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Polyion complex micelles for photodynamic therapy: Incorporation of dendritic photosensitizer excitable at long wavelength relevant to improved tissue-penetrating property

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Abstract

A polymeric micelle (DPcZn/m) system, which is formed via an electrostatic interaction of anionic dendrimer phthalocyanine (DPcZn) and poly(ethylene glycol)-poly(L-lysine) block copolymers (PEG-*b*-PLL), was prepared for use as an effective photosensitizer for photodynamic therapy. DPcZn/m exhibited strong Q band absorption around 650 nm, a useful wavelength for high tissue penetration. Dynamic light scattering studies indicated that the DPcZn/m system has a relevant size of 50 nm for intravenous administration. Under light irradiation, either DPcZn or DPcZn/m exhibited efficient consumption of dissolved oxygen in a medium to generate reactive oxygen species and an irradiation-time-dependent increase in photocytotoxicity. The photodynamic efficacy of the DPcZn was drastically improved by the incorporation into the polymeric micelles, typically exhibiting more than two orders of magnitude higher photocytotoxicity compared with the free DPcZn at 60-min photoirradiation.
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Keywords: Dendrimer; Photosensitizer; Phthalocyanine; Polymeric micelle; Photodynamic therapy

1. Introduction

Photodynamic therapy is based on the accumulation of a photosensitizer in malignant tissue after its administration usually through intravenous route [1–5]. Subsequent illumination with laser light of an appropriate wavelength generates reactive oxygen species (ROS) which results in tissue destruction. For an effective photodynamic effect, several ideal properties of photosensitizers should be needed. From the chemical point of view, the materials should be pure and have a high quantum yield of singlet oxygen generation. From the biological point of view, it should have no dark toxicity and have high solubility in an aqueous medium for the easy administration. High tumor localization and long wavelength absorption are also very important for effective medical treatment.

In this context, we have recently reported ionic dendrimer porphyrin as an efficient photosensitizer for photodynamic therapy [6–9]. To obtain high quantum yields and effective energy absorption, photosensitizers must generally have large π-conjugation domains. Therefore, most of photosensitizers easily form aggregates, which provide a self-quenching effect of the excited state in aqueous medium due to their π–π interaction and hydrophobic characteristics [10,11]. To overcome these problems, the structure of ionic dendrimer porphyrin is promising, because the substitution of large dendritic wedges

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sufficiently prevents the formation of aggregates and provides high solubility in the aqueous medium. Furthermore, a charged ion surface can form polyion complex micelles by means of electrostatic interaction with an oppositely charged block copolymer. These types of polyion complex micelles [12] with a PEG shell were demonstrated to accumulate effectively and specifically in solid tumor tissue due to the hyperpermeability of tumor capillaries. However, the dendrimer porphyrin has a relatively short wavelength absorption, where the absorption maximum is 430 nm, which is a limitation to improvement for practical PDT application. In relation to this fact, several phthalocyanine molecules, including the one with a dendritic architecture, are of interest as a potential photosensitizer with appropriate wavelength absorption for practical PDT application [13–16]. Herein, we report the first example of dendritic phthalocyanine-incorporated polyion complex micelle formation and demonstrate an order-of-magnitude enhancement in photodynamic efficacy of the phthalocyanine dendrimer through the micelle encapsulation at an excitation wavelength with clinical relevance (~600 nm).

2. Materials and methods

2.1. Materials

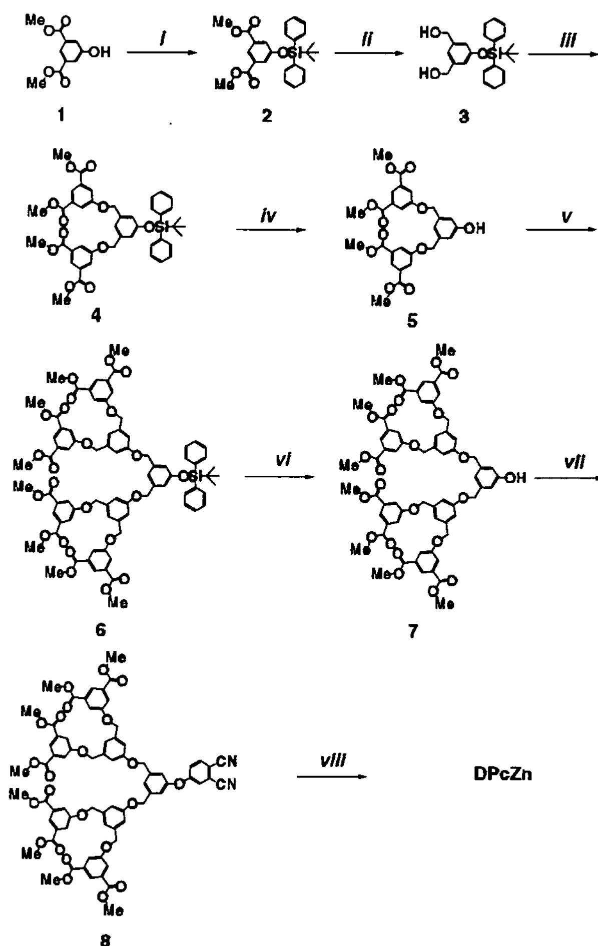
N^{ε}-Z-L-lysine and bis(trichloromethyl) carbonate (triphosgene), for the synthesis of polyethylene glycol-block-poly-L-lysine (PEG-*b*-PLL), were purchased from Tokyo Kasei Co., Ltd. α-Methoxy-ω-amino-poly(ethylene glycol) (MeO-PEG-NH_2, Mw = 12 kg/mol) was a kind gift from Nippon Oil and Fats Co., Ltd. Chemicals for dendrimer synthesis were purchased from Tokyo Kasei Co., Ltd. or Aldrich Chemical Co., Ltd. Tetrahydrofuran and hexane, used as a solvent for the synthetic reactions, were distilled from sodium benzophenone ketyl under Ar just before use. *n*-Pentanol and 1,8-diazabicyclo-(5,4,0)-undec-7-ene (DBU) for phthalocyanine dendrimer synthesis, were used as received without further purification.

2.2. Synthesis of block copolymer

MeO-PEG-NH_2 was precipitated in diethylether from chloroform, dried under reduced pressure and subsequently freeze-dried from benzene prior to use in the block copolymer synthesis. PEG-*b*-PLL was synthesized by a previously reported procedure [17]. Briefly, the *N*-carboxy anhydride of N^{ε}-Z-L-lysine was polymerized by initiation with CH_3O-PEG-NH_2 (12 000 g/mol) in DMF under Ar, followed by deprotection of the Z group. GPC measurement of PEG-*b*-PLL exhibited single sharp peak at Mw of 16,600 and Mn of 16,300 based on PEG standards. From the ^{1}H NMR measurement in D_2O, the polymerization degree of the PLL segment was determined to be 39.

2.3. Synthesis of dendrimer phthalocyanine

Dendrimer phthalocyanine (DPcZn) was prepared from dimethyl-5-hydroxyisophthalate and 4-nitrophthalonitrile



Scheme 1. Synthesis of phthalocyanine dendrimer. Reagents and conditions; (i) *tert*-butyldiphenylsilylchloride, imidazole, in DMF at 0 °C for 12 h; (ii) $LiAlH_4$ in THF at 0 °C for 12 h; (iii) 1, diethylazodicarboxylate (DEAD), PPh3 in THF at 0 °C 12 h; (iv) tetrabutylammoniumfluoride (TBAF) in THF at 0 °C for 1 h; (v) 1, DEAD, PPh3 in THF at 0 °C 12 h; (vi) TBAF in THF at 0 °C for 1 h; (vii) K_2CO_3, 4-nitrophthalonitrile, 18-crown in DMF at 60 °C for 12 h; (viii) Zn $(OAc)_2$, DBU in Pentanol reflux for 24 h.

according to the literature method (Scheme 1) [15]. Briefly, the hydroxy group of dimethyl-5-hydroxyisophthalate (**1**) was protected with a tert-butyldiphenylsilyl chloride to obtain 3-*tert*-butyldiphenylsilyloxy-dimethylisophthalate (**2**), and then the methyl ester groups were reduced to obtain 3-*tert*-butyldiphenylsilyloxy-5-hydroxymethyl benzyl alcohol (**3**), which was reacted with **1** using Mitsunobu's coupling reaction to obtain a silyl-protected G1 dendron (**4**). G1 dendron with phenol core (**5**) was obtained from **4** by deprotection reaction using tetrabutylammonium fluoride (TBAF). A silyl-protected G2 dendron (**6**) was synthesized from **5** by Mitsunobu's coupling reaction, and then deprotected to obtain G2 dendron with phenol core (**7**). The alkali mediated coupling reaction of **6** with 4-nitrophthalonitrile gave phthalonitrile-cored G2 dendron (**8**). A mixture of **8** and Zn $(OAc)_2$ in *n*-pentanol was heated at 90 °C, and then a few drops of 1,8-diazabicyclo[5,4,0]undec-7-ene (DBU) were added. The mixture was refluxed with stirring overnight. The reaction mixture was chromatographed with silica gel to obtain DPcZn.

2: yield 95%, ^1H NMR δ 8.18 (m, 1H, Ar–*H* in phthalate), 7.70 (m, 4H, *o-H* in C_6H_5), 7.57 (m, 2H, Ar–*H* in phthalate), 7.40 (m, 6H, *m,p-H* in C_6H_5), 3.86 (s, 6H, –OCH_3), 1.13 (s, 9H, –C(CH_3)$_3$). **3**: yield 89%, ^1H NMR δ 7.64 (m, 4H, *o-H* in C_6H_5), 7.32 (m, 6H, *m,p-H* in C_6H_5), 6.80 (m, 1H, Ar–*H* in C_6H_3), 6.58 (m, 2H, Ar–*H* in C_6H_3), 4.41 (s, 4H, –CH_2–), 1.03 (s, 9H, –C(CH_3)$_3$). **4**: yield 64%, ^1H NMR δ 8.27 (s, 2H, Ar–*H* in outer C_6H_3), 7.73 (s, 4H, Ar–*H* in outer C_6H_3), 7.69 (m, 4H, *o-H* in C_6H_5), 7.40 (m, 6H, *m,p-H* in C_6H_5), 7.02 (s, 1H, Ar–*H* in inner C_6H_3), 6.58 (s, 2H, Ar–*H* in inner C_6H_3), 4.97 (s, 4H, –CH_2–), 3.94 (s, 12H, –CH_3), 1.01 (s, 9H, –C(CH_3)$_3$). **5**: yield 90%, ^1H NMR δ 8.27 (s, 2H, Ar–*H* in outer C_6H_3), 7.80 (d, 4H, Ar–*H* in outer C_6H_3), 7.05 (s, 1H, Ar–*H* in inner C_6H_3), 6.92 (s, 2H, Ar–*H* in inner C_6H_3), 6.13 (s, 1H, –O*H*), 5.08 (s, 4H, –CH_2–), 3.94 (s, 12H, –CH_3). **6**: yield 79%, ^1H NMR δ 8.26 (s, 4H, Ar–*H* in outer C_6H_3), 7.79 (s, 8H, Ar–*H* in outer C_6H_3), 7.69 (m, 4H, *o-H* in –C_6H_5), 7.35 (m, 6H, *m,p-H* in –C_6H_5), 7.04 (s, 2H, Ar–*H* in inner C_6H_3), 6.98 (s, 1H, Ar–*H* in inner C_6H_3), 6.94 (s, 4H, Ar–*H* in mid C_6H_3), 6.81 (s, 2H, Ar–*H* in mid C_6H_3) 5.10 (s, 8H, outer –CH_2–), 5.00 (s, 4H, inner –CH_2–), 3.93 (s, 24H, –CH_3), 1.08 (s, 9H, –C(CH_3)$_3$). **7**: yield 91%, ^1H NMR δ 8.27 (s, 4H, Ar–*H* in outer C_6H_3), 7.79 (s, 8H, Ar–*H* in outer C_6H_3), 7.36 (s, 2H, Ar–*H* in inner C_6H_3), 7.08 (s, 1H, Ar–*H* in inner C_6H_3), 7.01 (s, 4H, Ar–*H* in mid C_6H_3), 6.87 (s, 2H, Ar–*H* in mid C_6H_3), 6.37 (s, 1H, –O*H*), 5.11 (s, 8H, outer –CH_2–), 5.09 (s, 4H, inner –CH_2–) 3.93 (s, 24H, –CH_3). **8**: yield 90%, ^1H NMR δ 8.28 (s, 4H, Ar–*H* in outer C_6H_3), 7.80 (s, 8H, Ar–*H* in outer C_6H_3), 7.68 (d, 1H, Ar–*H* in phthalonitrile), 7.45 (s 1H, Ar–*H* in inner C_6H_3) 7.28–7.23 (m, 2H, Ar–*H* in phthalonitrile), 7.15 (s, 2H, Ar–*H* in inner C_6H_3), 7.14 (s, 2H, Ar–*H* in mid C_6H_3), 7.04 (s, 4H, Ar–*H* in mid C_6H_3), 5.16 (s, 4H, outer –CH_2–), 5.13 (s, 8H, inner –CH_2–) 3.93 (s, 24H, –CH_3). DPcZn: yield 32%, ^1H NMR δ 9.22–8.88 (m, 8H, Ar–*H*), 8.2–7.7 (m, 28H, Ar–*H*), 7.6–6.9 (m, 60H, Ar–*H*), 5.2–4.9 (m, 48H, ArOCH_2–), 4.2–4.0 (m, 64H, –CO_2CH_2–), 1.7–1.1 (m, 256H, –CH_2–), 0.9–0.7 (m, 96H, –CH_3), MALDI-TOF-MS for $C_{416}H_{496}N_8O_{92}Zn$ *m/z*: calcd.: 7139 [M^+]; found 7150.

2.4. Preparation of polyion complex micelle

Polyion complex micelles were made from charged DPcZn with PEG-*b*-PLL. In a typical procedure, the PEG-*b*-PLL was dissolved in an aqueous NaH_2PO_4 solution and added to an aqueous solution of DPcZn in Na_2HPO_4 to give a solution containing polyion complex micelles. The ratio of positive charge to negative charge was fixed at 1:1.

2.5. Measurements

The DLS measurements were performed using a Photal dynamic laser scattering DLS-7000 spectrometer (Otsuka Electronics Co., Ltd., Osaka, Japan) equipped with GLG3050 488 nm Ar laser (NEC Co., Ltd., Japan) and/or Zetasizer Nano ZS-90 (Malvern Co., Ltd., USA) with 532 nm laser irradiation. The UV–Vis and fluorescence spectra were measured using a V-550 spectrophotometer (JASCO, Tokyo, Japan) and Type 850 spectrofluorometer (Hitachi, Tokyo, Japan), respectively. MALDI-TOF-MS was performed on a Bruker model Protein TOF mass spectrometer with dithranol as the matrix. ^1H NMR spectroscopy was performed in $CDCl_3$ or D_2O on a JEOL GSX-270 spectrometer operating at 270 MHz. GPC was performed with TOSOH HLC-8220 equipped with TSK-gel G4000HHR and G3000HHR column (eluent: DMF + 10 mM LiCl, temperature: 40 °C, detector: RI).

2.6. Oxygen consuming measurement

The oxygen consumption amount was measured using a Clark-type oxygen microelectrode with a tip diameter of 200 μm (PO_2-100DW, Eikou Kagaku Co., Ltd., Tokyo, Japan). The microelectrode was inserted into the PBS, which contained 3.13 μM of DPcZn or DPcZn/m and 10% FBS as a singlet oxygen acceptor, so that the tip was 100 μm above the bottom of the solution. Semiconductor laser light (660 nm; FWHM 6 nm, 25 mW/cm^2) was used for light irradiation. The solution was static and exposed to the atmosphere. Before each measurement, the system was calibrated in saline bubbled with air, in which the partial oxygen pressure was assumed to be 150 mm Hg.

2.7. Cell culture

HeLa cells were used in the cell culture studies. In the cytotoxicity assay, different concentration of DPcZn or DPcZn/m in Dulbecco's modified Eagle's medium (DMEM + 10% FBS) were added to cells in 96-wll culture plates (n = 4). After a 24 h incubation at 37 °C, the photosensitizers were removed, and then plates were photoirradiated for 15–60 min with broad-band visible light using a halogen lamp (150 W) equipped with a filter passing light of 400–700 nm (fluence energy; 27–107 kJ/m^2). The viability of the cells was evaluated using mitochondrial respiration via the 3-(4,5-dimethyl thiazole-2-yl)-2,5-diphenyltetrazolium bromide cleavage assay (MTT assay) following incubation for 48 h after photoirradiation or removing the photosensitizers by washing in the case of the dark toxicity investigation.

2.8. Cellular uptake amount

After incubation of HeLa cells with 10 μM of DPcZn or DPcZn/m for 24 h in 60-mm dishes, the cells were washed three times with PBS, and then dissolved in 20% SDS solutions for 24 h to give a homogenous solution. As a control experiment, HeLa cells were incubated without DPcZn or DPcZn/m addition and then dissolved in 20% SDS solutions include fixed amount of DPcZn or DPcZn/m. The homogeneous solution thus obtained was put into a quartz cell to measure fluorescence. Before measuring samples, it was confirmed that DPcZn or DPcZn/m has comparable intensity of fluorescence in the 20% SDS solution. Quantitative analysis of uptake amount of DPcZn and DPcZn/m by HeLa cells was performed on a fluorescence spectrophotometer (Type 850, Hitachi, Tokyo, Japan). The excitation wavelength was 630 nm, and the emission wavelength was measured from 650 to 900 nm. The number of HeLa cells was 60,000.

3. Results and discussion

3.1. Synthesis of dendrimer phthalocyanine and preparation of polyion complex micelle

The synthesis of the ionic dendrimer phthalocyanine was accomplished by the method of Ng's group [15]. 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 dendrimer phthalocyanine. Each step of synthesis was characterized by MALDI-TOF-MS and ^1H NMR measurement, and reaction yields were almost comparable to the literature. The dendrimer phthalocyanine thus obtained was treated with a THF/H_2O mixture solution of NaOH to obtain ionic dendrimer phthalocyanine (DPcZn; Fig. 1). DPcZn exhibited significantly high solubility at various pHs of the aqueous medium (over pH 4.3).

A cationic block copolymer (poly(ethyleneglycol)-*block*-poly-L-lysine: PEG-*b*-PLL; Fig. 1) was synthesized by the polymerization of the *N*-carboxy anhydride of N^{ε}-Z-L-lysine, initiated by ω-aminated poly(ethyleneglycol) (CH_3O-PEG-NH_2:12,000 g/mol) in DMF, followed by deprotection of the Z group according to a previously reported method [17]. The degree of polymerization was determined to be 39, which was confirmed by ^1H NMR. GPC measurement exhibited single sharp peak and relatively small molecular weight value compare to ^1H NMR result because of the interaction between PLL segment and GPC column.

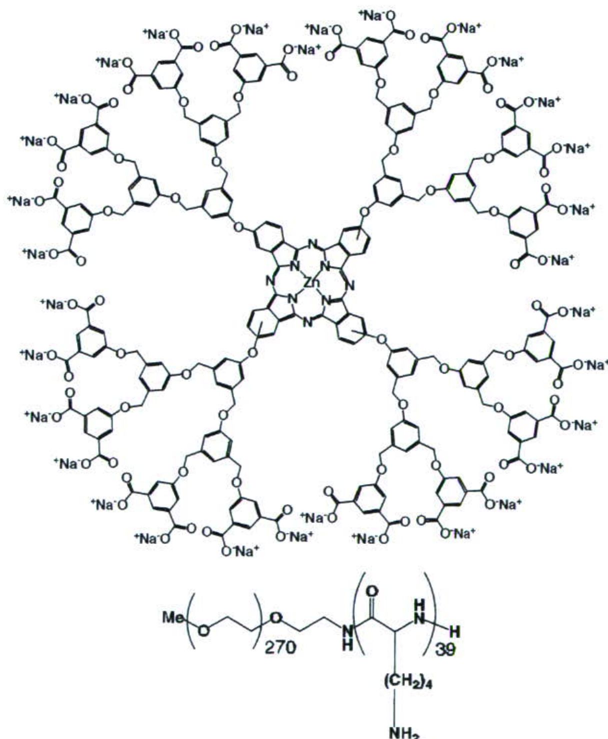


Fig. 1. Structures of DPcZn and PEG-*b*-PLL.

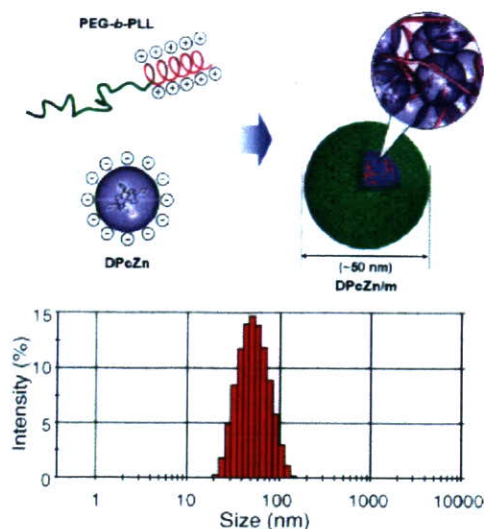


Fig. 2. Formation and DLS histogram analysis of polyion complex micelle (DPcZn/m).

Polyion complex micelles were prepared from negatively charged DPcZn with oppositely charged PEG-*b*-PLL. In a typical procedure, the PEG-*b*-PLL (14.2 mg) was dissolved in an aqueous NaH_2PO_4 (10 mM, 6.15 mL) and added to a solution of DPcZn (5 mg) in aqueous Na_2HPO_4 (10 mM, 13.85 mL) to give a solution containing polyion complex micelles encapsulating ionic DPcZn (Fig. 2). The ratio of positive charge to negative charge was fixed at 1:1. After mixing the two solutions, the pH of the solution becomes 7.3 (10 mM PBS). The resulting micelle has a diameter of ca. 50 nm with a narrow size distribution (unimodal, $\mu^2/\Gamma^2 = 0.12$), determined by a dynamic light scattering measurement (Zetasizer Nano ZS-90, Malvern Co., Ltd., USA) (Fig. 2). Furthermore, the diffusion coefficient of the resulting micelle was independent of the detection angle of the DLS measurement, suggesting that the polyion complex micelle of DPcZn with PEG-*b*-PLL is a narrowly dispersed spherical assembly.

3.2. Electronic absorption of dendrimer and micelle

Electronic absorption spectra of the dendrimer and micelle were measured (Fig. 3). DPcZn exhibits B band absorption at

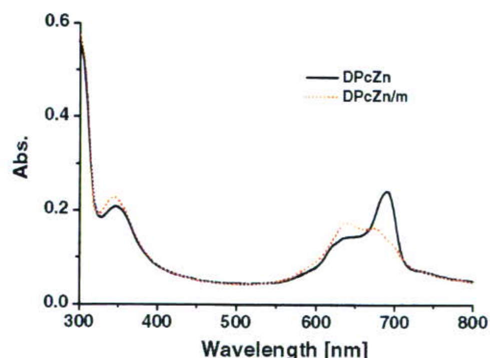


Fig. 3. Electronic absorption of DPcZn (15.3 μM) and DPcZn/m (15.3 μM) in 10 mM PBS.

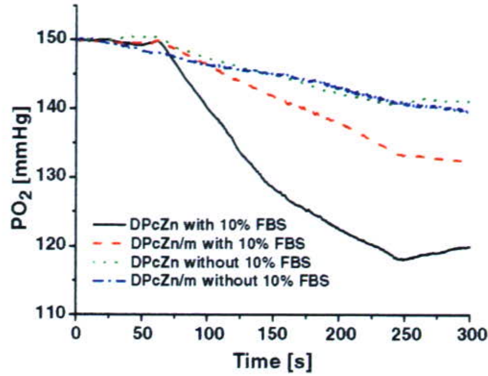


Fig. 4. Experimental setup for the measurement of oxygen consumption and the results obtained.

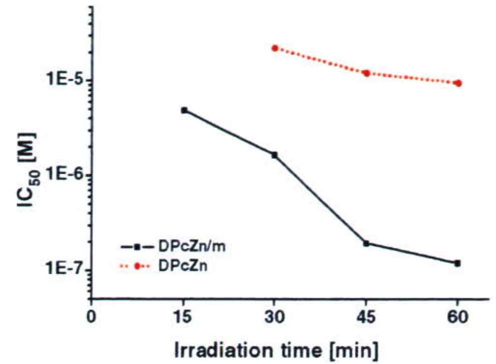


Fig. 6. Photoirradiation-time-dependent IC50 changes of DPcZn/m and DPcZn against HeLa Cells.

350 nm and strong Q band absorption at 685 nm, indicating successful dispersion as a monomeric species in the aqueous solution [16]. According to the formation of the polyion complex micelle, the absorption maximum of Q band absorption was slightly changed to 630 nm, indicating the possibility of slight aggregate formation of the core phthalocyanine units. Also, fluorescent intensity of DPcZn was drastically decreased by inclusion into the micelle (date not shown). The relatively small dendritic wedges may not perfectly prevent the aggregate formation of the phthalocyanine core units especially in the densely packed micellar core. Note that DPcZn (Mw = 4901) is smaller than that of previously reported ionic dendrimer porphyrin (Mw = 8029).

The absorption of light by tissue increases as the wavelength decreases and that the most efficient photosensitizers are those that have strong absorption bands between 600 and 800 nm. Therefore, although the relatively small dendritic wedges may not perfectly prevent collisional quenching, DPcZn has the potential for use as an effective photosensitizer in photodynamic therapy.

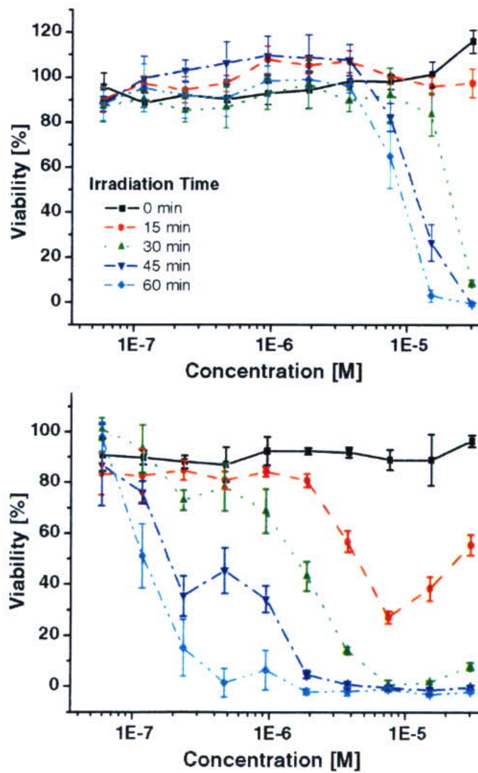


Fig. 5. Photocytotoxic profiles of DPcZn (top) and DPcZn/m (bottom) against HeLa cells.

3.3. Oxygen consumption ability of the dendrimer phthalocyanine and micelle

The oxygen consumption amount was measured to evaluate ROS generation under photoirradiation [18]. Note that the DPcZn/m was sufficiently stable in the 10 mM PBS with 10% FBS, where the size and polydispersity of DPcZn/m in the 10 mM PBS with 10% FBS were almost comparable to those without FBS (data not shown). The oxygen partial pressure (PO_2) of DPcZn solution is significantly reduced by the irradiation of laser light (660 nm; FWHM 6 nm, 25 mW/cm^2) (Fig. 4). Although the consumption ability of DPcZn/m was lower than that of DPcZn, the PO_2 of DPcZn/m solution was also effectively reduced, indicating that either DPcZn or DPcZn/m can take part in the photochemical reaction to generate ROS. On the other hand, either DPcZn or DPcZn/m solution without 10%

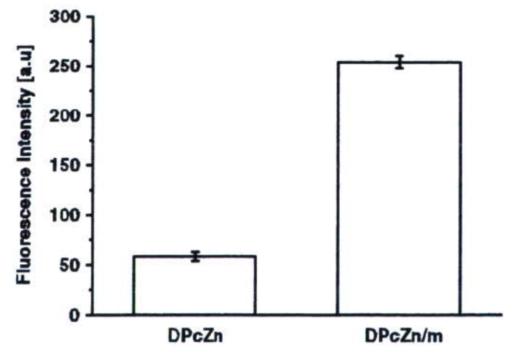


Fig. 7. Relative fluorescence intensities of uptaken DPcZn and DPcZn/m into HeLa cells. 10 μM of DPcZn or DPcZn/m was incubated with HeLa cells for 24 h. The excitation wavelength was 630 nm, and the fluorescence intensity was recorded from 650 to 900 nm.

FBS shows almost negligible change in the PO_2 upon the photoirradiation, indicating that the proteins in FBS act as sacrificial acceptors of ROS. In other words, if proteins do not exist in the medium, once generated, ROS promptly revert to oxygen molecules because of their short lifetimes.

3.4. *Cytotoxicity of dendrimer phthalocyanine and micelle*

The cytotoxicity of phthalocyanine dendrimer was assessed against HeLa cells (Fig. 5). The viability of cells upon photoirradiation was evaluated by MTT assay and determined to be a function of concentration and photoirradiation time with DPcZn and its micelle. DPcZn and DPcZn/m were incubated with the cells for 24 h and then fully washed with PBS to remove non-associated photosensitizers prior to photoirradiation. Under dark conditions, toxicities of DPcZn and DPcZn/m were negligible. However, either DPcZn or DPcZn/m exhibited photoinduced cytotoxicity upon the photoirradiation, where the cells were photoirradiated for 15–60 min with broad-band visible light using a halogen lamp (150 W) equipped with a filter passing light of 400–700 nm (fluence energy: 27–107 kJ/m^2). According to the exposure time increase, either DPcZn or DPcZn/m exhibited an increase in photocytotoxicity (Fig. 5). Very interestingly, the aspect of the photocytotoxicity increase is significantly different between DPcZn and DPcZn/m. As shown in Fig. 6, DPcZn exhibits a relatively small time-dependency, whereas DPcZn/m exhibits a remarkable change in the cell viability depending on the photoirradiation time. Typically at 60-min photoirradiation, DPcZn/m exhibited almost 100 times higher photocytotoxicity than free DPcZn. Although electronic absorption and oxygen consumption behaviours exhibited quenching signature, DPcZn/m have significantly high PDT efficacy compare to DPcZn alone. On the other hand, the cell viability exhibits abnormal increase with increase in the concentration of DPcZn/m at the 15-min light irradiation. There is various reasons can be considerable such as compositional change of micellar structure or microenvironment change around photosensitizers. To understand this phenomenon, we need further investigation.

In view of the negatively charged surface of mammalian cells, charge neutralization of DPcZn by formulation of micelle possibly improves the cellular uptake. In fact, DPcZn/m showed a 4 times higher cellular uptake compared to DPcZn alone when HeLa cells were incubated with 10 μM of DPcZn or DPcZn/m for 24 h (Fig. 7). Nevertheless, the enhancement of photocytotoxicity by the micelle formulation is much larger than the improvement in cellular uptake. Furthermore, this result is quite controversial to the quenching signature of DPcZn within the micellar core.

This phenomenon presumably suggests that the PEG shell layer of the DPcZn/m and micro environment around DPcZn may have a role in altering the intracellular mechanism of DPcZn to increase the photocytotoxicity. Also, in the case of DPcZn/m, a large amount of ROS can be generated at once within the micellar core. Therefore, the higher local concentration of ROS around the micelle may easily exceed the threshold of photodamage against typical cellular organelles.

The remarkably enhanced photocytotoxicity of the micellar system may be very advantageous point for practical applications. Because the most of photosensitizers have large π-conjugation domain and hydrophobic skeleton, photosensitizers easily form aggregates within the highly concentrated micellar core via π–π and hydrophobic interactions. The formation of aggregates result in the collisional quenching of the excitation state, photocytotoxicity will be impaired when the micellar structure occurs [19]. In contrast, because DPcZn shows less collisional quenching by micelle formation due to the large dendritic wedges, photocytotoxicity will be maintained, or even enhanced in the micelle form. The DPc-incorporated micelle is assumed to gradually dissociate into the constituent DPc and block copolymer in the body by dilution; therefore, eventually long-term phototoxicity due to non-specific uptake of photosensitizers in normal tissue may be avoidable after PDT using this micelle system [20]. Actually, our recent experiment showed that the dendritic photosensitizers have almost no skin toxicity under light irradiation compared to the clinically used photosensitizer formulation Photofrin® [21,22].

4. Conclusions

The first example of polyion complex micelle formation of DPcZn and its photodynamic efficacy were demonstrated. DPcZn/m exhibited long wavelength absorption around 650 nm, which is very advantageous for the treatment of deep lesions, because the long wavelength light is less absorbed by melanin dyes in skin tissue or heme proteins in blood. Furthermore, the micellar formulation may improve the longevity in blood circulation that achieves cumulative accumulation in the lesion with hyperpermeability, such as a macular degeneration [23], due to the enhanced permeation and retention (EPR) effect [24]. The in vivo PDT efficacy of DPcZn/m is now under investigation in our research group using disease models, such as cancer and macular degeneration.

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

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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.
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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

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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 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.

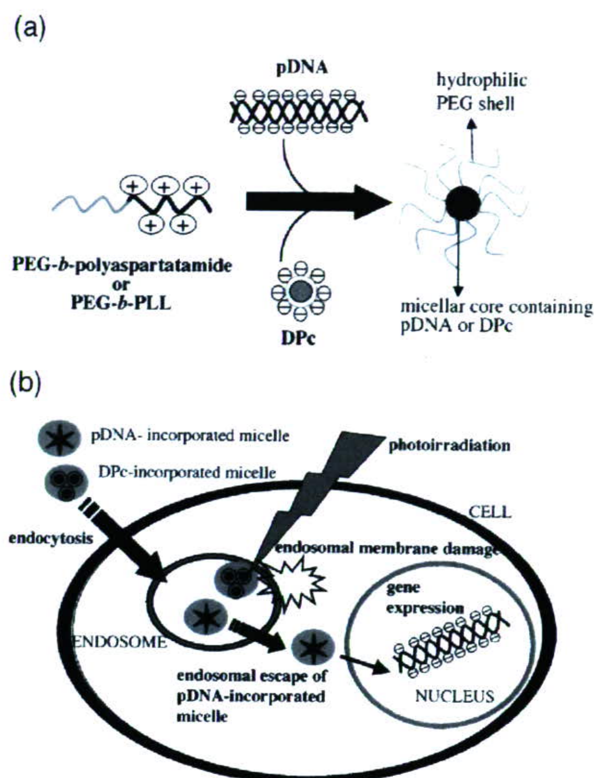

Fig. 1. Schematic illustration of (a) formation of pDNA- and DPc-incorporated micelles through electrostatic interaction between PEG-polycatiomer and pDNA or negatively charged DPc; (b) intracellular trafficking of the pDNA- and DPc-incorporated micelles in the PCI-mediated transfection. Both polymeric 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.

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)

($MeO\text{-}PEG\text{-}NH_2$, 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 catiomers 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].

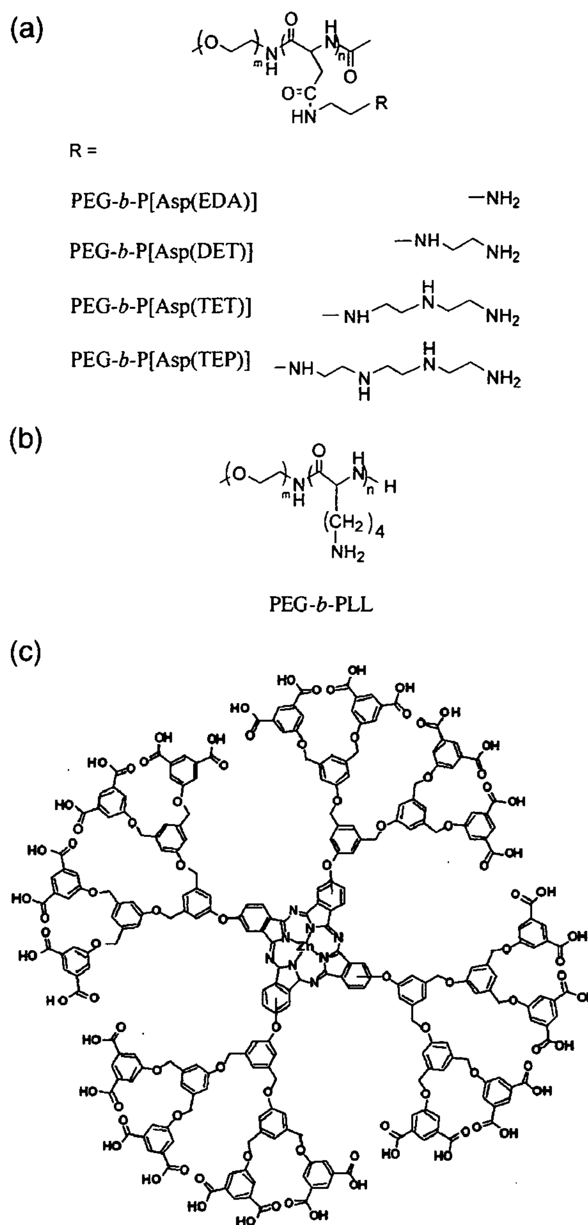

Fig. 2. (a) Chemical structures of PEG-*b*-P[Asp(EDA)], PEG-*b*-P[Asp(DET)], PEG-*b*-P[Asp(TET)], and PEG-*b*-P[Asp(TEP)]; (b) chemical structure of PEG-*b*-PLL; (c) chemical structure of anionic dendrimer phthalocyanine (DPc).

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 catiomer 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 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

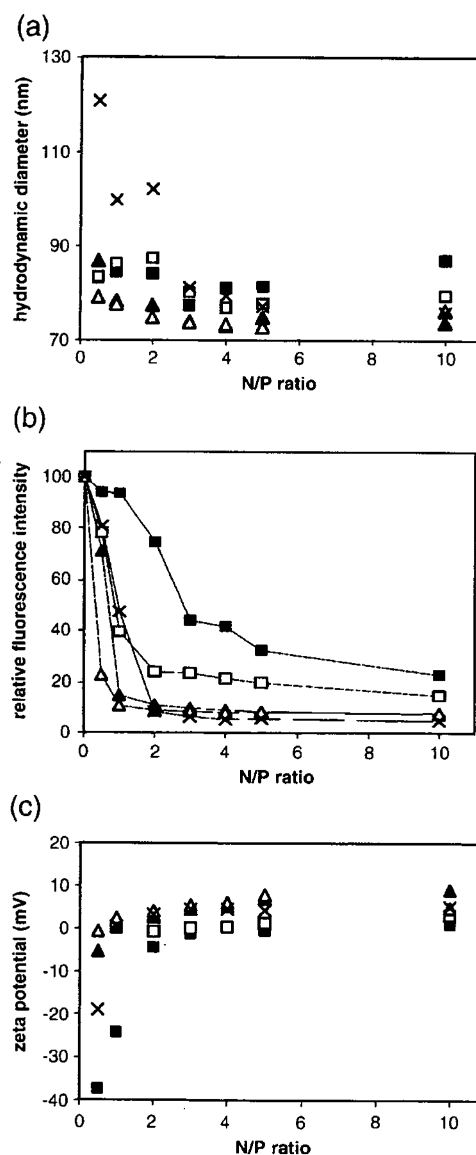

Fig. 3. Properties of PEG-*b*-polycation/pDNA polyplex micelles: (a) change in the cumulant diameter, (b) fluorescence intensity of EtBr and (c) zeta potential value of the polyplexes prepared at different mixing ratios. (■) PEG-*b*-P[Asp(EDA)]; (□) PEG-*b*-P[Asp(DET)]; (▲) PEG-*b*-P[Asp(TET)]; (△) PEG-*b*-P[Asp(TEP)];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

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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

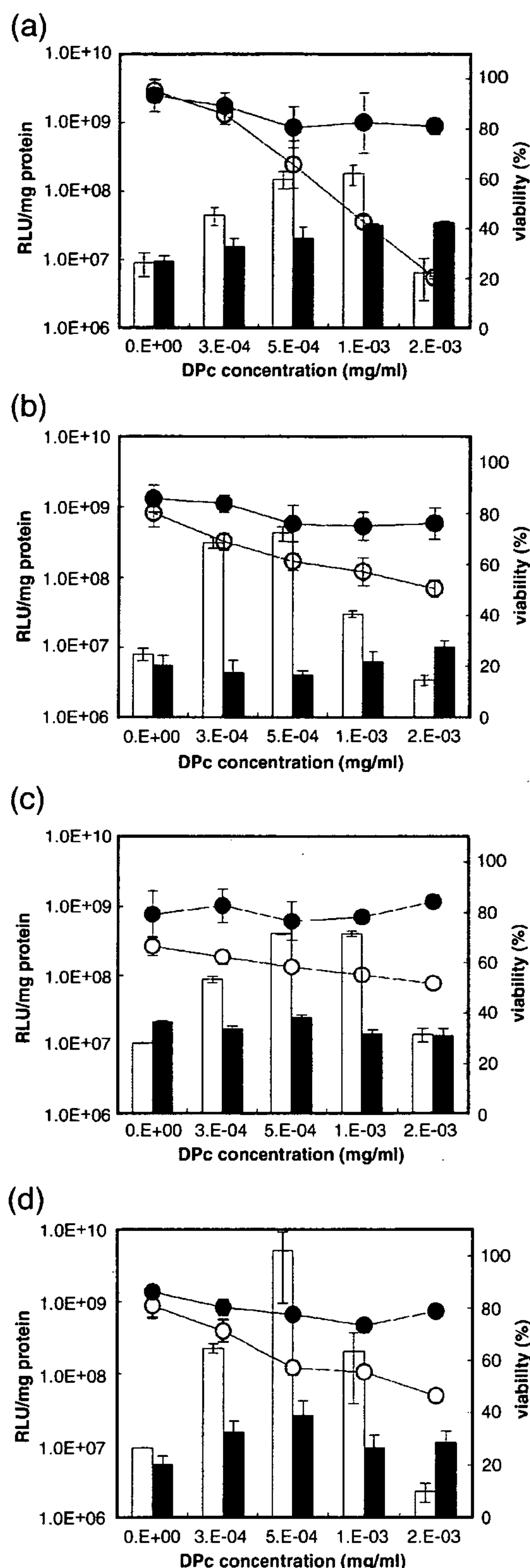

Fig. 4. The effect of DPc concentration on the transfection efficiency and cytotoxicity of the PCI-mediated transfection using the combination of pDNA- and DPc-incorporated micelles. (a) PEG-*b*-P[Asp(EDA)], (b) PEG-*b*-P[Asp (DET)], (c) PEG-*b*-P[Asp(TET)] and (d) PEG-*b*-P[Asp(TEP)] as pDNA vector.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

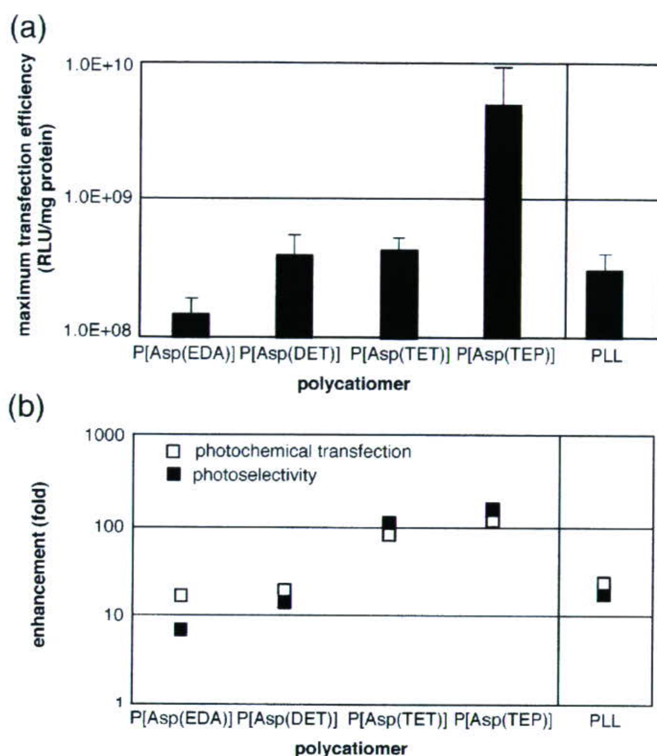

Fig. 5. Comparison of (a) transfection efficiency and (b) photochemical transfection enhancement and photoselectivity at optimal DPc concentration between the polyplex micelles from PEG-*b*-P[Asp(EDA)], PEG-*b*-P[Asp(DET)], PEG-*b*-P[Asp(TET)], PEG-*b*-P[Asp(TEP)] and PEG-*b*-PLL.

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

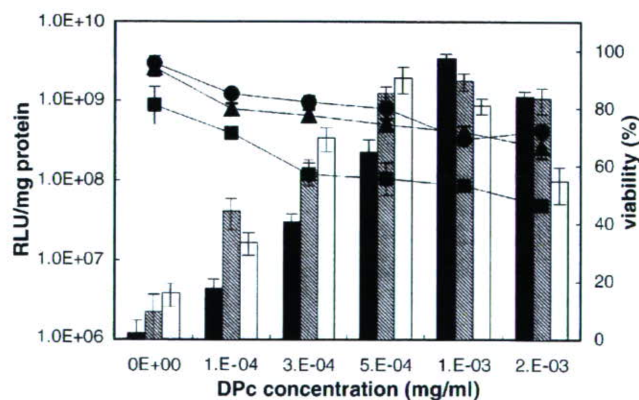

Fig. 6. The effect of irradiation times on photochemical transfection [solid bar: 20 min; hatched bar: 30 min; open bar: 45 min irradiation] and photocytotoxicity [●: 20 min; ▲: 30 min; ■: 45 min irradiation] of PEG-*b*-P[Asp(TEP)] polyplex micelle at different DPc concentrations.

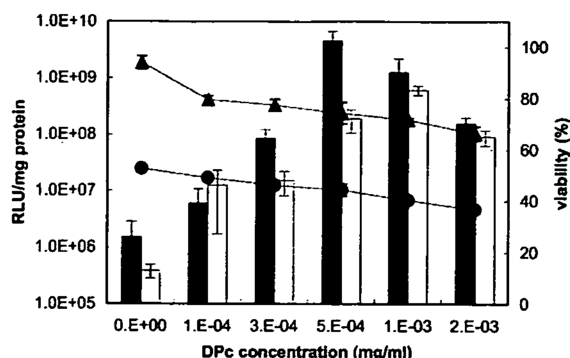

Fig. 7. Comparison of photochemical transfection efficiency (solid bar: BPEI; open bar: PEG-*b*-P[Asp(TEP)]) and photocytotoxicity (●: BPEI; ▲: PEG-*b*-P [Asp(TEP)]) between the polyplex micelles from PEG-*b*-P[Asp(TEP)] and BPEI 25 kDa at various concentrations of DPc and 20 min irradiation.

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.

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GENE DELIVERY

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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Photochemical enhancement of transgene expression by polymeric micelles incorporating plasmid DNA and dendrimer-based photosensitizer

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Abstract
The development of synthetic gene carriers has recently received much attention; however, they might lack the ability to control the transgene expression. The use of external stimuli for enhancement of the transgene expression may be a promising approach for the site-directed transfection *in vivo*. In this regard, a new technology of "photochemical internalization (PCI)" has recently been reported, in which the endosomal escape of gene carriers is assisted by photodamage of the endosomal membrane with co-incubating photosensitizers. To apply this technology for systemic gene delivery, the development of appropriate carrier systems for both the plasmid DNA (pDNA) and photosensitizer is of crucial importance. Also, the photocytotoxicity accompanied by the photochemical enhancement of the gene expression may need to be reduced. In this study, the combinational formulation of polymeric micelles incorporating pDNA and a dendrimer-based photosensitizer (DP) (dendrimer phthalocyanine (DPc)) was applied in the PCI-mediated transfection *in vitro* and then, estimating its potential utility for *in vivo* applications. The PCI using the polymeric micelle system achieved a remarkable photochemical enhancement of the transgene expression while maintaining an approximate 80% cell viability over a wide range of the DPc concentrations and light doses. Thus, this system may be promising for *in vivo* PCI-mediated gene delivery.

Keywords: *Gene therapy, non-viral gene carriers, polymeric micelles, photochemical internalization (PCI), dendrimer*

Introduction

Recently, non-viral gene carriers based on cationic lipids and synthetic polymers have received much attention as an attractive alternative to viral vectors in gene therapy (Merdan et al. 2002; Ogris and Wagner 2002; Pack et al. 2005). In addition to several advantages, such as safety, simplicity of use and ease of mass production, the variety of chemical designs of the constituent lipids and polymers is a strong motivation to develop novel gene carriers. In particular, considerable efforts have been devoted to the development of biocompatible gene carriers, which might show longevity during blood circulation and effectively accumulate at the target site (Ogris and Wagner 2002; Pack et al. 2005). In this regard, a promising approach is the use of poly(ethylene glycol) (PEG)–polycation block copolymers, since they spontaneously associate with plasmid DNA (pDNA) to form the nano-scaled polyplex micelles, in which the pDNA/polycation polyplex core is surrounded by a dense and hydrophilic PEG palisade (Katayose and Kataoka 1997; Harada-Shiba et al. 2002; Itaka et al. 2003; Miyata et al. 2004; Wakebayashi et al. 2004; Fukushima et al. 2005). Indeed, the polyplex micelles formed between pDNA and PEG–poly(L-lysine)

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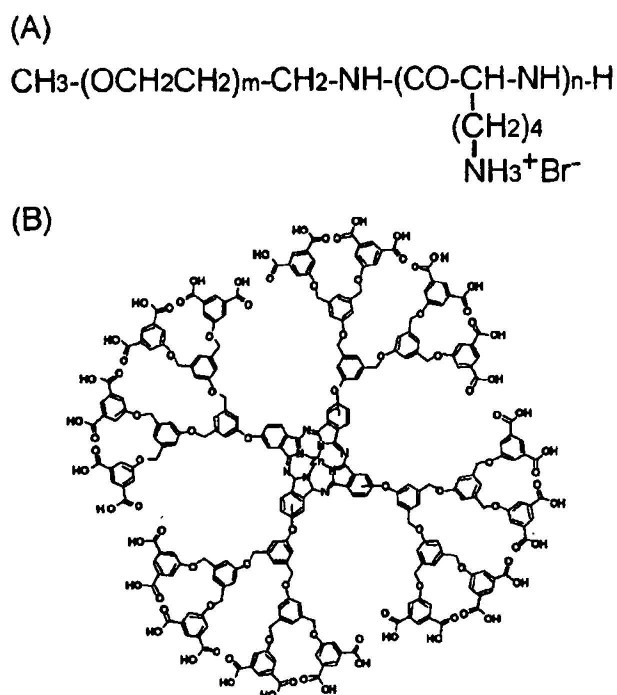

Figure 1. Chemical structures of PEG–PLL block copolymer (A) and DPc (B).

(PEG–PLL) block copolymers (Figure 1(A)) showed an improved stability in proteinous media (Itaka et al. 2003) and prolonged blood circulation in mice (Harada-Shiba et al. 2002). However, the limited transfection ability of the pDNA/PEG–PLL polyplex micelle is a major issue in their applications for *in vivo* gene therapy (Harada-Shiba et al. 2002; Itaka et al. 2003; Miyata et al. 2004). It has been suggested that such a low gene transferring ability of the polyplex micelles might be due to their inefficient transport from the endosome/lysosome to the cytoplasm (Itaka et al. 2003; Miyata et al. 2004).

Other than a high transfection efficiency, site-specific gene transfer has been strongly desired for gene vectors to ensure the safety and effectiveness for *in vivo* gene therapy. However, the existing vectors including viral and non-viral ones still have the problem of non-specific gene transfection. Recently, a smart approach called "photochemical internalization (PCI)" was introduced by Høgset and Berg et al. to overcome both the limited transfection efficiency and the lack of specificity of non-viral gene vectors. The PCI using hydrophilic photosensitizers allows the photochemical disruption of the endosomal/lysosomal membranes, facilitating the cytoplasmic delivery of macromolecular compounds such as genes and proteins (Berg et al. 1999; Høgset et al. 2000, 2002, 2004; Prasmickaite et al. 2001). This approach indeed achieved an appreciable increase in the transfection efficiency upon light illumination under *in vitro* conditions; however, it was accompanied by the problem of photocytotoxicity (Høgset et al. 2000). It should be noted that the photocytotoxicity might not be directly correlated with the photodamage to the endosomal/lysosomal membranes, but the photodamaging of other susceptible organelles may account for the cytotoxicity involved in the PCI (Macdonald and Dougherty 2001; Moan et al. 1994). Hence, the spatially localized photodamage limited to the endosome/lysosome is assumed to lead to the enhanced photochemical transfection with reduced cytotoxicity (Nishiyama et al. 2005). Also, in addition to controlled localization in the intracellular compartment, photosensitizers might need to be systemically delivered to the target tissue to achieve a successful PCI-mediated gene delivery *in vivo*.

In this study, the PCI using polymeric micelles incorporating DPs (dendrimer phthalocyanine (DPc) illustrated in Figure 1(B)) was carried out to enhance the gene transferring ability of the aforementioned pDNA/PEG–PLL polyplex micelle in a light-selective manner (Figure 2). DPc possesses a center phthalocyanine molecule surrounded by a second generation of aryl ether dendrons and 32 carboxyl groups on the periphery of the DPc allow the formation of polyion complex (PIC) micelles through an electrostatic interaction with the PEG–PLL block copolymers

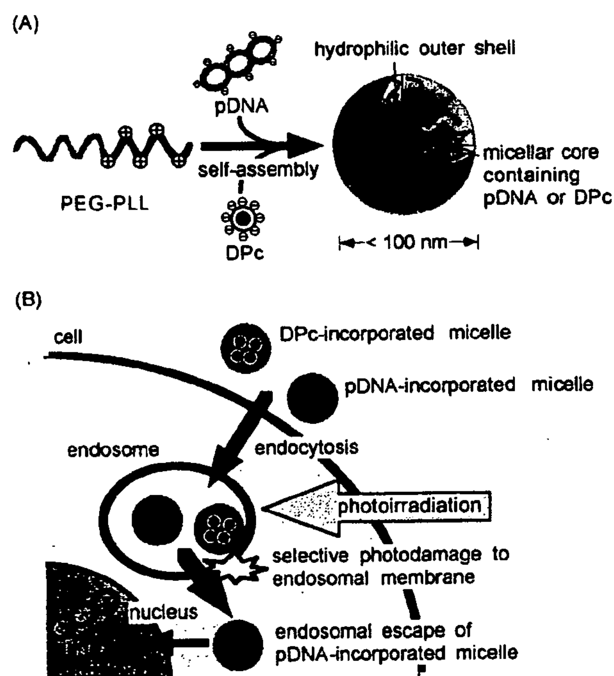

Figure 2. (A) Formation of polymeric micelles through the electrostatic interaction between PEG–PLL and pDNA or DPc. (B) Scheme for itinerary of the pDNA- and DPc-incorporated micelles in the PCI-mediated transfection. Both polymeric micelles are assumed to be taken up by the cell through the endocytic pathway and the localization of the DPc-incorporated micelles in the endosome may allow the selective photodamaging of the endosomal membrane upon photoirradiation, thereby inducing the cytoplasmic delivery of the pDNA-incorporated micelles.

(Stapert et al. 2000; Ideta et al. 2005; Jang et al. 2005). In this strategy, both the pDNA- and DPc-incorporated polymeric micelles are expected to exhibit prolonged blood circulation after intravenous administration and selectively accumulate in the target tissues such as solid tumors as previously reported (Kwon et al. 1994; Nishiyama et al. 2003a,b; Bae et al. 2005). Also, both micelles are assumed to show the same subcellular localization at the target site due to similar particle sizes and surface properties. The control of the localization of the photosensitizers and gene carriers at both the tissue and subcellular levels appears to be a prerequisite for the PCI-mediated gene delivery *in vivo*. Furthermore, the DPc-incorporated micelles may cause highly selective photodamage to the endosomal/lysosomal membranes, because polymeric micelles are assumed to be taken up by the cell through the endocytic pathway (Figure 2(B)). Thus, the combinational use of the pDNA- and DPc-incorporated micelles may be a promising approach to the PCI-mediated gene delivery. In the present study, the feasibility of our strategy was confirmed by the enhanced *in vitro* transfection with limited cytotoxicity under different conditions, thus featuring the possibilities for future *in vivo* applications.

Materials and methods

Materials

N^{ε}-Z-L-Lysine and bis(trichloromethyl) carbonate (triphosgene), for the synthesis of PEG–PLL, were purchased from Sigma-Aldrich Co., Inc. (St Louis, MO) and Tokyo Kasei Co., Ltd (Tokyo, Japan), respectively. α-Methoxy-ω-amino-poly(ethylene glycol) (MeO–PEG–NH_2, MW = 12 kg/mol) was purchased from Nippon Oil and Fats, Co., Ltd. Chemicals for the dendrimer synthesis were purchased from Tokyo Kasei and Sigma-Aldrich. *n*-Pentanol and 1,8-diazabicyclo-(5,4,0)-undec-7-ene (DBU) were purchased from Tokyo Kasei and used without further purification. All solvents for the polymer syntheses were distilled just before use.

A pDNA, pCAcc + Luc, containing a firefly luciferase cDNA driven by a CAG promoter (Niwa et al. 1991) was provided by the RIKEN Bioresource Center (Tsukuba, Japan). pDNA was amplified in competent DH5α *Escherichia coli* and purified by a HiSpeed Plasmid Maxi Kit from Qiagen Co., Inc. (Valencia, CA). Sulfonated aluminum phthalocyanine ($AlPcS_{2a}$) (aluminum phthalocyanine with two sulfonate groups on adjacent phthalate rings) was purchased from Frontier Scientific Co., Inc. (Logan, UT).

Polymer synthesis and characterization

The synthesis of the ionic DPc was performed according to the method reported by Ng's group (Ng et al. 1999). 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 DPc. The obtained DPc was treated with a THF/H_2O mixture solution containing NaOH to obtain the ionic DPc (MW: 4904). The absorption spectra in an aqueous solution revealed that DPc exhibits a B band absorption at 350 nm and a strong Q band absorption at 685 nm, indicating a monomeric dispersion without agglomerization.

PEG–PLL block copolymers (PEG: MW = 12 kg/mol) having different polymerization degrees of the PLL segment (49 and 73; the code names are 12–49 and 12–73, respectively) were synthesized as previously reported (Harada and Kataoka 1995). Briefly, the *N*-carboxy anhydride of N^{ε}-Z-L-lysine was polymerized from the ω-NH_2 group of CH_3O–PEG–NH_2 in DMF under Ar to obtain PEG–PLL(Z), followed by deprotection of the Z group. The polymerization degree of the PLL segments and the narrowly distributed nature of the synthesized PEG–PLL(Z) were determined by the ^{1}H-NMR and the gel permeation chromatography (GPC), respectively.

Preparation of DPc-incorporated micelles

The DPc-incorporated micelles were prepared by mixing DPc and PEG–PLL 12–49 at a stoichiometric charge ratio. In a typical procedure, PEG–PLL was dissolved in a 10 mM NaH_2PO_4 solution (0.457 ml) and added to DPc in a 10 mM Na_2HPO_4 solution (1.0 ml) to give the solution containing the DPc-incorporated micelles in a 10 mM phosphate buffered solution (pH 7.4). The size and size distribution (polydispersity index) of the micelles were measured by dynamic light scattering (DLS) measurements using a DLS-7000 instrument with a vertically polarized incident beam of 488 nm wavelength from an Ar ion laser (Otsuka Electronics Co., Ltd, Osaka, Japan).

Preparation of pDNA/PEG–PLL polyplex micelles

pDNA and PEG–PLL 12–73 were separately dissolved in a 10 mM Tris–HCl buffer (pH 7.4). The PEG–PLL solution with varying concentrations was then added to the pDNA solution to form the polyplex micelles with different N/P ratios, which denote the ratio of the molar concentration of the cationic amino groups in PEG–PLL to that of the phosphate groups in DNA. The polyplex micelle solution was maintained overnight at ambient temperature before use.

The size and size distribution of the polyplex micelles were measured by the DLS measurement using the DLS-7000. The effect of the N/P ratios on the pDNA condensation in the polyplex micelles was estimated from a decrease in the fluorescence intensity

of ethidium bromide (EtBr) due to the exclusion from the DNA double strand. The polyplex micelle solutions at various N/P ratios were adjusted to 20 μg pDNA/ml with 0.4 μg EtBr/ml by adding 10 mM Tris–HCl buffer containing EtBr. The ratio of the residual molar concentration of EtBr to that of the base pair in pDNA was 0.033. The fluorescence measurements (Ex: 510 nm; Em: 590 nm) were carried out at 25°C using a FP-777 spectrofluorometer from Jasco Co., Ltd (Tokyo, Japan). The results were expressed as the relative fluorescence intensity to the intensity of the free pDNA solution with EtBr.

In vitro *transfection and cytotoxicity assays*

Human cervical carcinoma HeLa cells or human hepatoma HuH-7 cells (10,000 cells) were seeded and cultured on a 24-well culture plate (BD Bioscience, Franklin Lakes, NJ) for 24 h prior to the transfection (cell seeding density: 1400 cells/cm^2). The pDNA/-PEG–PLL polyplex micelle solution prepared at a defined N/P ratio containing 1 μg pDNA and the photosensitizer solutions (i.e. $AlPcS_{2a}$, DPc and DPc-incorporated micelle) with various concentrations were added to the cells in 0.5 ml of Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), followed by a 6 or 24 h incubation and medium replacement with a fresh one. The culture plates were photoirradiated using a 300 W halogen lamp (fluence rate: 3.0 mW/cm^2) equipped with a band-pass filter (400–700 nm) with increased fluence (2.7–8.1 J/cm^2). After a 48 h post-incubation, the transfection efficiency and cell viability were examined. In the transfection assay, the cells were lysed and the luciferase activity of the lysate was measured using the Luciferase Assay System (Promega, Madison, WI) and a Lumat LB9507 luminometer (Berthold Technologies, Bad Wildbad, Germany). The results are expressed as light units per milligram of cell protein determined by a BCA assay kit (Pierce, Rockford, IL). On the other hand, the cell viability was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Dojindo Laboratories, Kumamoto, Japan) assay.

Results and discussion

In this study, we investigated the feasibility of using polymeric micelles as a nanocarrier for the pDNA and photosensitizer in the PCI-mediated gene delivery *in vitro*. As previously described, a simple mixing of pDNA and PEG–PLL results in the formation of the polyplex micelles (Katayose and Kataoka 1997; Itaka et al. 2003). The size and polydispersity indices of the mixtures prepared at various N/P ratios are shown in Figure 3(A) and (B), indicating the formation of the polyplex micelles with sizes of 90–140 nm and a moderate polydispersity over a broad range of N/P ratios (0.5–6). The histogram analysis in the DLS measurement revealed that the polyplex micelles possess a unimodal size distribution (data not shown). Figure 3(C) shows the effect of the N/P ratios

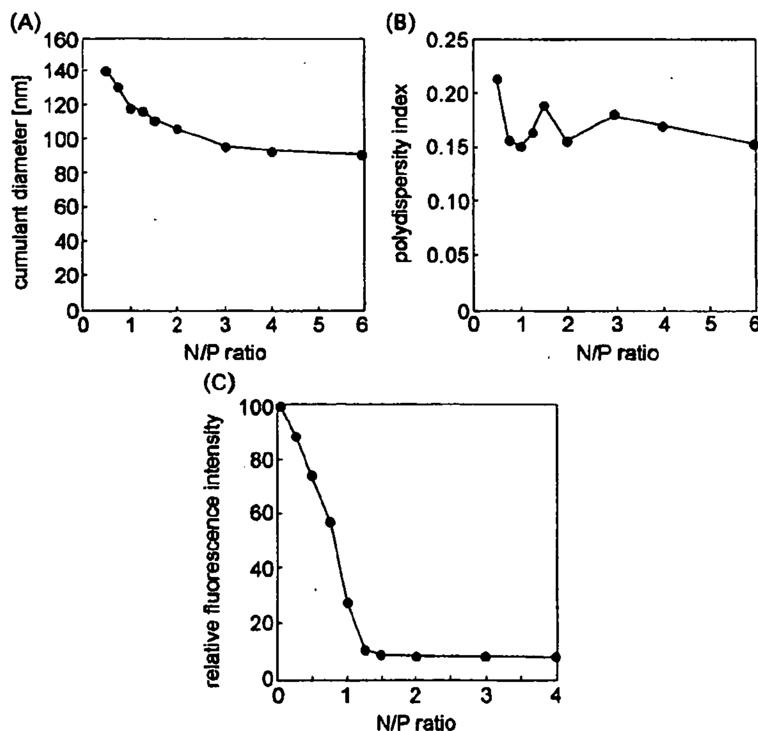

Figure 3. Changes in the cumulant diameter (A), the polydispersity index (μ_2/Γ^2) (B) and the fluorescence intensity of EtBr (C) for the pDNA/PEG–PLL polyplex micelles prepared at different N/P ratios.

on the exclusion of EtBr from the pDNA/PEG-PLL complexes. Apparently, the fluorescence intensity of EtBr leveled off above the N/P ratio of 1.2, suggesting this to be a minimum N/P ratio to fully condense pDNA. On the other hand, it is generally difficult to incorporate conventional photosensitizers into nanocarriers, because they have a hydrophobic structure with a large π-conjugation domain that easily forms aggregates. Recently, we reported DPs as a promising photosensitizer applicable for drug delivery (Nishiyama et al. 2003a,b). DPs have a focal sensitizer core segregated by a 3D dendritic architecture, which might allow effective photochemical reactions even at a high concentration and the periphery with tailored functional groups. Ionic DPs with cationic or anionic peripheral groups show a good solubility in aqueous media and form a PIC micelle with oppositely charged block copolymers (Stapert et al. 2000). We have demonstrated that polymeric micelles incorporating DPs are promising photosensitizer formulations for photodynamic therapy (Ideta et al. 2005; Jang et al. 2005). In the present study, the DPc-incorporated micelles were spontaneously formed through an electrostatic interaction between DPc and PEG-PLL. The DLS measurement revealed that the DPc-incorporated micelles had a diameter of 50 nm with a narrow size distribution (unimodal, polydispersity index (μ_2/Γ^2): 0.12). The DPc-incorporated micelles have strong Q-band absorptions at 630 and 685 nm for excitation of the DPc, thus, expecting a deeper tissue penetration of light for *in vivo* applications.

This study's objective is to investigate the feasibility of the PCI-mediated gene delivery using polymeric micelles as nanocarriers for pDNA and DPc. First, the optimal N/P ratio of the pDNA/PEG-PLL polyplex micelles on the photochemical transfection was determined. Figure 4(A) and (B) show the photochemical enhancement of the transfection of the pDNA/PEG-PLL polyplex micelles prepared at the N/P ratio of 1.2 or 2.0 and the concomitant photocytotoxicity, respectively. In this experiment, HeLa cells were photoirradiated at the fluence of 5.4 J/cm^2 after a 6 h incubation with the combination of the pDNA- and DPc-incorporated micelles and medium replacement with a fresh one, followed by a 48 h post-incubation. The polyplex micelles prepared at N/P = 1.2 achieved more than a 100-fold photochemical enhancement of the gene expression with 20-25% decreases in cell viability, whereas those prepared at N/P = 2.0 showed only a 10-30-fold gene expression enhancement with comparable decreases in cell viability. Thus, the polyplex micelles prepared at N/P = 1.2 might be more efficient in the PCI-mediated transfection compared with those prepared at N/P = 2.0. It should be noted that naked plasmid did not show any detectable gene transfection, regardless of the utilization of the PCI (data not shown), suggesting the necessity of appropriate gene

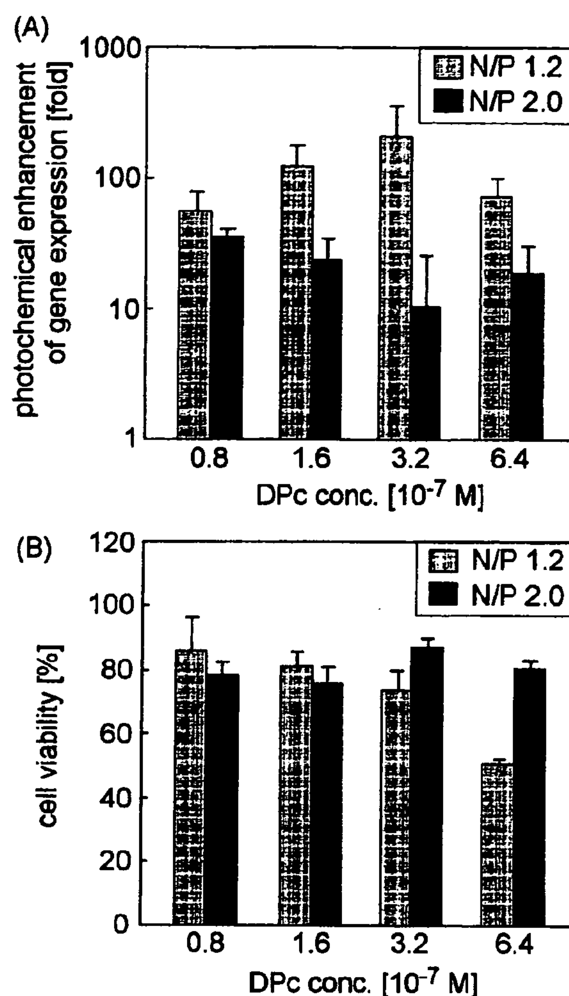

Figure 4. The effect of the N/P ratio of the polyplex micelles on the photochemical enhancement of the gene expression (A) and photocytotoxicity (B) in the PCI-mediated transfection using DPc-incorporated micelle. The light irradiation (fluence: 5.4 J/cm^2) was performed 6 h after incubation with the pDNA- and DPc-incorporated micelles, followed by 48 h post-incubation in a fresh medium.

carriers for the PCI-mediated gene delivery. Interestingly, the polyplex micelles prepared at the N/P ratios of 1.2 and 2.0 showed similar transfection efficiencies in the absence of a photosensitizer and light (5,08,000 ± 1,49,000 vs. 4,17,000 ± 1,69,000 RLU/mg protein (mean ± SD)). These results suggest that an excess of PEG PLL might decrease the efficacy of the photochemical transfection. In the PCI-mediated transfection, the efficient cytoplasmic delivery of the polyplex micelles can be achieved by photochemical rupture of the endosomal membrane; therefore, the release of pDNA from the polyplex micelles in the cytoplasm or nuclei may be a rate-limiting process in the gene transfection. The polyplex micelles at N/P = 1.2 are expected to show more efficient pDNA release than those at N/P = 2.0, thereby showing a higher photochemical enhancement of the transfection. Based on these results,