [21]. The second generation of dendritic phenol was reacted with 4-nitrophthalonitrile by an alkali-mediated coupling reaction to obtain the corresponding dendritic phthalonitrile, which was then treated with Zn $(OAc)_2$ and DBU in *n*-pentanol to give the dendrimer phthalocyanine. The dendrimer phthalocyanine thus obtained was treated with a THF/H_2O mixture solution containing NaOH to obtain ionic dendrimer phthalocyanine (DPc; Mw: 4904). The chemical structure of DPc was shown in Fig. 2c.

2.1.2. Preparation of DPc-incorporated polyion complex

The given amount of DPc and PEG-*b*-PLL (Mw of PEG=12,000, repeating unit of PLL=49) (Fig. 2b) was separately dissolved in NaH_2PO_4 (10 mM, pH 4.81 and Na_2HPO_4 (10 mM, pH 9.20) solutions, respectively, and then mixed at a stoichiometric ratio to give a final concentration of DPc 1 mg/ml [22]. The resulting micelle is spherical, with a diameter of ca. 50 nm and narrow size distribution [22].

2.1.3. Preparation of pDNA-incorporated polyplex micelles

Block copolymer and plasmid pCAcc+Luc (pDNA) [23] were dissolved separately in 10 mM Tris–HCl buffer (pH 7.4). Then, polymer solution was added to pDNA solution at a fixed concentration (50 μg/ml) to form complex with various N/P ratios. N/P ratio is defined as the ratio of molar concentration of cationic moieties in diblock cationer to that of phosphate group in pDNA. Final concentration of pDNA was fixed at 33.3 μg/ml. Complexes were kept at room temperature overnight before use.

2.1.4. Dynamic light scattering measurement

The size of pDNA polyplex micelles was evaluated by dynamic light scattering (DLS) measurement. Sample solutions

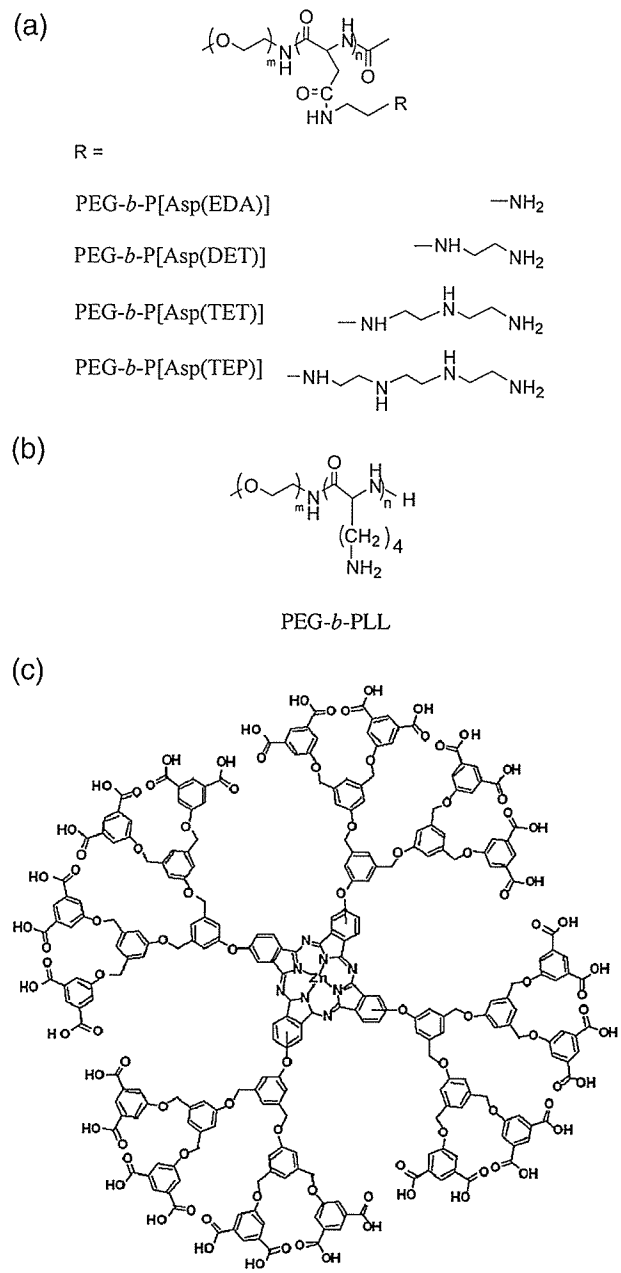

Fig. 2. (a) Chemical structures of PEG-*b*-P[Asp(EDA)], PEG-*b*-P[Asp(DET)], PEG-*b*-P[Asp(TET)], and PEG-*b*-P[Asp(TEP)]; (b) chemical structure of PEG-*b*-PLL; (c) chemical structure of anionic dendrimer phthalocyanine (DPc).

with various N/P ratios in 10 mM Tris–HCl buffer (pH 7.4) were adjusted to pDNA concentration of 33.3 μg/ml. DLS measurements were carried out at 25.0±0.2 °C using a DLS-7000 instrument (Otsuka Electronics, Osaka, Japan) with a vertically polarized incident beam of 488 nm wavelength from an Ar ion laser. A scattering angle of 90° was used in these measurements. Data were analyzed by a cumulant method as reported in detail previously [24].

2.1.5. Laser-doppler electrophoresis measurement

Laser-doppler electrophoresis measurements were carried out using a ELS-6000 (Otsuka Electronics Co., Ltd., Osaka, Japan) at 25 °C. From the electrophoretic mobility, the zeta

potential (ζ) was calculated using Smoluchowski equation as follows:

$$\zeta = 4\pi\eta u/\varepsilon$$

where u is the electrophoretic mobility, η is the viscosity of the solvent, and ε is the dielectric constant of the solvent.

2.1.6. Ethidium bromide exclusion assay

The effect of the N/P ratio on the degree of pDNA condensation in PIC micelle was estimated from the reduction in fluorescence intensity of ethidium bromide (EtBr) due to the exclusion from DNA. PIC micelle solutions (33.3 μg of pDNA/mL) prepared at various N/P ratios were adjusted to 20 μg of pDNA/mL with 0.4 μg of EtBr/mL by adding 10 mM Tris–HCl buffer containing EtBr. The ratio of residual molar concentration of EtBr to that of the base pair in pDNA was 0.033. The solutions were incubated at ambient temperature overnight. Fluorescence measurement of sample solution was carried out at 25±0.2 °C using a spectrofluorometer (JASCO, FP-6500). Excitation (Ex) and emission (Em) wavelengths were 510 and 590 nm, respectively. Results were expressed as relative fluorescence intensity. The fluorescence of pDNA solution with EtBr was set at 100%, and measured against a background of EtBr without pDNA.

2.1.7. Cell culture and photochemical transfection

Human hepatoma Huh7 cells were cultured in DMEM containing 10% fetal bovine serum (FBS) and 10 mM HEPES buffer. The cells were grown at 37 °C in humidified atmosphere containing 5% CO_2. In the experiment, 10,000 cells per well were plated on 24-well plate 24 h before transfection. The cells then were incubated in a medium containing DPc-loaded micelles and pDNA polyplex micelles prepared at an N/P ratio of 2. The amount of pDNA was adjusted to 1 μg per well while DPc concentrations were varied. After 6 h incubation at 37 °C, the medium was replaced with fresh medium to remove non-associated photosensitizers. The cells then were irradiated for 45 min (except for irradiation times variation experiment) using halogen lamp equipped with a band-pass filter (400–700 nm) at a fluence rate of 3 mW/cm^2. After another 48 h incubation at 37 °C, the luciferase gene expression was measured using LB940, Berthold Technologies (Bad Wilbad, Germany). The amount of protein in each well was determined using Micro BCA Protein Assay Reagent Kit, Pierce Chemical Co., Inc. (Rockford, USA).

2.1.8. Cytotoxicity measurement

Cell culture was carried out as described above. After 48 h incubation, the viability of the cells was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [25]. Briefly, 40 μl of MTT solution (5 mg/ml in PBS) was added to each well, followed by 4 h incubation at 37 °C. Then, 400 μl SDS solution (20% w/v in PBS) was added to dissolve the formed formazan. After 2 days incubation at room temperature, the absorbance from each well was measured at 570 nm. Results were expressed as percentage relative to control.

3. Results and discussion

The PEG-*b*-polyaspartatamide catiomers with different amino functionality at the side chain used for pDNA delivery were synthesized through the new synthetic procedure of PEG-*b*-polycatiomer developed in our laboratory [20]. It based on the recent finding that the benzyl ester group of poly(β-benzyl-L-aspartate) (PBLA) can undergo aminolysis reaction with the primary amino group of polyamine compounds in a selective and quantitative manner under a mild condition, allowing the preparation of cationic polyaspartatamides with different amino functionality but the same molecular weight. In our previous study, we found that PEG-*b*-polycatiomer having the ethylenediamine structure at the side chain showed sufficient buffering

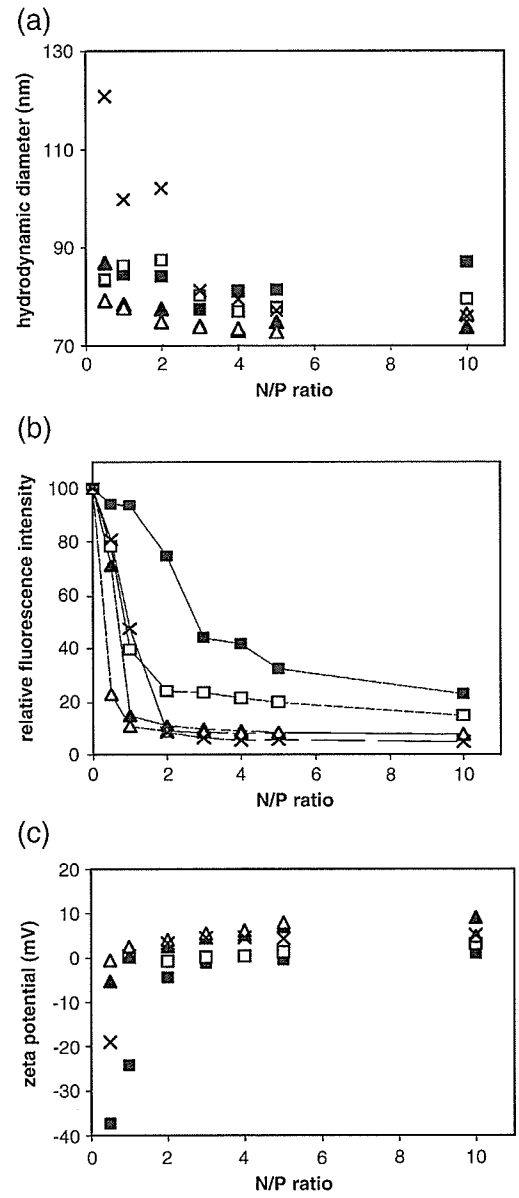

Fig. 3. Properties of PEG-*b*-polycation/pDNA polyplex micelles: (a) change in the cumulant diameter, (b) fluorescence intensity of EtBr and (c) zeta potential value of the polyplexes prepared at different mixing ratios. (■) PEG-*b*-P[Asp(EDA)]; (□) PEG-*b*-P[Asp(DET)]; (▲) PEG-*b*-P[Asp(TET)]; (△) PEG-*b*-P[Asp(TEP)]; (×) PEG-PLL.

capacity and therefore enhanced transfection efficiency based on the aforementioned proton sponge effect [20]. In this study, we hypothesized that the use of the buffering polycations might facilitate the PCI-mediated gene delivery, and aimed to optimize the chemical structure of PEG-*b*-polycatiomers for this purpose. Therefore, we synthesized a series of PEG-*b*-polycatiomers carrying the ethylenediamine repeating units (PEG-*b*-P[Asp (EDA)]), PEG-*b*-P[Asp(DET)], PEG-*b*-P[Asp(TET)] and PEG-*b*-P[Asp(TEP)]) at the side chain with fixed composition of 12–68 (Mw of PEG=12,000, repeating unit of cationic segment=68). The chemical structures of these block catiomers were confirmed by ^{1}H NMR and size exclusion chromatography measurements (Figs. S1, S2, S3, S4, S5 and Table S1 in Supporting information), and are shown in Fig. 2a.

The polyplex micelles were prepared by mixing the solution of each polymer and pDNA at various N/P ratios. The size of the polyplexes was then evaluated by DLS measurement. The hydrodynamic diameter of the polyplexes was revealed to be 70–90 nm through the range of examined N/P ratios as shown in Fig. 3a. Increasing N/P ratio decreases the hydrodynamic diameter of the polyplexes, suggesting the formation of more compact pDNA micelles probably due to the increased densities of positive charges surrounding pDNA polyplexes. From the practical point of view, the polyplex micelles need to be stable under physiological salt concentrations. Therefore, the DLS measurements of the polyplex micelles were carried out after 24-h incubation in 150 mM NaCl solutions. Consequently, the polyplex micelles maintained the particle size of 100 nm with a narrow distribution above the N/P ratio of 2.0, whereas the branched polyethylenimine (BPEI) polyplexes showed appreciable increases in the particle size and polydispersity index (Fig. S6 in Supporting information). This result suggests high stability of polyplex micelles under physiological conditions for *in vivo* use.

Ethidium bromide (EtBr) is a DNA intercalator and exhibits approximately 10-fold greater fluorescence emission upon binding to DNA. Condensation of DNA by cationic component displaces EtBr, resulting in the reduced fluorescence intensity. Hence, EtBr exclusion assay was frequently used to estimate the degree of pDNA condensation in polyplexes [26]. We found that in the order of PEG-*b*-P[Asp(EDA)], PEG-*b*-P[Asp(DET)], PEG-*b*-P[Asp(TET)] and PEG-*b*-P[Asp(TEP)], pDNA condensation ability increased (i.e., the N/P ratio required to reduce the EtBr fluorescent intensity decreased) as shown in Fig. 3b. It seems that longer polyamine side chain more effectively interacts with DNA double strands, thereby replacing the dye molecule. Except for PEG-*b*-P[Asp(EDA)], all the PEG-*b*-polycatiomers induced EtBr quenching to be leveled off at N/P ratio of 2, suggesting the completion of pDNA condensation at N/P ratio less than 2. PEG-*b*-P[Asp(EDA)]/pDNA polyplex retained 75% of its fluorescence intensity at N/P ratio of 2. As known, P[Asp(EDA)] has shorter spacer between the primary amino group and backbone polymer, and this may explain the lower condensation ability of DNA.

Zeta potential values of the polyplex micelles increased with increasing N/P ratio and were almost leveled off at N/P ratio of 2 (Fig. 3c). EtBr exclusion assay also indicated that at N/P ratio

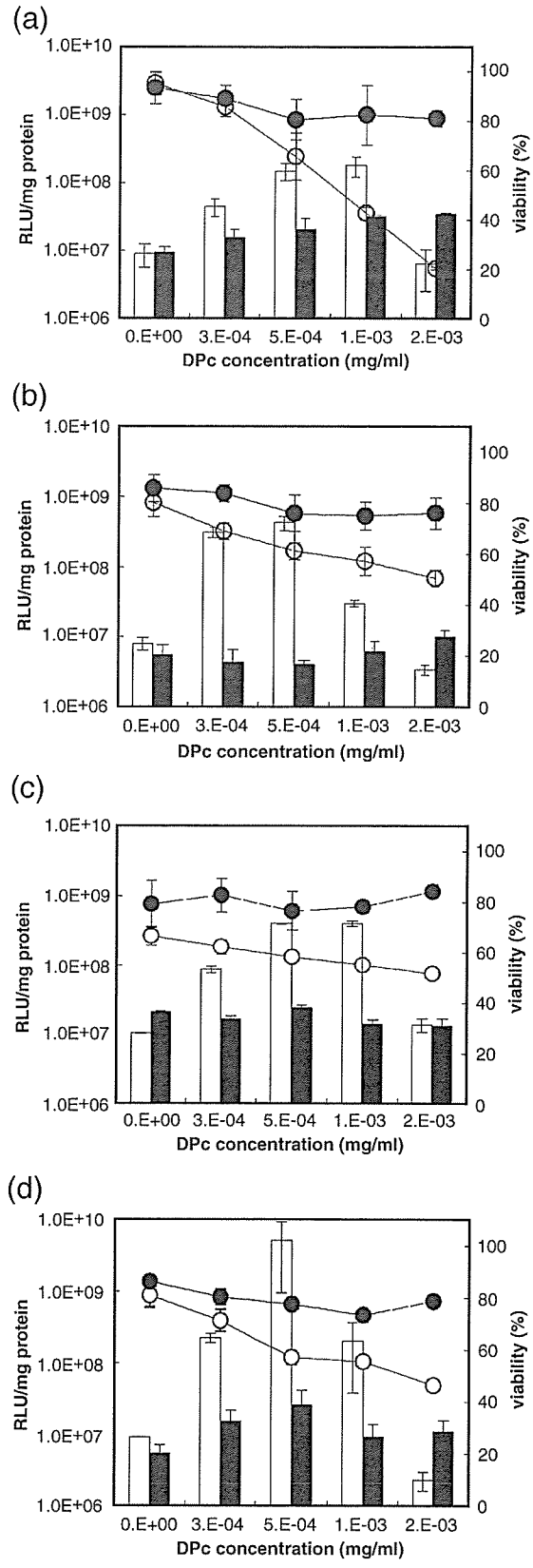

Fig. 4. The effect of DPc concentration on the transfection efficiency and cytotoxicity of the PCI-mediated transfection using the combination of pDNA- and DPc-incorporated micelles. (a) PEG-*b*-P[Asp(EDA)], (b) PEG-*b*-P[Asp (DET)], (c) PEG-*b*-P[Asp(TET)] and (d) PEG-*b*-P[Asp(TEP)] as pDNA vector. Open bar: transfection efficiency with irradiation; solid bar: transfection efficiency without irradiation; O: photocytotoxicity; ●: dark cytotoxicity.

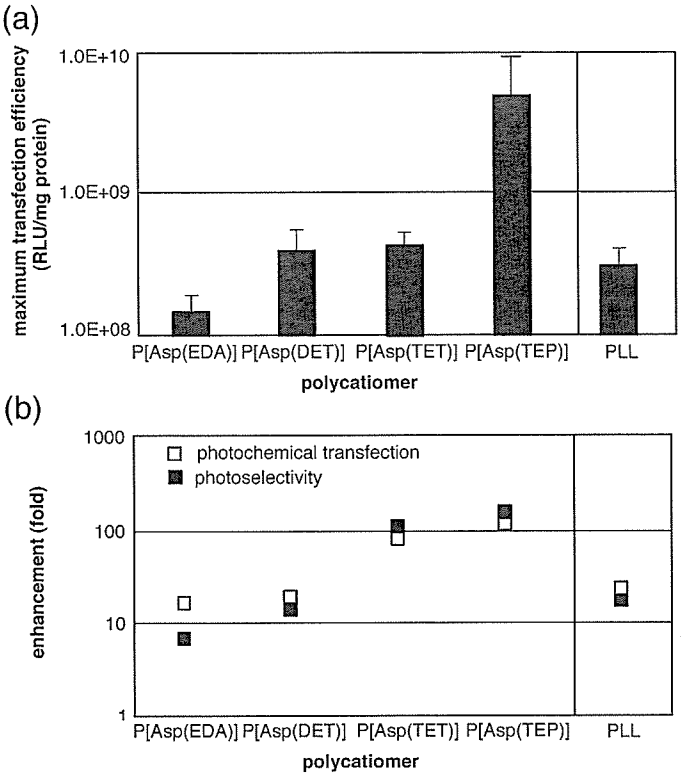

Fig. 5. Comparison of (a) transfection efficiency and (b) photochemical transfection enhancement and photoselectivity at optimal DPc concentration between the polyplex micelles from PEG-*b*-P[Asp(EDA)], PEG-*b*-P[Asp (DET)], PEG-*b*-P[Asp(TET)], PEG-*b*-P[Asp(TEP)] and PEG-*b*-PLL.

of 2, the pDNA was fully condensed in the core. A gradual increase of the zeta potential suggests adsorption of polymer onto the polyplex surface. Above N/P ratio of 2, the polyplexes have relatively small absolute zeta potential values, suggesting the formation of the core-shell structured polyplex micelles covered with PEG palisades. The sterically repulsive character of the neutral PEG layers prevents the micelles from secondary aggregation, keeping their highly dispersive nature in aqueous medium. These characters are expected to provide the polyplexes with prolonged blood circulation by avoiding interaction with serum proteins and cellular components [17]. Another advantage of the presence of neutral PEG layers at the outer part of the micelle might be the minimization of toxicity of the polycatiomers due to its binding with various types of RNA and genomic DNA that impairs the normal cellular functions of these polyanions [27].

In this study, the PCI using polymeric micelles incorporating dendrimer-based photosensitizers [dendrimer phthalocyanine (DPc) illustrated in Fig. 2c] was carried out to enhance the gene transferring ability of the aforementioned polyplex micelles in a light-selective manner. DPc has longer excitation wavelengths (i.e., strong Q-band absorptions at 630 and 685 nm), facilitating deeper tissue penetration of light therefore preferable for clinical use [28,22]. The rationale for the use of polymeric micelles as a nanocarrier of DPc is similar to that for the delivery of DNA, which are to prolong blood circulation after intravenous administration and to selectively accumulate them in the target tissues such as solid tumors as previously reported [22,29,30]. Also, both micelles are assumed to show the same subcellular localization in the endosome due to similar particle sizes and surface properties, which might be a key to success in the PCI-mediated gene delivery [14,19].

To evaluate the ability of the combinatorial use of the newly developed polyplex micelles and DPc-incorporated micelles for the PCI-mediated gene delivery, *in vitro* transfection was performed on human hepatoma Huh7 cells with a luciferase (Luc) reporter gene in the presence and absence of photoirradiation. The photochemical transfection and cytotoxicity were examined using the combination of polyplex micelles at N/P ratio of 2, where the fluorescence intensity was found to be leveled off for most of the complexes (Fig. 3b), and the DPc-incorporated micelles with different concentrations of DPc. The photoirradiation was carried out using a broad band light of 400–700 nm with 3 mW/cm^2 of fluence rate for 45 min. The PCI-mediated gene transferring ability and cytotoxicity of the polyplexes from PEG-*b*-P[Asp(EDA)], PEG-*b*-P[Asp(DET)], PEG-*b*-P[Asp(TET)] and PEG-*b*-P[Asp(TEP)] are shown in Fig. 4a, b, c and d, respectively. All the polyplexes showed the photochemical enhancement of the gene expression with optimum doses of DPc ($5*10^{-4}$ mg/ml), above in which the transfection efficiency decreased. The decrease of transfection efficiency with increasing DPc concentration is most likely to be caused by increased photocytotoxicity (open symbols in Fig. 4). Hence, the PCI-mediated transfection with the DPc-incorporated micelles should be performed based on a careful balance between transfection efficiency and cytotoxicity. But there is also another possibility that the lowered transfection may be due to the damage of the plasmid DNA induced by photochemical treatment [31].

The maximum transfection efficiencies achieved by the PCI-mediated gene delivery using polyplex micelles from different block catiomers are summarized in Fig. 5a. Also, the photochemical transfection enhancement and photoselectivity are shown in Fig. 5b. Photochemical transfection enhancement is defined as the ratios of the above-mentioned maximum transfection efficiency to the transfection efficiency of the polyplex

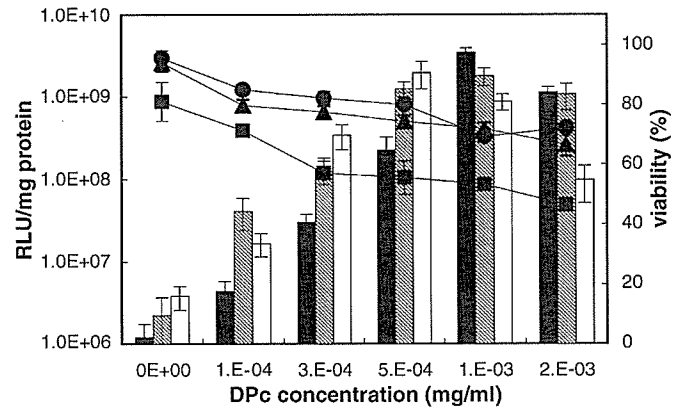

Fig. 6. The effect of irradiation times on photochemical transfection [solid bar: 20 min; hatched bar: 30 min; open bar: 45 min irradiation] and photocytotoxicity [●: 20 min; ▲: 30 min; ■: 45 min irradiation] of PEG-*b*-P[Asp(TEP)] polyplex micelle at different DPc concentrations.

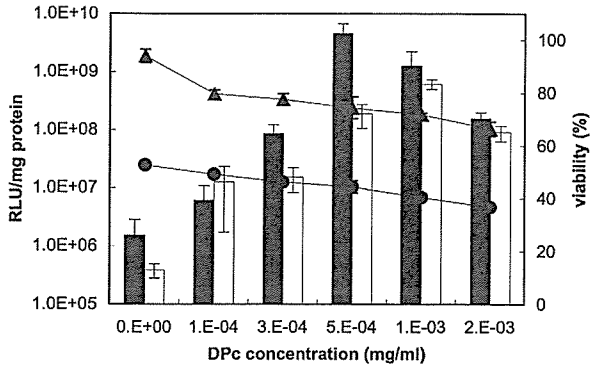

Fig. 7. Comparison of photochemical transfection efficiency (solid bar: BPEI; open bar: PEG-*b*-P[Asp(TEP)]) and photocytotoxicity (●: BPEI; ▲: PEG-*b*-P[Asp(TEP)]) between the polyplex micelles from PEG-*b*-P[Asp(TEP)] and BPEI 25 kDa at various concentrations of DPc and 20 min irradiation.

micelles alone, while photoselectivity is the ratios of the maximum PCI-mediated transfection efficiency to the transfection efficiency of the combination of the pDNA- and DPc-incorporated micelles without photoirradiation. As shown in Fig. 5a, increasing the length of ethylenediamine structure at the side chain of polycation segment of diblock copolymer led to an appreciable increase in the photochemical transfection efficiency even at the same N/P ratio. Also, the polyplexes from PEG-*b*-polyaspartatamide catiomer gave a higher photochemical transfection than those of PEG-*b*-PLL, except for PEG-*b*-P[Asp(EDA)], which lacks both the ability to condense DNA and buffering capacity [20]. Notably, PEG-*b*-P[Asp(TEP)] bearing 3 times-repeated ethylenediamine units showed a 1000-fold photochemical transfection enhancement, which is in a marked contrast with those obtained by PEG-*b*-P[Asp(TET)] bearing 2 times-repeated ethylenediamine units (76-fold) and PEG-*b*-P[Asp(DET)] bearing mono-ethylenediamine unit (19-fold) (Fig. 5b). Thus, the photochemical transfection enhancement of the polyplex micelles increased as the length of the ethylenediamine structure at the side chain of block catiomer strand increased. The mechanisms of the enhanced PCI-mediated transfection dependent on the length of the ethylenediamine side chain remain to be clarified. It is possible that the increasing number of a relatively low pK_a secondary amino groups may contribute to such transfection enhancement. Increasing the length of the ethylenediamine side chain increased the pDNA condensation ability (Fig. 3b), which may also contribute to the enhanced PCI-mediated transfection. However, this factor is unlikely to be solely effective in the photochemical transfection, since PEG-*b*-PLL showed lower PCI-mediated transfection efficiency than those of PEG-*b*-P[Asp(DET)], PEG-*b*-P[Asp(TET)] and PEG-*b*-P[Asp(TEP)] despite its high DNA condensation ability. Thus, as we hypothesized, the use of block catiomers having the ethylenediamine side chain with a high buffering capacity may assist the photochemical disruption of the endosomal membrane due to the proton sponge mechanism [5], thereby accelerating the cytoplasmic delivery of the polyplex micelles upon photoirradition. The synergistic effect between photochemical reaction and proton sponge effect on the PCI-mediated transfection has been successfully demonstrated in this study.

PEG-*b*-P[Asp(TEP)] gave the highest and remarkable photochemical transfection enhancement. In this experiment, the effect of the irradiation times on the PCI-mediated transfection using polyplex micelles from PEG-*b*-P[Asp(TEP)] was further studied. In particular, we tried to find an optimum irradiation condition for high transfection enhancement without a trade on the viability of the cells. Fig. 6 shows the photochemical transfection and photocytotoxicity of PEG-*b*-P[Asp(TEP)] polyplex micelle at different irradiation times. As the irradiation time increased, the optimum condition for photochemical transfection was shifted to a lower concentration of DPc while the photocytotoxicity increased. The highest photochemical transfection enhancement was achieved at DPc concentration of 0.001 mg/ml and irradiation times of 20 min with around 72% of the cells remained alive. Thus, this condition might be an optimum irradiation condition for photochemical transfection enhancement for PEG-*b*-P[Asp(TEP)] polyplex micelle.

Further, polyethylenimine (PEI) consisting of ethylenediamine units, which is one of the most highly transfectable polycatiomers, was used as pDNA vector for the PCI-mediated transfection. In this study, we compared the photochemical transfection efficiency and photocytotoxicity between PEG-*b*-P[Asp(TEP)] and 25 kDa branched PEI (BPEI) as pDNA vector (Fig. 7). BPEI showed approximately 7-fold higher transfection enhancement compared to PEG-*b*-P[Asp(TEP)] at the optimum DPc concentration. Nevertheless, the transfection with BPEI was accompanied by significant cytotoxicity. The PEG-*b*-P[Asp(TEP)] polyplex micelle showed significantly lower cytotoxicity despite its efficient PCI-mediated transfection ability.

In conclusion, we have studied on the relationship between chemical structure of block catiomers and photochemical transfection efficiencies. The light-inducible transfection activity was significantly enhanced as the length of the ethylenediamine side chain of PEG-*b*-polycatiomer increased. PEG-*b*-P[Asp(TEP)] gave the highest photochemical enhancement and photoselectivity of the transfection. The PEG-*b*-polyaspartatamide catiomers, except for PEG-*b*-P[Asp(EDA)], are more effective than PEG-*b*-PLL, which was used in the previous study [19], as a pDNA vector for the light-selected gene transfer. It is worth mentioning that all the polyplex micelles used in this study were prepared under the conditions with minimal free polymers (i.e., at the N/P ratio of 2.0), facilitating their use for the systemic administration. PEG-*b*-polyaspartatamide catiomers as pDNA vector might become vectors for the PCI-mediated gene transfer *in vivo* after systemic delivery.

Acknowledgments

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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.2006.07.014.

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A PEG-Based Biocompatible Block Catiomer with High Buffering Capacity for the Construction of Polyplex Micelles Showing Efficient Gene Transfer toward Primary Cells

Naoki Kanayama,[a, d] Shigeto Fukushima,[a, c] Nobuhiro Nishiyama,*[b] Keiji Itaka,[b] Woo-Dong Jang,[a] Kanjiro Miyata,[a] Yuichi Yamasaki,[a, d] Ung-il Chung,[b] and Kazunori Kataoka*[a, b, d]

Nonviral gene vectors from synthetic catiomers (polyplexes) are a promising alternative to viral vectors. In particular, many recent efforts have been devoted to the construction of biocompatible polyplexes for in vivo nonviral gene therapy. A promising approach in this regard is the use of poly(ethylene glycol) (PEG)-based block catiomers, which form a nanoscaled core–shell polyplex with biocompatible PEG palisades. In this study, a series of PEG-based block catiomers with different amine functionalities were newly prepared by a simple and affordable synthetic procedure based on an aminolysis reaction, and their utility as gene carriers was investigated. This study revealed that the block catiomers carrying the ethylenediamine unit at the side chain are capable of efficient and less toxic transfection even toward primary cells, highlighting critical structural factors of the cationic units in the construction of polyplex-type gene vectors. Moreover, the availability of the polyplex micelle for transfection with primary osteoblasts will facilitate its use for bone regeneration in vivo mediated by nonviral gene transfection.

Introduction

Gene therapy is a promising approach for the treatment of genetic and intractable diseases and for tissue engineering; however, its success still strongly depends on the development of useful gene vectors.[1] Recently, nonviral vectors based on the complexation of plasmid DNA (pDNA) with synthetic cationic polymers (catiomers) have attracted a great deal of attention as an alternative to viral vectors.[2–4] These vectors, the so-called polyplexes, are aimed toward both efficient transfection and decreased cytotoxicity.[5,6] In particular, there has recently been a strong impetus toward engineering the constituent catiomers to construct biocompatible polyplexes suitable for gene delivery in vivo.[2,5] A promising approach in this regard is the block copolymerization of catiomers with poly(ethylene glycol) (PEG) to obtain PEG-*block*-catiomers, as they spontaneously associate with pDNA to form polyplex micelles at the sub-100-nm scale with a dense and hydrophilic PEG palisade surrounding the polyplex core (Figure 1).[7–10] These polyplex micelles with PEG palisades showed high colloidal stability under physiological conditions and afforded appreciable levels of reporter-gene expression to various cell lines even after preincubation in a serum-containing medium.[7] Notably, the polyplex micelles demonstrated longevity in blood circulation,[11] offering the possibility of their use in systemic gene delivery. Nevertheless, a major obstacle to the successful application of this biocompatible nonviral vector system remains: the limited transfection efficacy toward primary cells.

Herein, we report a novel approach to obtain PEG-*block*-catiomers with remarkably high transfecting activity even toward primary cells, which are known to be sensitive to the toxicity induced by conventional polyplexes. The synthetic strategy for novel block catiomers is based on our unprecedented finding that the flanking benzyl ester groups of poly(β-benzyl L-aspar-

[a] *Dr. N. Kanayama, S. Fukushima, Dr. W.-D. Jang, K. Miyata, Dr. Y. Yamasaki, Prof. Dr. K. Kataoka*
Department of Materials Engineering
Graduate School of Engineering
The University of Tokyo
Tokyo 113-8656 (Japan)
Fax: (+81) 3-5841-7139
E-mail: kataoka@bmw.t.u-tokyo.ac.jp

[b] *Dr. N. Nishiyama, Dr. K. Itaka, Prof. Dr. U.-i. Chung, Prof. Dr. K. Kataoka*
Center for Disease Biology and Integrative Medicine
Graduate School of Medicine
The University of Tokyo, Tokyo 113-0033 (Japan)
Fax: (+81) 3-5841-7139
E-mail: nishiyama@bmw.t.u-tokyo.ac.jp

[c] *S. Fukushima*
R&D Division, Pharmaceuticals Group
Nippon Kayaku Co., Ltd. (Japan)

[d] *Dr. N. Kanayama, Dr. Y. Yamasaki, Prof. Dr. K. Kataoka*
Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Agency (JST) (Japan)

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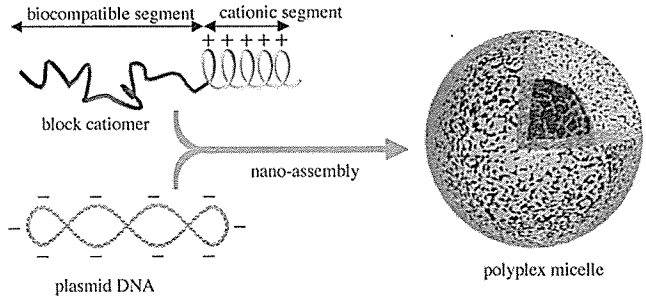
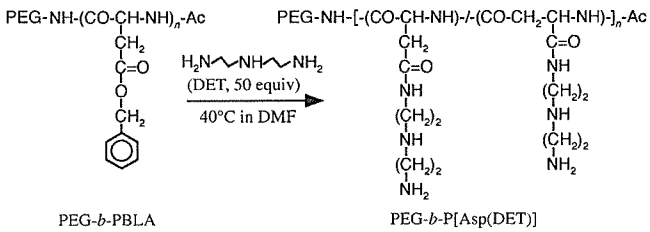


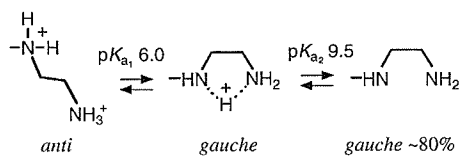
Figure 1. Formation of polyplex micelles through the electrostatic interaction between block catiomers and plasmid DNA.

tate) (PBLA) can undergo a quantitative aminolysis reaction with various polyamine compounds under mild anhydrous conditions at 40 °C, thus allowing the preparation of cationic polyaspartamides with different amine functionalities, yet with the same molecular weight and distribution (Scheme 1). In par-



Scheme 1. Synthesis of PEG-*b*-P[Asp(DET)] block catiomer through the aminolysis of PEG-*b*-PBLA. DMF = *N*,*N*-dimethylformamide.

ticular, this study is focused on the unique properties of the ethylenediamine unit integrated into the polyaspartamide side chain. Notably, ethylenediamine is known to undergo a clear two-step protonation with a distinctive *gauche–anti* conformational transition as depicted in Scheme 2,[12] and is thus expected to provide an effective buffering function in the acidic en-



Scheme 2. Two-step protonation of the ethylenediamine unit with a distinctive *gauche–anti* conformational transition.

dosomal compartment (pH 5). It has been suggested that catiomers with a low pK_a value such as polyethylenimine could buffer endosomal acidification and cause an increase in osmotic pressure in the endosome, leading to the disruption of the endosomal membrane to facilitate polyplex transport into the cytoplasm (the so-called proton sponge effect[13]). Indeed, PEG-*block*-polyaspartamide with an ethylenediamine unit at the side chain (PEG-*b*-P[Asp(DET)]) showed a remarkably high transfection efficacy to various cancer cells as well as mouse primary osteoblast cells. Importantly, this block catiomer was found to have remarkably low toxicity, facilitating its use for in vivo gene therapy.

Results and Discussion

PEG-*b*-polyaspartamide carrying the *N*-(2-aminoethyl)aminoethyl group $-(CH_2)_2NH(CH_2)_2NH_2$ as the side chain (PEG-*b*-P[Asp(DET)]) was prepared by the aminolysis of PEG-*b*-PBLA in dry DMF at 40 °C for 24 h in the presence of a molar excess (50 equiv relative to benzyl groups) of diethylenetriamine (DET) (Scheme 1). The ^{1}H NMR spectrum of PEG-*b*-P[Asp(DET)] is shown in Figure 2, and the ^{13}C NMR spectrum is available in

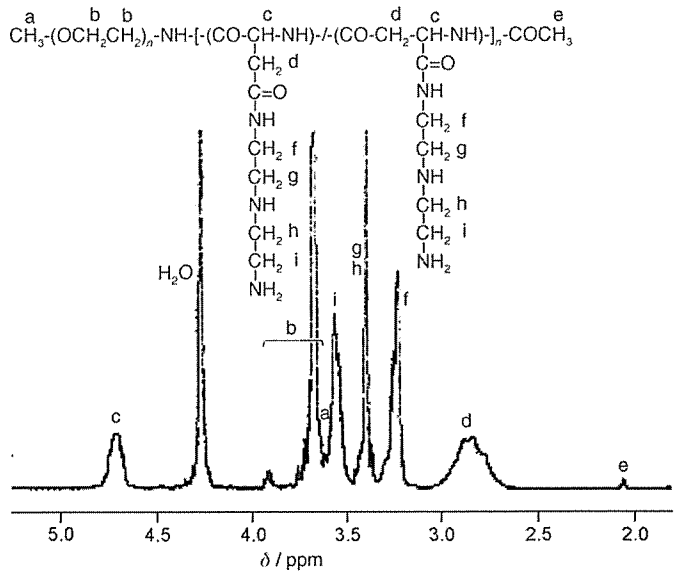


Figure 2. ^{1}H NMR spectrum of PEG-*b*-P[Asp(DET)] (solvent: D_2O, T = 80 °C); the polymer is in a salt form.

the Supporting Information. These data indicate that the aminolysis of the PBLA benzyl groups proceeded in a selective manner to the primary amine moiety of DET. Also, comparison of the integration ratio of the proton peaks (b and f–i) in Figure 2 reveals quantitative introduction of DET into the side chain of PBLA, and a unimodal molecular weight distribution of the obtained polymer was revealed by size-exclusion chromatography (SEC) measurement (Supporting Information). These results suggest a minimal occurrence of inter- or intrapolymer cross-linking by DET during aminolysis. Note that the peaks from the carbonyl and methylene groups of the aspartamide units in the ^{13}C NMR spectrum (Figure S1, Supporting Information) are split into two peaks, suggesting that the aminolysis of PBLA might induce intramolecular isomerization of the aspartamide units to form β-aspartamide. Figure 3 shows the time course of the aminolysis reaction of PBLA with DET, which was evaluated from the change in the ratio of the proton peak integration (f over b) in the ^{1}H NMR spectrum (Figure 2). This result indicates a fast and quantitative aminolysis of PBLA, which is in marked contrast to the lack of aminolysis with poly(γ-benzyl L-glutamate) (PBLG) under the same re-

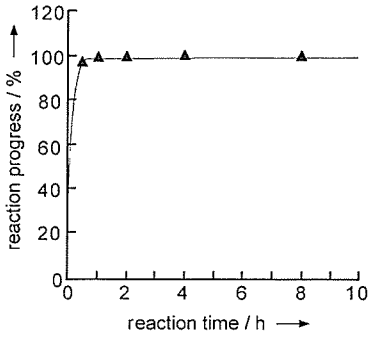


Figure 3. Time course of the aminolysis of PEG-*b*-PBLA with DET in DMF at 40 °C. The reaction progress was estimated from the change in the ratio of the proton peak integration (f over b) in the ^{1}H NMR spectrum (Figure 2).

action conditions (data not shown), highlighting a unique mechanism involved in the aminolysis of PBLA under mild conditions. Presumably, the amide groups of the main chain interact with the carbonyl group of the side chain, which may facilitate the aminolysis reaction.[14] The details of the mechanism of this unique aminolysis reaction are now under investigation in our research group and will be reported elsewhere.

The pH-dependent protonation of PEG-*b*-P[Asp(DET)] in media containing 150 mM NaCl was evaluated by potentiometric titration. The α/pH curve shown in Figure 4 clearly indicates

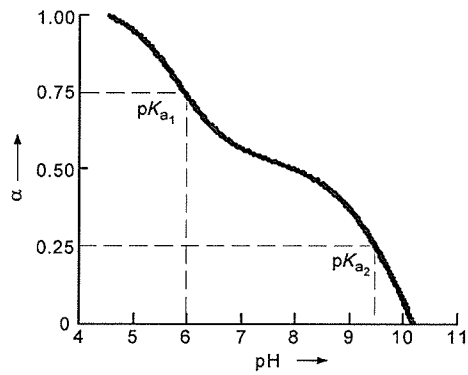


Figure 4. Degree of protonation (α) as a function of pH (α/pH curve) for the PEG-*b*-P[Asp(DET)] block catiomer (150 mM NaCl, aq, 25 °C).

the two-step protonation behavior of PEG-*b*-P[Asp(DET)], which is attributable to the two-step protonation of the ethylenediamine moiety with a distinctive *gauche–anti* conformational transition as indicated in Scheme 2. The two distinct pK_a values of the ethylenediamine moiety in the side chain of polyaspartamide were determined to be 6.0 and 9.5. Notably, this group remains nearly 100% populated by the mono-protonated state (*gauche* form) at pH 7.4, and is capable of exerting a substantial buffering effect in the pH range down to 5.0, at which point the equilibrium shifts to the di-protonated state (*anti* form) (Scheme 2).

The polyplex micelle was prepared by mixing solutions of PEG-*b*-P[Asp(DET)] and pDNA in various ratios of *N/P*, for which *N* is the total number of amine groups in the block catiomer and *P* represents the number of phosphate units in the pDNA. The formation of the polyplex, which accompanies pDNA condensation, was followed by an ethidium bromide (EtBr) dye-exclusion assay at different pH values. As shown in Figure 5, the

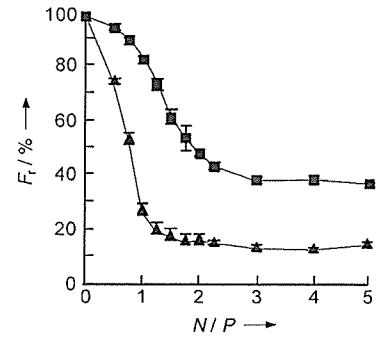


Figure 5. Effect of pH (▲: pH 5.0, ■: pH 7.4) on the relative fluorescence intensity (F_r) of EtBr in solution with pDNA and PEG-*b*-P[Asp(DET)] at various *N/P* ratios.

fluorescence intensity of EtBr decreases with an increase in the *N/P* ratio. At pH 5.0, the fluorescence of EtBr levels off at *N/P* = 1, which is consistent with approximately 95% protonation of the ethylenediamine unit, as expected from the α/pH curve in Figure 4. On the other hand, at pH 7.4, substantial quenching occurred at *N/P* ≈ 2.0, which is consistent with the hypothesis that the mono-protonated form of the ethylenediamine unit in PEG-*b*-P[Asp(DET)] might be maintained even inside the polyplex. It is possible that the stabilized *gauche* conformation (Scheme 2) of the mono-protonated form may prevent the ethylenediamine unit from further protonation facilitated by the zipper effect or the local electrostatic field effect in the complexation process with anionic pDNA at pH 7.4.[15] The cumulant diameters and ζ potentials of the polyplexes prepared at different *N/P* ratios are shown in Figure 6. The cumulant diameters of the polyplex micelles were determined to be 70–90 nm throughout the range of the examined *N/P* ratios of 1–20, and the ζ potentials of the polyplexes increased with *N/P* ratios and leveled off at *N/P* = 2 (Figure 6). At *N/P* > 2, the polyplexes were observed to have small absolute ζ potentials (~8 mV), suggesting a core–shell architecture with a hydrophilic and neutral PEG shell surrounding the polyplex core.

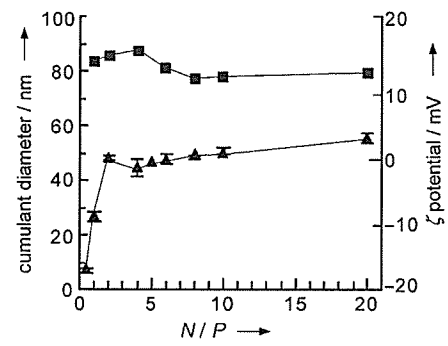


Figure 6. Cumulant diameter (■) and ζ potential (▲) of the PEG-*b*-P[Asp(DET)] polyplex micelles as a function of *N/P* ratio.

The in vitro transfection efficiency (TE) against human hepatoma HuH-7 cells was assessed by a luciferase assay (Figure 7). Notably, a similar trend in TE was also observed for human kidney 293T cells (Supporting Information). In this experiment,

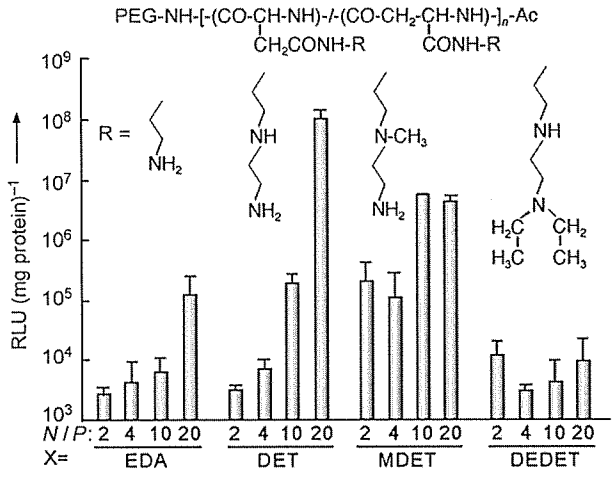


Figure 7. In vitro transfection of the luciferase gene into HuH-7 cells by polyplex micelles from PEG-*b*-polyaspartamides carrying various polyamine components in the side chain (PEG-*b*-P[Asp(X)]) with varying *N/P* ratios. Transfection is reported in relative light units (RLU) per mg protein. The cells were incubated with each polyplex in the medium containing 10% serum for 24 h, followed by incubation for a further 24 h in the absence of polyplex.

the PEG-*b*-P[Asp(DET)]–pDNA micelle was compared with the polyplex micelles from various PEG-*b*-polyaspartamide catiomers made by the similar aminolysis of PEG-PBLA with different amine compounds, with the aim to highlight the unique nature of the P[Asp(DET)] segment. Note that the polyplex micelles from each block catiomer prepared in this study showed sizes and ζ potentials similar to those of the polyplex micelle from PEG-*b*-P[Asp(DET)] (data not shown). The polyplex micelle from the block catiomer with the 2-aminoethyl group $-(CH_2)_2NH_2$ (pK_a 9.4) in the side chain (PEG-*b*-P[Asp(EDA)]), which was prepared through the aminolysis of PEG-*b*-PBLA with ethylenediamine (EDA), showed only 1/10000 of the TE compared with the PEG-*b*-P[Asp(DET)] polyplex micelle at $N/P = 20$. This is presumably due to the impaired buffering capacity of the $-(CH_2)_2NH_2$ unit with the high pK_a value of 9.4 in the experimental pH range as well as to the weak ability of PEG-*b*-P[Asp(EDA)] to condense pDNA based on the EtBr exclusion assay (data not shown).

The TE of the PEG-*b*-P[Asp(DET)] polyplex micelle was further compared with those of the polyplex micelles from the PEG-*b*-polyaspartamide catiomers carrying the N-alkylated ethylenediamine units in the side chain to explore the structural features of the polyplex micelles that are important for effective gene transfection (Figure 7). These block catiomers, PEG-*b*-P[Asp(MDET)] and PEG-*b*-P[Asp(DEDET)], are prepared by the aminolysis reaction of PEG-PBLA with the corresponding amine compounds, 4-methyldiethylenetriamine (MDET) and *N,N*-diethyldiethylenetriamine (DEDET), respectively. Both the PEG-*b*-P[Asp(MDET)] and PEG-*b*-P[Asp(DEDET)] polyplex micelles showed an appreciably lower TE than the PEG-*b*-P[Asp(DET)] polyplex micelle, particularly at higher *N/P* ratios (Figure 7). This result, which highlights the critical sensitivity of TE toward subtle changes in catiomer structure, indicates that additional structural factors, besides distinct pK_a values, play a substantial role in determining the TE of the polyplex micelles constructed from the PEG-*b*-polyaspartamide catiomers carrying the derivatized ethylenediamine units as a side chain; further study is needed to clarify the detailed mechanisms.

The cytotoxicity of the polyplex-forming catiomers is also a crucial aspect for successful nonviral gene therapy. In this regard, all the polyplex micelles from each block catiomer shown in Figure 7 elicited no appreciable cytotoxicity toward HuH-7 cells under the same conditions used for gene transfection (data not shown). Notably, the polyplex micelle from PEG-*b*-P[Asp(DET)] showed remarkably low cytotoxicity despite its efficiency in gene transfection. Therefore, the intrinsic cytotoxicity of PEG-*b*-P[Asp(DET)] catiomer was further assessed against HuH-7 cells, and compared with that of branched polyethylenimine (BPEI, 25 kDa, Aldrich Chemical, USA) and linear polyethylenimine (LPEI, 22 kDa, ExGen500, MBI Fermentas, Germany). As shown in Figure 8, the PEG-*b*-P[Asp(DET)] catiomer showed >20-fold higher 50% growth-inhibitory concentration (IC_{50}) than BPEI and LPEI, highlighting the remarkably low cytotoxicity of the block catiomers synthesized in this study.

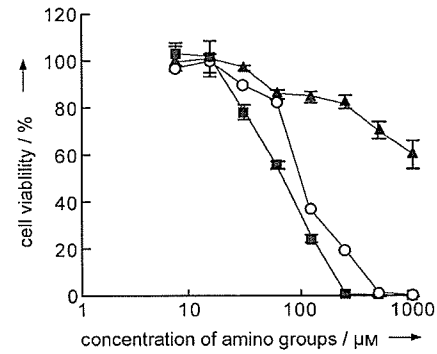


Figure 8. Cytotoxicity of branched (BPEI, ○) and linear (LPEI, ■) polyethylenimines and PEG-*b*-P[Asp(DET)] (▲) against HuH-7 cells. The cells were incubated with each catiomer with different concentrations for 48 h.

The major challenge for the practical use of synthetic vectors in gene therapy is the effective and non-cytotoxic gene transfer to primary cells with therapeutic interest. To evaluate the feasibility of the PEG-*b*-P[Asp(DET)] polyplex micelles toward such primary cells, mouse primary osteoblasts, which are the focus of clinical interest in bone regeneration,[16] were challenged with the polyplex micelles. The luciferase plasmid was transfected, and the resulting TE and cytotoxicity profiles are shown in Figure 9. Notably, the PEG-*b*-P[Asp(DET)] system with $N/P = 80$ gave a TE similar to the polyplexes from ExGen500, the most effective transfection reagent based on LPEI,[17] with the optimal *N/P* ratios (Figure 9A). Nonetheless, the PEG-*b*-P[Asp(DET)] system exhibited no appreciable cytotoxicity under the conditions of gene transfection (Figure 9B).

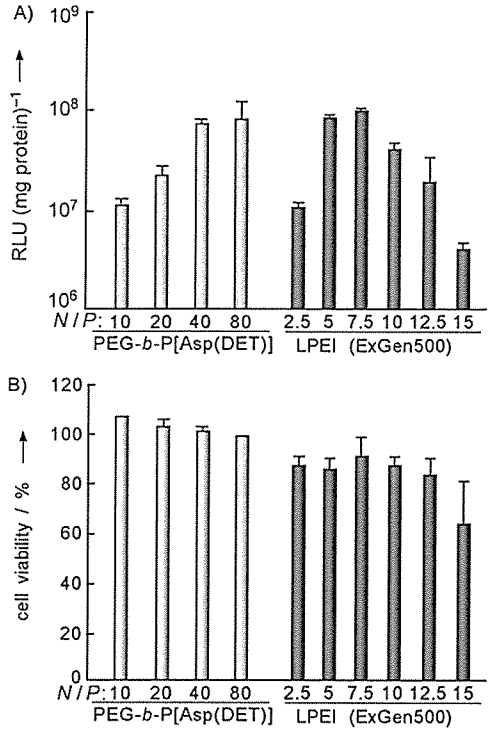


Figure 9. A) In vitro transfection efficiency and B) cytotoxicity of the PEG-*b*-P[Asp(DET)] polyplex micelles and LPEI polyplexes with varying *N/P* ratios toward mouse primary osteoblasts after a 48 h incubation.

Thus, we have successfully obtained highly transfection-efficient and less toxic polyplex micelles in this study. Particularly, the less toxic nature of the block catiomers compared with conventional catiomers of high transfection efficiency, as observed in Figure 8, should be of great significance for in vivo nonviral gene therapy. Indeed, bone regeneration in critical-size cranial defects based on in vivo transduction of osteogenic factors was recently carried out by our research group by using the PEG-*b*-P[Asp(DET)] polyplex micelle with plasmids expressing the optimized combination of osteogenic factors to facilitate cellular differentiation in situ.[18] Furthermore, polyplex micelles with the PEG palisade seem to be suitable for systemic gene delivery,[7,11] and the engineering the constituent block catiomers to construct polyplex micelles with integrated smart functions such as environment sensitivity[8,10] and tissue targetability[9] will maximize the efficacy of nonviral gene therapy. Thus, the PEG-*b*-P[Asp(DET)] polyplex micelle is expected to be a biocompatible vector system applicable toward various aspects of gene medicine.

Conclusion

We have established a simple and novel synthetic route for the generation of biocompatible block catiomers through the quantitative aminolysis of PEG-*b*-PBLA. The construction of a library of block catiomers, the PEG-*b*-polyaspartamides carrying a series of amine compounds in the side chain, revealed the importance of the ethylenediamine unit for enhanced and less toxic gene transfection by the polyplex micelles made from pDNA and the block catiomers. The availability of the polyplex micelles developed in this study for the transfection of primary osteoblasts will facilitate the use of this type of block catiomer for the construction of synthetic vectors suitable for nonviral gene therapy.

Experimental Section

Materials: β-Benzyl-L-aspartate *N*-carboxyanhydride (BLA-NCA) and α-methoxy-ω-amino poly(ethylene glycol) (MeO-PEG-NH_2) (M_r = 12000) were obtained from Nippon Oil and Fats (Tokyo, Japan). Ethylenediamine (EDA), diethylenetriamine (DET) and 4-methyldiethylenetriamine (MDET) were purchased from Tokyo Kasei Kogyo (Tokyo, Japan) and distilled over CaH_2 under decreased pressure. *N,N*-Diethyldiethylenetriamine (DEDET) was purchased from Lancaster Synthesis, (Lancashire, England) and distilled over CaH_2 under decreased pressure. *N,N*-Dimethylformamide (DMF), dichloromethane, and acetic anhydride were purchased from Wako Pure Chemical Industries, (Osaka, Japan) and purified by general methods before use.

Synthesis of PEG-*b*-polyaspartamide catiomers: The PEG-*block*-poly(β-benzyl L-aspartate) (PEG-*b*-PBLA) copolymer was prepared as previously reported.[19] Briefly, BLA-NCA was polymerized in DMF at 40 °C by the initiation from the terminal primary amino group of MeO-PEG-NH_2, followed by acetylation of the *N*-terminus of PBLA to obtain PEG-*b*-PBLA. PEG-*b*-PBLA was confirmed to have a unimodal molecular weight distribution (M_w/M_n: 1.17) by gel-permeation chromatography (GPC) measurement (columns: TSK-gel G4000HHR + G3000HHR, eluent: DMF + 10 mM LiCl, T = 40 °C, detector: RI) (data not shown). The degree of polymerization (DP) of PBLA was calculated to be 68 based on ^{1}H NMR spectroscopy (data not shown).

Lyophilized PEG-*b*-PBLA (300 mg, 11.6 μmol) was dissolved in DMF (10 mL), followed by reaction with DET (50 equiv to benzyl group of PBLA segment, 4.0 g, 39.4 mmol) under mild anhydrous conditions at 40 °C to obtain PEG-*b*-P[Asp(DET)]. After 24 h, the reaction mixture was slowly added dropwise into a solution of acetic acid (10% *v/v*, 40 mL) and dialyzed against a solution of 0.01 N HCl and distilled water (M_r cutoff: 3500 Da). The final solution was lyophilized to obtain the polymer as the chloride salt form, and the yield was approximately 90%. Similarly, other block catiomers, PEG-*b*-P[Asp(EDA)], PEG-*b*-P[Asp(MDET)], and PEG-*b*-P[Asp(DEDET)] were synthesized by the aminolysis reaction of PEG-*b*-PBLA with EDA, MDET, and DEDET, respectively. The structures of these block catiomers were confirmed by ^{1}H and ^{13}C NMR measurements and size-exclusion chromatography (SEC).

Potentiometric titration of block catiomers: PEG-*b*-P[Asp(DET)] (30 mg) was dissolved in 50 mL 0.01 N HCl and titrated with 0.01 N NaOH. An automatic titrator (TS-2000, Hiranuma, Kyoto, Japan) was used for titration. In this experiment, the titrant was added in quantities of 0.063 mL after the pH values were stabilized (minimal interval: 30 s). The α/pH curves were determined from the obtained titration curves.

Dye exclusion assay: Polyplex solutions with a pDNA concentration of 33 μg mL^{-1}, prepared by mixing pDNA and block catiomers at different *N/P* ratios (N = total amines in block catiomer; P = total phosphate anions in pDNA), were diluted to 10 μg pDNA mL^{-1} with ethidium bromide (EtBr, 2.5 mg mL^{-1}) in 10 mM Tris–HCl (pH 7.4) or 10 mM sodium acetate (pH 5.0) buffer. The sample solutions were incubated at ambient temperature overnight. The fluo-

rescence intensity of the samples at $\lambda = 590$ nm (excitation at $\lambda = 510$ nm) was measured at 25 °C with a spectrofluorometer (FP-6500, Jasco, Tokyo, Japan). The relative fluorescence intensity was calculated as: $F_r = (F_{sample} - F_0)/(F_{100} - F_0)$, for which F_{sample}, F_{100}, and F_0 represent the fluorescence intensity of the samples, free pDNA, and background, respectively.

Dynamic light scattering (DLS) and ζ potential measurements: In the DLS measurements, polyplex solutions with various *N/P* ratios in 10 mM Tris–HCl buffer (pH 7.4) were adjusted to have pDNA concentrations of 33.3 μg mL^{-1}. DLS measurements were then performed at 25 ± 0.2 °C with a DLS-7000 instrument (Otsuka Electronics, Osaka, Japan) with a vertically polarized incident beam of $\lambda = 488$ nm from an Ar ion laser. The ζ potential of the polyplex micelles was measured at 25 ± 0.2 °C with an ELS-6000 instrument (Otsuka Electronics, Osaka, Japan) equipped with a He–Ne ion laser ($\lambda = 633$ nm). The scattering angle was fixed at 20°. From the obtained electrophoretic mobility, the ζ potential was calculated by using the Smoluchowski equation: $\zeta = 4\pi\eta\upsilon/\varepsilon$ in which η is the electrophoretic mobility, υ is the viscosity of the solvent, and ε is the dielectric constant of the solvent. The results are expressed as the average of five experiments.

In vitro transfection of HuH-7 cells: Human hepatoma HuH-7 cells were seeded on 6-well culture plates and incubated overnight in 1.5 mL Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) before transfection. The cells were then incubated with the polyplex micelles from PEG-*b*-P[Asp(EDA)], PEG-*b*-P[Asp(DET)], PEG-*b*-P[Asp(MDET)], and PEG-*b*-P[Asp(DEDET)] (3 μg pDNA/well) with various *N/P* ratios in DMEM containing 10% FBS for 24 h, followed by an additional incubation for 24 h in the absence of polyplexes. Luciferase gene expression was evaluated using the Luciferase Assay System (Promega, Madison, USA) and a Lumat LB9507 luminometer (Berthold Technologies, Bad Wildbad, Germany). The results were expressed as light units per milligram of cell protein determined by a BCA assay kit (Pierce, Rockford, USA).

Mouse primary osteoblast culture and transfection: Osteoblasts were isolated from calvariae of neonatal littermates. The experimental procedures were handled in accordance with the guidelines of the Animal Committee of the University of Tokyo. Calvariae were digested for 10 min at 37 °C in an enzyme solution containing 0.1% collagenase and 0.2% dispase for five cycles. Cells isolated by the final four digestions were combined as an osteoblast population and cultured in DMEM containing 10% FBS. For luciferase transfection assays, primary osteoblasts were inoculated at a density of 2×10^4 cells/well in a 24-multiwell plate, cultured for 24 h, and, after changing to fresh culture medium containing 10% FBS, pDNA polyplex solution (33.3 μg mL^{-1}, 22.5 μL) was applied to each well. Luciferase gene expression was measured 48 h later by using the Luciferase Assay System (Promega) and a Lumat LB9507 luminometer (Berthold). For the cytotoxicity assay, primary osteoblasts were plated into a 96-multiwell plate (6×10^3 cells/well). After 24 h incubation, 6 μL of each pDNA polyplex solution was added, followed by further incubation for 24 h. The viability of the cells was evaluated by an MTT assay (Cell Counting Kit-8, Dojindo, MTT = 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide). Each well was measured by reading the absorbance at $\lambda = 450$ nm according to the protocol provided by the manufacturer.

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Keywords: aminolysis · block copolymers · gene delivery · polycations · polymeric micelles

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Improvement of cancer-targeting therapy, using nanocarriers for intractable solid tumors by inhibition of TGF-beta signaling

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Improvement of cancer-targeting therapy, using nanocarriers for intractable solid tumors by inhibition of TGF-β signaling

Mitsunobu R. Kano*[††], Younsoo Bae[‡§], Caname Iwata*[¶], Yasuyuki Morishita*, Masakazu Yashiro[‖], Masako Oka*, Tomoko Fujii*, Akiyoshi Komuro*, Kunihiko Kiyono*, Michio Kaminishi[¶], Kosei Hirakawa[‖], Yasuyoshi Ouchi[†], Nobuhiro Nishiyama[§**], Kazunori Kataoka[‡§**††], and Kohei Miyazono*[‡‡]

Departments of *Molecular Pathology, [†]Geriatrics, [¶]Gastrointestinal Surgery, and [§]Center for Disease Biology and Integrative Medicine, Graduate School of Medicine; **Department of Materials Engineering, Graduate School of Engineering; and [‡]Center for Nano-Bio Integration, University of Tokyo, Tokyo 113-0033 Japan; and [‖]Department of Surgical Oncology, Osaka City University Graduate School of Medicine, Osaka 545-8585, Japan

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Transforming growth factor (TGF)-β plays a pivotal role in regulation of progression of cancer through effects on tumor microenvironment as well as on cancer cells. TGF-β inhibitors have recently been shown to prevent the growth and metastasis of certain cancers. However, there may be adverse effects caused by TGF-β signaling inhibition, including the induction of cancers by the repression of TGF-β-mediated growth inhibition. Here, we present an application of a short-acting, small-molecule TGF-β type I receptor (TβR-I) inhibitor at a low dose in treating several experimental intractable solid tumors, including pancreatic adenocarcinoma and diffuse-type gastric cancer, characterized by hypovascularity and thick fibrosis in tumor microenvironments. Low-dose TβR-I inhibitor altered neither TGF-β signaling in cancer cells nor the amount of fibrotic components. However, it decreased pericyte coverage of the endothelium without reducing endothelial area specifically in tumor neovasculature and promoted accumulation of macromolecules, including anticancer nanocarriers, in the tumors. Compared with the absence of TβR-I inhibitor, anticancer nanocarriers exhibited potent growth-inhibitory effects on these cancers in the presence of TβR-I inhibitor. The use of TβR-I inhibitor combined with nanocarriers may thus be of significant clinical and practical importance in treating intractable solid cancers.

angiogenesis | gastric cancer | molecular targeting therapy | pancreatic cancer

Chemotherapy that uses nanocarriers has been developed to improve the clinical treatment of solid tumors by obtaining high accumulation of drugs in tumor tissues but limited accumulation in normal organs. Doxil (1), a liposomal adriamycin (ADR), is one such drug that has already been used clinically (2). Doxil has exhibited therapeutic effects on some cancers with hypervascular characteristics (3, 4), including Kaposi sarcoma and ovarian cancers. Another promising formulation of nanocarriers is polymeric micelles (5, 6), which are already being used in clinical trials (7, 8).

However, despite the urgent need for effective chemotherapy for intractable solid tumors, including pancreatic adenocarcinoma (9) and diffuse-type gastric carcinoma (10), nanocarriers of any design have not been successful yet in exhibiting significant therapeutic effects on these cancers. Pancreatic cancer is the fourth leading cause of cancer-related death in the United States and the fifth in Japan (9), and the median survival period of patients who suffer from advanced pancreatic adenocarcinoma is still extremely short ($\approx$6 months), despite recent progress in development of conventional chemotherapies (11). Although cancer cells derived from these tumors are sufficiently sensitive *in vitro* to conventional anticancer agents such as ADR (12), most of these agents have failed to exhibit sufficient therapeutic effects *in vivo*, regardless of formulation, whether encapsulated in nanocarriers or not. The theoretical basis of the specific accumulation of nanocarriers in tumor tissues is leakiness of tumor vessels to the macromolecular agents, termed the "enhanced permeability and retention (EPR) effect," which was demonstrated and named by Maeda *et al.* (13, 14). The major obstacles to treatment of these cancer cells could thus be insufficient EPR effect because of certain characteristics of their cancer microenvironment, including hypovascularity and thick fibrosis (15, 16). However, methods of regulating this effect have not been well investigated.

Transforming growth factor (TGF)-β signaling plays a pivotal role in both the regulation of the growth and differentiation of tumor cells and the functional regulation of tumor interstitium (17). Because TGF-β is a multifunctional cytokine that inhibits the growth of epithelial cells and endothelial cells and induces deposition of extracellular matrix, inhibition of TGF-β signaling in cancer cells and fibrotic components has been expected to facilitate the effects of anticancer therapy. TGF-β binds to type II (TβR-II) and type I receptors (TβR-I), the latter phosphorylates Smad2 and -3. Smad2 and -3 then form complexes with Smad4, translocate into the nucleus, and regulate the transcription of target genes (18). Several small-molecule TβR-I inhibitors have been reported to prevent metastasis of some cancers (19). However, there may be adverse effects of TGF-β inhibition, including potential progression of some cancers because of the repression of TGF-β-mediated growth inhibition of epithelial cells (20).

In this study, we show that administration of the small-molecule TβR-I inhibitor (LY364947) (21) at a low dose, which could minimize the potential side effects of TβR-I inhibitor, can alter the tumor microenvironment and enhance the EPR effect. This effect of low-dose TβR-I inhibitor was demonstrated with two of nanocarriers, i.e., Doxil and a polymeric micelle incorporating ADR (micelle ADR) that we have recently developed (22) [supporting information (SI) Fig. 7]. The present findings strongly suggest that our method, which uses a combination of

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The authors declare no conflict of interest.

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Abbreviations: ADR, adriamycin; EPR, enhanced permeability and retention; PECAM, platelet/endothelial cell adhesion molecule; TβR-I, type I transforming growth factor β receptor.

[††]To whom correspondence may be addressed at: Department of Material Engineering, Graduate School of Engineering, University of Tokyo, Tokyo 113-8656, Japan. E-mail: kataoka@bmw.t.u-tokyo.ac.jp.

[‡‡]To whom correspondence may be addressed. E-mail: miyazono-ind@umin.ac.jp.

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