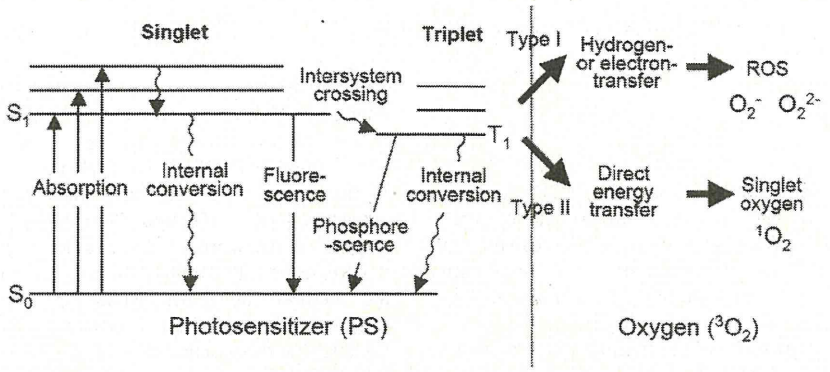

Fig. 1. Jablonski diagram showing the energy transfer from photosensitizers (PSs) to molecular oxygen.

state energy level (S_0) absorb a photon of the light of a specific wavelength to enter an excited state (S_1). PSs in the short-lived excited state return to the ground state by emitting fluorescence or by internal conversion with energy loss as heat, and also can be converted to the excited triplet state (T_1) with a longer lifetime via an intersystem crossing process. PSs in the triplet state return to the ground state by emitting phosphorescence, and also can transfer energy to other molecules by type I and type II reaction processes. In the type I reaction, free radicals formed by the hydrogen- or electron-transfer from PSs react with oxygen, thereby producing reactive oxygen species, including superoxide (O_2^-) and peroxide anions (O_2^{2-}). In the type II reaction, PSs in the excited triplet state directly transfer energy to molecular oxygen in a triplet ground state ($^3O_2$), resulting in the formation of highly reactive singlet oxygen ($^1O_2$). In PDT, these reactive oxygen species (ROS) oxidize (photodamage) subcellular organelles and other biomolecules, leading to light-induced cell death.

PDT has been shown to be effective against early-stage superficial cancers [1–4]. Also, PDT is non-invasive and has little or no effect on organ functions, so that it is recognized as a useful initial treatment for malignant tumors. Indeed, PDT using porfimer sodium (Photofrin®) has been approved for the treatment of oesophageal cancer in the United States and Canada, early- and late-stage lung cancer in the Netherlands, bladder cancer in Canada, and early-stage lung, oesophageal, gastric and cervical cancers in Japan [4]. However, PDT is often accompanied by long-lasting skin toxicity, which is a major limitation in the clinical application [1–4]. This is mainly due to the unfavorable biodistribution of PSs [5]. Therefore, a challenge in PDT is to create tumor-specific PSs. In this regard, the use of nanocarriers is a promising method for the tumor-specific delivery of PSs. To date, a variety of nanocarriers, including polymer-PS conjugates [6,7], long-circulating liposomes [8] and polymeric micelles [9–24], have been developed, because they have been demonstrated to show tumor-selective accumulation due to the enhanced microvascular permeability and impaired lymphatic drainage in the tumor tissue, a phenomenon which Maeda et al. termed the enhanced permeability and retention (EPR) effect [25–28]. Recently, we also developed a new PS formulation based on polymeric micelles encapsulating dendrimer photosensitizers, and demonstrated its remarkable efficacy in vitro and in vivo [17–24,29]. This review deals with general concepts and considerations of PDT with nanocarrier-encapsulated PSs, and our recent progress in research on polymeric micelles encapsulating dendrimer photosensitizers.

2. Photodynamic therapy (PDT) with nanocarrier-encapsulated photosensitizers (PSs)

2.1. Biodistribution of nanocarrier-encapsulated PSs

In general, most PSs intravenously administered are rapidly cleared from the circulation, although some of the molecules bind to serum proteins such as albumin and low-density lipoprotein (LDL) and remain in circulation for a longer period [3]. PSs show some specificity to the tumor cells. This may be partially due to the facilitated uptake of LDL-bound PSs by tumor cells expressing a large number of LDL receptors on their surface [3,30,31]. However, such LDL-bound PSs can also be taken up by macrophages; therefore, localization of macrophages containing LDL-bound PSs in the skin may be responsible for the skin hyperphotosensitivity [3,30,31]. To circumvent such a side effect, considerable efforts have been devoted to the development of new PSs that accumulate quickly in the tumor tissue and show rapid clearance from the body. For example, taporfin sodium (Talaporfin), a second generation PS, shows rapid clearance from the skin and has been approved for the treatment of early-stage lung cancers in Japan [32,33]. Thus, PDT with Talaporfin can reduce the skin phototoxicity; however, the patient still needs to stay in a darkened room for at least 2 weeks.

The use of long-circulating nanocarriers for PS delivery seems to be contradictory to the aforementioned recent trends in the development of new PSs. Nevertheless, long-circulating nanocarriers preferentially accumulate in the tumor tissue due to the EPR effect. It is known that long-circulating nanocarriers can achieve a tumor/normal tissue (muscle) accumulation ratio higher than 10 [34], whereas most conventional PSs including chlorin e_6 and Photofrin have been reported to exhibit a value of less than 2 for this ratio [5]. Buchholz et al. reported that free meta-tetrahydroxyphenylchlorin (m-THPC) and a stealth liposomal formulation of m-THPC showed tumor/skin accumulation ratios of 1.55 ± 0.88 and 6.50 ± 3.18, respectively [35]. Thus, the nanocarrier-mediated PS delivery can achieve a higher tumor/skin ratio compared with administration of PS alone. In general, low molecular weight PSs are assumed to penetrate the endothelium by passive diffusion and eventually accumulate in various organs and tissues to some extent. Such non-specific PS accumulation in the skin and normal tissues seems to be inevitable, even if PSs show rapid clearance. In contrast, nanocarriers might not pass through the tight junctions of the vasculature in the tissues (with the exception of the liver, spleen, and tumor tissues, which possess leaky vasculatures), which could account for their ability to achieve reduced PS accumulation in the skin and normal tissues. Although the underlying mechanisms of this effect remain to be clarified, the high tumor-specificity of nanocarrier-encapsulated PSs ensures the effectiveness and safety of PDT.

At the same time, the prolonged retention of nanocarrier-encapsulated PSs in the tumor tissue enables PDT with multiple irradiations. Kopecek et al. demonstrated that, in the PDT with *N*-(2-hydroxypropyl) methacrylamide copolymer-mesochlorin e_6 conjugates (PHPMA-Me_6), multiple irradiation can achieve a higher antitumor effect than single irradiation with an equivalent light dose [6]. Since the PDT effect depends on the oxygen concentration in the tumor tissue, a single excessive photoirradiation may consume considerable amounts of

oxygen, resulting in limited therapeutic efficacy. In contrast, double irradiation with a time lag allows oxygen to be reaccumulated in the tumor tissue, enhancing the efficacy of PDT.

2.2. In vivo mechanisms of PDT

The in vivo mechanisms of the anti-tumor effect of PDT are rather complicated. As illustrated in Fig. 2, several different but mutually related effects are involved. First, ROS generated from PSs can directly kill tumor cells. Second, PDT can damage the tumor vasculature, leading to vascular collapse and embolization [1]. And finally, PDT can activate anti-tumor immunity [40].

In regard to the first mechanism, the killing of tumor cells by ROS, it is difficult to achieve complete tumor eradication by this mechanism alone due to the generally non-uniform distribution of PSs in the tumor tissue. Such uneven distribution may be due to at least two causes: the difficulty of delivering therapeutic agents to tumor cells distant from the vasculature [36,37], and the restriction of drug penetration into solid tumors by increased interstitial fluid pressure (IFP) in the tumor tissue [37]. Both these problems may also be relevant to PDT using nanocarriers. To address them, we have recently explored the tumor-penetrability of polymeric micelles and found that polymeric micelles incorporating doxorubicin (Dox) showed penetrability into multi-cellular tumor spheroids [38] as well as solid tumors after intravenous administration [39]. These results were in contrast to those for the intratumoral distribution of Doxil®, a stealth liposomal formulation of Dox, which showed localized accumulation in perivascular regions and stromal tissues [39], although the underlying mechanisms remain to be clarified. Thus, there is a possibility that the use of polymeric micelles may overcome the limitations in the penetration into solid tumors. In addition to a non-uniform intratumoral distribution of PSs, limited oxygen supply due to the above-mentioned features of solid tumors as well as oxygen consumption during PDT might also decrease the PDT efficacy. This can be partially solved by multiple light irradiation. In this regard, nanocarriers might have a great advantage due to their prolonged retention property in solid tumors as described in Section 2.1.

In the second mechanism, PDT-induced vascular collapse and embolization abolishes the supply of oxygen and nutrients to the tumor cells, resulting in tumor destruction. We previously reported that the vessels treated with PDT using dendrimer porphyrin (DP)-loaded micelles regressed to collagen tubes without endothelial cells or were occluded by erythrocytes [21]. Such vascular collapse and embolization by PDT may also affect the accumulation of nanocarriers in the tumor tissue—i.e., this effect may shut out the outward convective flow by elevated IFP in the tumor tissue and improve the retention of nanocarriers in the tumor tissue. In this regard, Kopecek et al. reported that combination therapy using PDT with PHPMA-Me_6 and chemotherapy with PHPMA-Dox conjugates was much more effective than the multiple chemotherapy with PHPMA-Dox [6]. It is possible that the vascular damage by PDT may improve the retention of PHPMA-Dox in the tumor tissue.

As stated above, the third mechanism involves the activation of anti-tumor immunity by PDT [40]. This is unusual in that most cancer therapies—including surgical resection and chemotherapy—are immunosuppressive, whereas PDT is immunostimulatory. PDT induces acute inflammation through the release of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin (IL)-1 and IL-6, and thereby attracts leukocytes such as dendritic cells (DCs) and neutrophils. On the other hand, PDT induces necrosis or apoptosis of tumor cells, thereby stimulating the production of tumor cell-derived antigens. Such alterations of the tumor micro-environment by PDT might increase the antigen cross-presentation efficiency of DCs and facilitate their maturation, leading to induction of $CD8^+$ cytotoxic T cells. Therefore, PDT can cause not only local destruction of primary tumors but also a systemic antitumor effect when used in combination with immunostimulatory therapies, ultimately leading to complete eradication of malignant tumors without recurrence and metastasis [41,42]. For example, Engleman et al. reported that local PDT followed by intratumoral injection of naive DCs induced systemic antitumor immunity that inhibited the growth of untreated tumors at the distant site, including multiple lung metastases. These effects were mediated mainly by $CD8^+$ cytotoxic T cells [41].

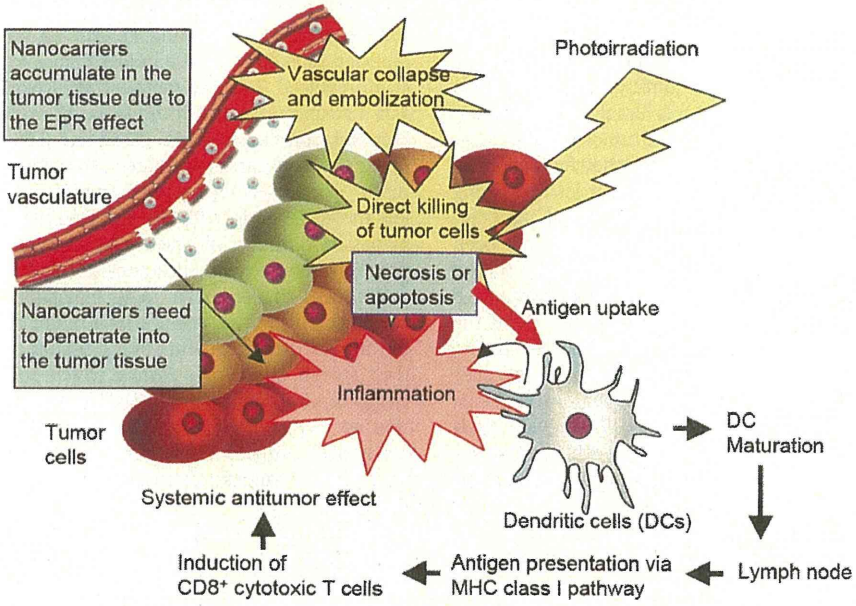

Fig. 2. In vivo mechanisms of PDT. The nanocarrier-encapsulated PSs accumulate in the tumor tissue by the enhanced permeability and retention (EPR) effect. Upon photoirradiation, reactive oxygen species (ROS) generated from PSs can directly kill tumor cells. PDT can also cause vascular collapse and embolization, leading to tumor destruction through a lack of oxygen and nutrients. Furthermore, PDT induces acute inflammation, attracting leukocytes such as dendritic cells (DCs). PDT might provide a tumor environment that facilitates antigen uptake by DCs and antigen presentation via the MHC class I pathway. As a result, PDT can induce $CD8^+$ cytotoxic T cells, thereby achieving a systemic antitumor effect.

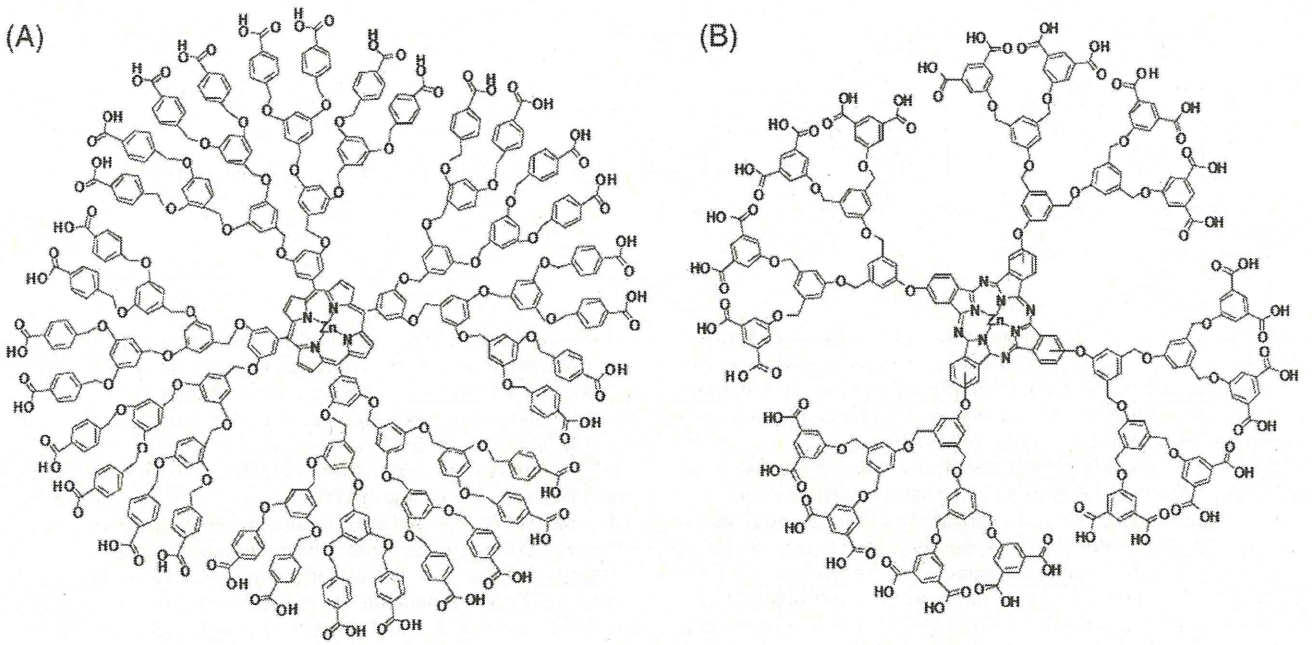

Fig. 3. Chemical structures of ionic dendrimer porphyrin (DP) and ionic dendrimer phthalocyanine (DPc).

The above-mentioned mechanisms indicate the great promise of PDT for use in therapy against malignant tumors. These mechanisms are also very important for the design of nanocarriers for PS delivery. Several key parameters, such as the intratumoral distribution of PSs and modality of cell death, may be controlled by using nanocarriers, which has further motivated us to develop innovative nanocarriers for PDT.

3. Polymeric micelles encapsulating dendrimer photosensitizers for enhanced PDT

3.1. Development of dendrimer photosensitizer-loaded micelles

To date, a number of PSs have been developed and explored in preclinical and clinical studies [1–4]. In general, potent PSs have large π-conjugation domains, such as porphyrin or phthalocyanine rings, that allow them to efficiently absorb energy in the region above 600 nm and a high quantum yield for singlet oxygen formation. Therefore, most PSs easily form aggregates in aqueous media through their π–π stacking and hydrophobic interactions. Such aggregate formation severely decreases ROS formation due to the self-quenching of the excited state [23,43–45]. These properties may hamper the encapsulation into nanocarriers such as liposomes and polymeric micelles. That is, the encapsulation of PSs into nanocarriers might induce the aggregation of PSs, resulting in their reduced PDT efficacy [23]. Also, it is generally difficult to incorporate such very hydrophobic compounds into nanocarriers without compromising the nanocarrier's properties such as the size and surface properties, which are critical to the prolonged blood circulation.

To solve the above-mentioned problems with conventional PSs, we developed ionic dendrimer photosensitizers in which the core of porphyrin or phthalocyanine is surrounded by large dendritic wedges (Fig. 3) [22,29]. It is assumed that dendrimer photosensitizers elicit effective ROS production even at extremely high concentrations, because the dendritic wedges sterically prevent or weaken aggregation of the center dye molecules [20,23]. Also, ionic groups on the dendrimer periphery allow their stable incorporation into polyion complex (PIC) micelles through the electrostatic interaction with oppositely charged poly(ethylene glycol)-polyelectrolyte block

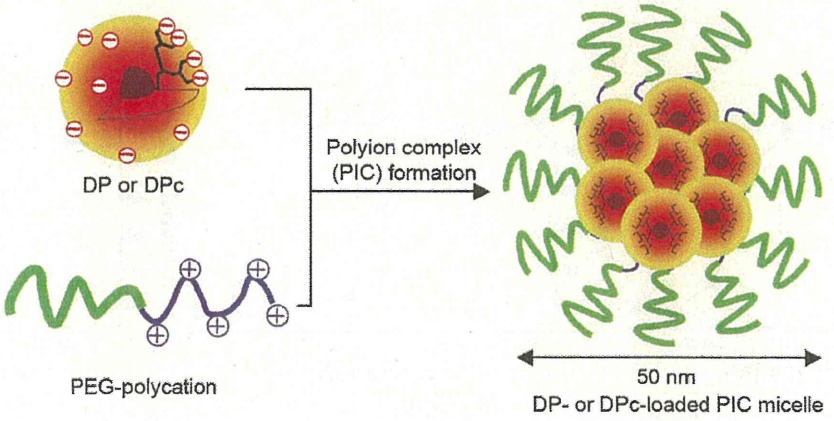

Fig. 4. Formation of DP- or DPc-loaded polyion complex (PIC) micelles through the electrostatic interaction between anionic dendrimers and PEG-polycations. The DP or DPc-loaded micelle is assumed to induce an effective photochemical reaction because the dendritic wedges can sterically prevent or weaken aggregation of the center dye molecules in the micellar core.

Table 1
Physicochemical properties of the DP-loaded micelles from DPs of different generations.

Generation	Hydrodynamic diameter (nm)[a]	Polydispersity index (μ_2/Γ^2)	$M_{w,app}$ (10^5 g/mol)	Association no. PEG-*b*-PLL/DP	CAC[b] (mg/mL)	ς-potential (mV)
G1	126.5	0.131	2240	8989/32585	<0.1	1.11 ± 0.072
G2	78.0	0.249	100	399/722	<0.1	0.48 ± 0.31
G3	44.0	0.030	9.26	39/38	<0.1	−0.20 ± 0.57

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[a] Cumulant diameter.
[b] Critical association concentration.

copolymers (Fig. 4) [17–24]. Polymeric micelles, which are characterized by a size of several tens of nanometers and a core–shell architecture, are potent nanocarriers for site-specific drug delivery, and several formulations of polymeric micelles containing antitumor agents have progressed to clinical trial [34,46–54].

Recently, we synthesized poly(benzyl ether) dendrimer porphyrins (DPs) with different generations (G*n* = *n*-generation dendrimer, *n* = 1–3) to study the effects of dendritic structure on the formation of PIC micelles and their photochemical properties [23]. Note that DPs have a singlet oxygen quantum yield comparable to protoporphyrin IX, a core constituent PS in DPs, which was measured by direct observation of singlet oxygen luminescence at 1270 nm in MeOD [29]. The PIC micelles (DP-loaded micelles) were prepared by mixing of DPs and poly(ethylene glycol)-poly(L-lysine) block copolymers (PEG-PLL) at a stoichiometric charge ratio, and characterized by dynamic and static light scattering measurements and zeta-potential measurement. As summarized in Table 1, G3 formed narrowly distributed particles with a diameter of 44 nm, which is consistent with the core–shell type micellar structure. On the other hand, G1 and G2 formed relatively larger aggregates with diameters of 126 and 78 nm, respectively. The relatively open architectures and small dendritic wedges of G1 and G2 may not perfectly prevent π–π interactions between DPs, which would account for the large aggregate formation. In regard to the efficiency of the photochemical reactions, we evaluated the fluorescence decay and oxygen consumption ability of DPs in a free or micelle form. The results showed that the G3-loaded micelles showed fluorescence decay and oxygen consumption profiles comparable to those of free G3, whereas the G1- and G2-loaded micelles showed a shortened fluorescence lifetime and decreased oxygen consumption rate compared with free G1 and G2, respectively [23]. It should be noted that the oxygen consumption rate, which was measured by a Clark-type oxygen microelectrode in phosphate-buffered saline containing fetal bovine serum (FBS) as an ROS acceptor, might reflect both the efficiency of ROS formation and the reactivity of the ROS to proteins of FBS [20]. Therefore, the size of dendritic wedges of G3 might be sufficiently large to prevent self-quenching of G3 in the micellar core. This effect contrasts with the fact that conventional PSs easily form aggregates, resulting in decreased ROS formation [23,35–43]. Such aggregate formation and concomitant self-quenching of PSs were also observed when they are incorporated into the liposomes [55,56]. Another important aspect is that the ROS produced from DPs reacted with serum proteins, which are immiscible with PEG chains. We assume that ROS produced in the micellar core may diffuse in the PEG layer and photo-oxidize biomolecules in the solution, although further studies should be performed. In conclusion, we demonstrated that ionic dendrimer photosensitizers with an appropriate dendrimer generation can be efficiently incorporated into polymeric micelles without compromising the efficiency of ROS formation and the reactivity of the ROS to biomolecules.

3.2. In vitro PDT effect of dendrimer photosensitizer-loaded micelles

The in vitro PDT effect (photocytotoxicity) of DPs of different generations (G1, G2 and G3) in a free or micelle form against human cervical adenocarcinoma HeLa cells is shown in Fig. 5. The G1-, G2- and G3-loaded micelles showed 7.5, 50 and 167 times higher photocytotoxicity compared with the corresponding free DPs, respectively. On the other hand, the G1-, G2- and G3-loaded micelles displayed 95, 53 and 15 times higher cellular uptake compared with the corresponding free DPs, respectively. Therefore, the PDT efficiencies, in which the phototoxicity ratio is normalized by the uptake ratio, are calculated to be 0.079, 0.94 and 11.1 for the G1-, G2- and G3-loaded micelles, respectively. The reduced PDT efficiency of the G1-loaded micelle may be responsible for the shortened fluorescence lifetime and decreased oxygen consumption ability. However, the G2-loaded micelle exhibited PDT efficiency comparable to that of free G2 despite the shortened fluorescence lifetime and decreased oxygen consumption. Notably, the G3-loaded micelle showed an 11-fold enhancement in PDT efficiency. We thus made an important observation—i.e., that incorporation of DPs into polymeric micelles enhanced the PDT efficiency [23].

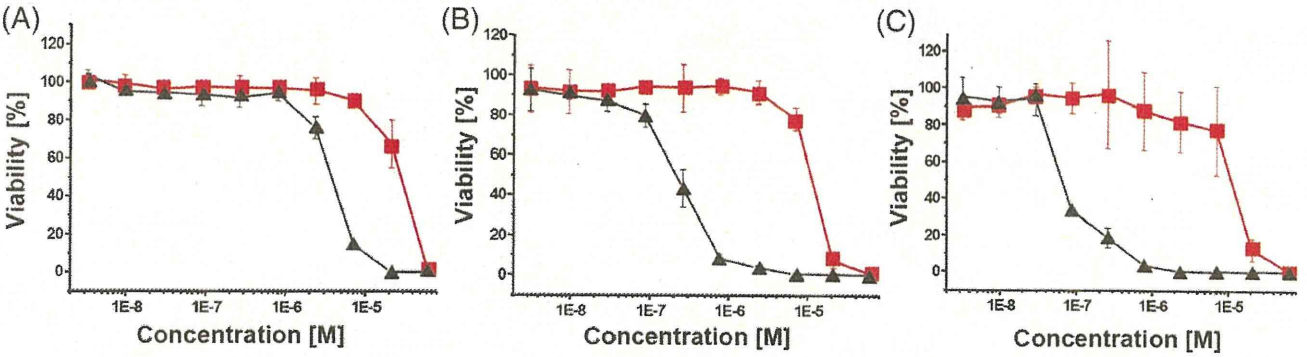

Fig. 5. Cell viability of HeLa cells treated with DPs (red) of different generations [(A): G1, (B): G2 and (C): G3] and the corresponding DP-loaded micelles (black) after photoirradiation. In this experiment, HeLa cells were incubated with DPs or DP-loaded micelles for 12 h, followed by photoirradiation for 30 min with broad-band visible light using a halogen lamp equipped with a filter passing only 400–700 nm light (fluence: 5.4 J cm^{-2}). After 24 h, the cell viability was evaluated by MTT assay. (Reprinted with permission from Ref. [23]. Copyright 2007 by the American Chemical Society).

Table 2
In vitro cytotoxicity of DPc, DPc-loaded micelle and Photofrin after photoirradiation.

Photoirradiation conditions		IC_{50} (μM)[a] of photosensitizing agent		
Time (min)	Fluence (J/cm^2)	DPc	DPc-loaded micelle	Photofrin
0	0	N.D.[b]	N.D.	N.D.
15	2.7	N.D.	1.0	1.0
30	5.4	N.D.	0.20	0.60
45	8.1	N.D.	0.20	0.35
60	10.8	7.0	0.090	0.35

In this experiment, A549 cells were incubated with photosensitizing agents for 12 h, followed by photoirradiation with broad-band visible light using a halogen lamp equipped with a filter passing only 400–700 nm light. After 24 h, the cell viability was evaluated by MTT assay. (Reprinted from Ref. [24]).

[a] 50% cell growth-inhibitory concentration.

[b] IC_{50} was higher than the highest examined concentration (20 μM).

Since in vivo applications of DPs are limited due to their relatively short excitation wavelengths (430 and 559 nm), we also developed an ionic dendrimer phthalocyanine (DPc) with strong Q-band absorption at 685 nm, a wavelength at which the light penetrates deeply into tissues [22]. The simple mixing of DPc and PEG-PLL resulted in the formation of a PIC micelle with a diameter of ca. 50 nm and a narrow size distribution. The photocytotoxicity of the DPc-loaded micelle was assessed against human lung adenocarcinoma A549 cells [24]. Table 2 summarizes the 50% cell growth inhibitory concentrations (IC_{50}) of DPc, the DPc-loaded micelle and clinically used Photofrin. The DPc-loaded micelle showed a fluence-dependent increase in photocytotoxicity, achieving 78 times higher photocytotoxicity than free DPc at 10.8 J/cm^2. It is noted that the DPc-loaded micelle was 3.9 times more effective than Photofrin on a molar basis of photosensitizing units, although the differences in the quantum yields and cellular uptake of photosensitizers were not taken into consideration. Similarly to the above-mentioned DP-loaded micelle, the enhancement of the photocytotoxicity of the DPc-loaded micelle cannot be explained by the intracellular concentration of DPc: the DPc-loaded micelle showed only 7.6 times higher cellular uptake than free DPc. We therefore hypothesize that different mechanisms may be involved in the light-induced cell death between free DPc and DPc-loaded micelle. Indeed, there were appreciable differences in the light-induced morphological changes occurring in the cells treated with IC_{99} of free DPc or DPc-loaded micelle (Fig. 6) [24]. The DPc-loaded micelle induced very rapid cell death accompanied by characteristic morphological changes including swelling and membrane blebbing, whereas free DPc induced gradual shrinkage of the cells. It is noted that the characteristic morphological changes of the cells induced by the DPc-loaded micelle appear to be similar to the characteristics of *oncosis*, which is reported to be induced by several pathological conditions, such as hypoxia, inhibition of ATP production, and increased permeability of the plasma membrane [57]. As shown in Fig. 6, we also monitored the fluorescence of DPc (red) and Rhodamine 123 (Rh123) (green), a dye that specifically stains mitochondria. The fluorescence of DPc became diffusive in both free DPc- and DPc-loaded micelle-treated cells. Since we confirmed that both DPc and the DPc-loaded micelle selectively accumulated in the endo-/lysosomes in separate experiments, the diffused DPc fluorescence in Fig. 6 might indicate that both DPc and the DPc-loaded micelle are translocated from the endo-/lysosomes to the cytoplasm upon photoirradiation. This translocation might arise due to the photochemical disruption of the endo-/lysosomal membranes by photosensitizing agents [58–60]. Indeed, we confirmed that DPc and the DPc-loaded micelle enabled light-induced cytoplasmic delivery of other co-incubated nanocarriers (see Section 5) [60]. In spite of the similar intracellular behaviors between DPc and DPc-loaded micelle, as indicated by the DPc fluorescence, only DPc-loaded micelle caused rapid disappearance of the Rh123 fluorescence in the cell (Fig. 6). This result suggests that the DPc-loaded micelle might affect the mitochondrial functions during photoirradiation. Since the fluorescent intensity of Rh123 is correlated with the amount of ATP in the cells [61], PDT using the DPc-loaded micelle may directly or indirectly clip the ATP in the cell. To determine whether or not the DPc-loaded micelle induces direct photodamage to the mitochondria, we detected the ROS production in the mitochondria by using MitoSOX Red, which rapidly accumulates in the mitochondria and exhibits fluorescence upon oxidation by superoxide and other ROS [24]. As shown in Fig. 7, the DPc-loaded micelle-treated cells displayed apparent fluorescence after 1 min of photoirradiation, whereas the DPc-treated cells showed no fluorescence even after prolonged photoirradiation. Thus, the DPc-loaded micelle was shown to induce photodamage to the mitochondria and thereby affect their functions. In summary, the light-induced cell death caused by the DPc-loaded micelle might involve the following steps (Fig. 8). (i) The DPc-loaded micelle is internalized through endocytosis and accumulates in the endo-/lysosomes. (ii) Upon photoirradiation, the DPc-loaded micelle might escape from the endo-/lysosomes to the cytoplasm by photodamaging the endo-/

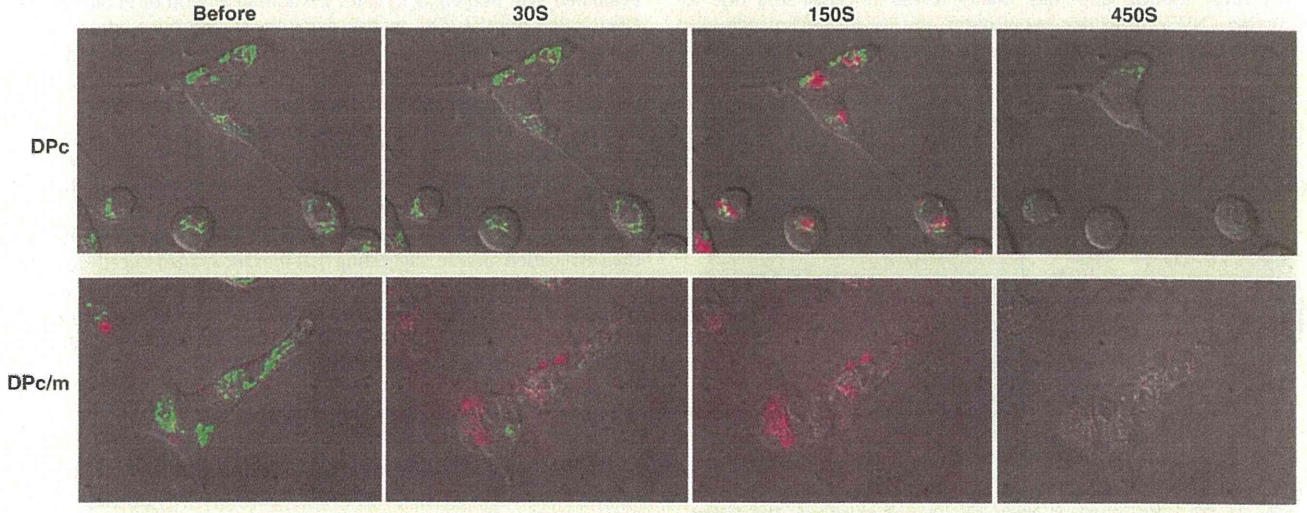

Fig. 6. Time-dependent morphological changes of A549 cells treated with DPc alone or DPc-loaded micelle during photoirradiation. In this experiment, A549 cells were incubated with DPc alone or DPc-loaded micelle for 24 h at a 99% growth inhibitory concentration (IC_{99}). After the medium replacement, the morphological changes in the cells during photoirradiation by the light source of a time-lapse sectioning fluorescent microscope were continuously monitored using the microscope's differential interference contrast (DIC) mode. In addition, the fluorescent images from DPc (red) and Rhodamine 123 (Rh123) (green), a dye that specifically stains mitochondria, were simultaneously monitored. (Reprinted from Ref. [24]).

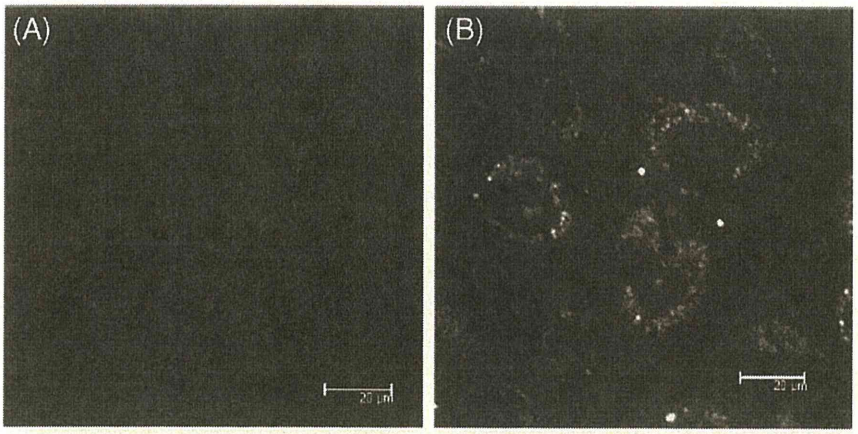

Fig. 7. Fluorescence of MitoSOX Red in A549 cells treated with DPc alone (A) or DPc-loaded micelle (B) after 1 min photoirradiation. MitoSOX Red rapidly accumulates in the mitochondria and exhibits fluorescence upon oxidation by superoxide and other ROS. (Reprinted from Ref. [24]).

lysosomal membranes. (iii) The DPc-loaded micelle might induce photodamage to the mitochondria, leading to *oncosis*-like cell death through exhaustion of ATP in the cell. Although free DPc was also translocated into the cytoplasm, it could not induce photodamage to the mitochondria. In addition to the unique intracellular localization and photochemical reactions, the characteristic structure of the DPc-loaded micelle, in which several tens of DPc molecules are concentrated in a nano-scaled micellar core, may also contribute to the high PDT efficiency. The high local concentration of DPc within the micellar core might generate a high concentration of ROS at a local site, achieving a high photochemical oxidation level that exceeds the threshold of cell death. Thus, spatially regulated incorporation of PSs into nanocarriers appears to greatly enhance the PDT efficiency.

3.3. In vivo PDT effect of dendrimer photosensitizer-loaded micelles

We applied the G3-loaded micelle for PDT of choroidal neovascularization (CNV) [21], which is the cause of exudative age-related macular degeneration (AMD), a leading cause of visual loss in developed countries [62]. It is noted that PDT with a liposomal formulation of verteporfin (Visudyne®) has been clinically used for the treatment of AMD; however, it often requires repeated treatments every three months and may finally result in visual loss due to recurring CNV [63]. In this experiment, the G3-loaded micelle was intravenously administered to rats with experimental CNV, which was created by laser photocoagulation. As a result, the G3-loaded micelle selectively accumulated in the CNV lesions [21], presumably because the CNV features a leaky vascular structure like that of solid tumors. When the PDT laser (fluence: 5–50 J/cm^2) was applied 4 h after i.v. administration of the G3-loaded micelle, 80% of the tested animals showed successful CNV occlusion lasting over 7 days [21]. It has been reported that PDT with Visudyne under the same experimental conditions achieved a less effective CNV occlusion, with CNV recurring 7 days after the laser irradiation [64]. In contrast, the G3-loaded micelle may not require repeated treatments and is expected to improve the therapeutic efficacy of PDT against AMD.

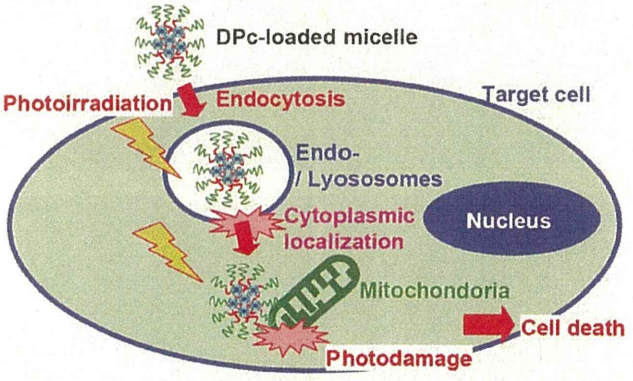

Fig. 8. Hypothetical mechanisms of the light-induced cell death by the DPc-loaded micelle. The DPc-loaded micelle is internalized through endocytosis. Upon photoirradiation, the DPc-loaded micelle might escape from the endo-/lysosomes to the cytoplasm by photodamaging the endo-/lysosomal membranes. Finally, the DPc-loaded micelle might induce photodamage to the mitochondria, leading to the *oncosis*-like cell death.

Recently, we examined the in vivo PDT efficacy of the DPc-loaded micelle against solid tumors [24]. Fig. 9A indicates the relative tumor volumes in the mice bearing subcutaneous A549 tumors after PDT using Photofrin (2.7 μmol/kg), DPc and DPc-loaded micelle (0.37 μmol/kg) through i.v. injection. The DPc-loaded micelle showed significantly higher antitumor activity compared with DPc and Photofrin. It is noted that the injected dose of the DPc-loaded micelle was 7.3 times lower than that of Photofrin on the basis of photosensitizing units (Fig. 9A). The enhanced antitumor activity of the DPc-loaded micelle might be attributed to the effective tumor accumulation based on the EPR effect as well as the enhanced photocytotoxicity. On the other hand, we also evaluated skin phototoxicity after i.v. administration of Photofrin and DPc-loaded micelle [24]. In this experiment, the mice administered higher doses of Photofrin (8.1 μmol/kg) and DPc-loaded micelle (4.2 μmol/kg) underwent white light irradiation to abdominal skin at 0.5, 1, 2 or 4 days after administration. Four days after irradiation, severe damage to the skin and liver was observed only in the Photofrin-administered mice (Fig. 9B). The reduced phototoxicity of the DPc-loaded micelle may be attributed to its lower accumulation in the skin and other normal organs, as described in Section 2.1. It should be noted that no skin damage was observed even 1 h after i.v. administration of the DPc-loaded micelle, suggesting that the DPc-loaded micelle may not be photoactivated during the circulation, probably due to strong absorption by a heme in red blood cells. Thus, the DPc-loaded micelle is expected to serve as an innovative photosensitizer formulation to improve the effectiveness and safety of current PDT.

4. Photodymamic diagnosis (PDD)-guided PDT

PSs such as porphyrins and related compounds have strong fluorescence and therefore can be used for in vivo fluorescent imaging. This optical diagnostic technology is known as photodynamic diagnosis (PDD). PDD can be exploited to confirm the accumulation of PSs in the target tissue before laser irradiation for PDT. PDD is particularly useful for the treatment of malignant glioma by surgical resection and/or PDT

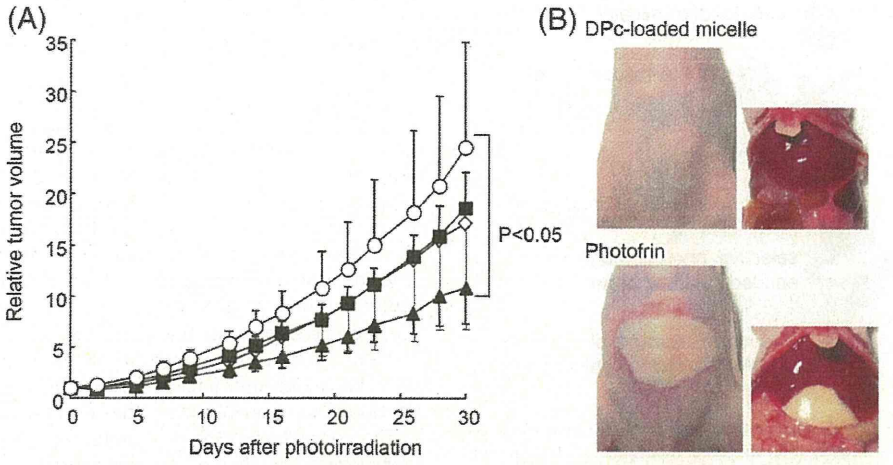

Fig. 9. (A) Growth curves of subcutaneous A549 tumors in control mice (open circles) and mice administered 0.37 μmol/kg DPc (closed squares), 0.37 μmol/kg DPc/m (closed triangles) and 2.7 μmol/kg Photofrin (open diamonds) ($n=6$). Twenty-four hours after administration of photosensitizing agents, the tumors were photoirradiated using a diode laser (fluence: 100 J/cm^2). (B) Macroscopic observation of the skin and organs in the mice treated with 4.2 μmol/kg DPc-loaded micelle and 8.1 μmol/kg Photofrin at 4 days after light irradiation to the abdominal skin using a halogen lamp (fluence: 60 J/cm^2). (Reprinted from Ref. [24]).

[65,66]. In general, it is difficult to selectively remove the glioma tissue without disturbing the normal brain tissue, because the glioma tissue invades and infiltrates into the normal brain tissue. Since 5-aminolevulinic acid (5-ALA) elicits the biosynthesis of protoporphyrin IX (PpIX) in various epithelial and cancer cells and PpIX emits fluorescence at 635 nm, oral administration of 5-ALA allows the glioma tissue to be visualized by fluorescent imaging [65,66]. Thus, intraoperative PDD using 5-ALA provides real-time information about surgical margins and enables selective removal of the tumor tissue without functional damage to the brain. In addition, laser irradiation to the glioma tissue might induce apoptosis of malignant glioma cells, because PpIX is a potent photosensitizing agent. Intraoperative PDD and PDT for the treatment of malignant glioma are now being studied in clinical trials [65]. PDD-guided PDT might also be applicable to the diagnosis and treatment of mucosal carcinoma and dysplasia, which are highly disseminated and thus difficult to completely remove by endoscopic mucosal resection (EMR). Thus, PDD-guided PDT has great potential for the treatment of intractable tumors due to the technical difficulties of surgical resection. In this regard, Hasan et al. performed PDT on an experimental model of disseminated abdominal metastases of ovarian cancers by i.p. administration of the polymer-chlorin$_{e6}$ conjugate [7].

Although most conventional PSs emit fluorescence, their excitation wavelengths are too short for their fluorescence to be visualized in deep tissues. For example, the largest absorption band of the above-mentioned PpIX is at 405 nm (Soret band). Furthermore, conventional PSs have an insufficient Stokes shift of 10–15 nm, making discrimination of the fluorescent light from the strong excitation light difficult. Therefore, PSs have been chemically modified with different fluorescent dyes to construct bifunctional PSs for PDD and PDT [67]. However, such bifunctional properties can also be achieved more simply by conjugating fluorescent dyes to nanocarriers for the PS delivery. Since nanocarriers preferentially accumulate in the tumor tissue due to the EPR effect, tumor-specific imaging might be realized by PDD using nanocarriers. Furthermore, the construction of nanocarriers integrated with smart functions to respond to specific environments or react to specific molecules in the target cell enables functional and molecular imaging such as detection of apoptosis after PDT [68].

5. Development of light-sensitive nanocarriers utilizing photochemical internalization (PCI)

In general, cell membrane-impermeable drugs and macromolecular compounds such as plasmid DNA (pDNA), siRNA and proteins are taken up by the cells through the endocytosis and end in degradation in the lysosomes. Therefore, enormous efforts have been devoted to the development of nanocarriers, which can escape from the endo-/lysosomes and move into the cytoplasm to reach the target organelles or interact with the target molecules. For example, cationic polymers, which can directly or indirectly perturb the endosomal membranes, have been studied as a vehicle to facilitate the cytoplasmic delivery of pDNA and siRNA [69–71]. In this regard, Berg and Høgset have recently developed a new technology called photochemical internalization (PCI), in which the endosomal escape of macromolecular compounds or nanocarriers is induced by the photochemical disruption of the endosomal membranes by co-incubated PSs, allowing their delivery into the cytoplasm in a light-inducible manner [58,59,72–75]. PCI has been demonstrated to be useful for the in vitro and in vivo delivery of pDNA, siRNA and immunotoxins [58–60,72–74,76–78]. This strategy is quite smart; however, the PCI is often accompanied by significant cytotoxicity. Such photocytotoxicity may be admissible for the purpose of cancer therapy to some extent but should be avoided for other applications. In this regard, the photocytotoxicity in the PCI may be attributed to the properties of PSs. It has been demonstrated that dissymmetric PSs such as AlPcS$_{2a}$ (aluminium phthalocyanine with two sulfonate groups on adjacent phthalate rings) and TPPS$_{2a}$ (meso-tetraphenylporphine with two sulfonate groups on adjacent phenyl rings) are effective in PCI-mediated delivery because they are internalized by the endocytosis due to high water solubility, while the hydrophobic moiety in those PSs causes them to preferentially interact with cell membranes [59,73,74]. However, such amphiphilic PSs may interact with not only endosomal membranes but also plasma membranes. In addition, it is also possible that PSs may interact with cytoplasmic organelles such as mitochondria and endoplasmic reticulum after their endosomal escape by the PCI. It is known that the photodamage to the plasma membrane or some cytoplasmic organelles such as mitochondria might induce efficient cell death, whereas the cell viability might be less affected by the photodamage to the endo-/lysosomes [3]. Therefore, the selective photodamage to the endo-/lysosomes is assumed to be a key to the reduced phototoxicity in the PCI-mediated delivery.

As described in Section 3.2., the DPc-loaded micelle might be internalized through the endocytosis and photodamage the endo-/lysosomal membranes, which motivated us to utilize the DPc-loaded micelle for the PCI-mediated delivery. We prepared the pDNA-loaded micelle through an electrostatic interaction between pDNA and PEG-

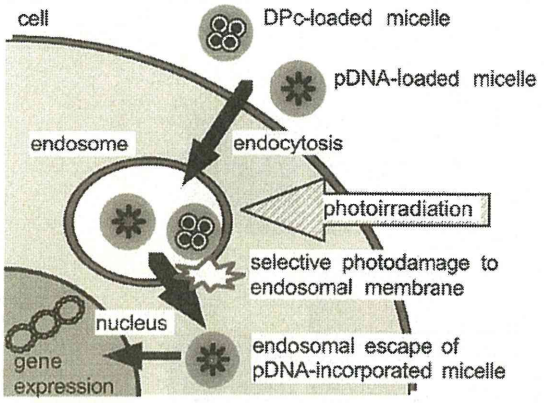

Fig. 10. Scheme for the itinerary of the plasmid DNA (pDNA)- and DPc-loaded micelles in the photochemical internalization (PCI)-mediated transfection. Both polymeric micelles are assumed to be taken up by the cell through the endocytic pathway, and the localization of the DPc-loaded micelle in the endosome may allow the selective photodamaging of the endosomal membrane upon photoirradiation, thereby inducing the cytoplasmic delivery of the pDNA-loaded micelle. (Reprinted with permission from Ref. [60]. Copyright 2006 by Taylor & Francis.)

PLL, and performed in vitro PCI-mediated transfection by using the combination of the pDNA- and DPc-loaded micelles [60]. In this strategy, both the pDNA- and DPc-loaded micelles might show the same localization in the endosome following the endocytic uptake, and upon photoirradiation the DPc-loaded micelle is assumed to photodamage the endosomal membrane, achieving the effective cytoplasmic delivery of the pDNA-loaded micelle (Fig. 10). Also, the DPc-loaded micelle might induce the selective photodamage to the endosomal membranes, allowing the PCI-mediated transfection without compromising the cell viability. Indeed, the combination of the pDNA- and DPc-loaded micelles achieved 100-fold photochemical enhancement of the transgene expression while maintaining high cell viability over a wide range of DPc concentrations and light doses. It is noted that the light dose necessary for PCI is much lower than that required for PDT, ensuring low photocytotoxicity in the PCI-mediated delivery. Thus, the use of nanocarriers for the PS delivery might improve the efficiency and cell viability in the in vitro PCI-mediated delivery.

The use of nanocarriers may also be beneficial for in vivo applications of the PCI-mediated delivery. That is, PSs need to be selectively delivered to the endosome in the target cells for successful in vivo PCI. However, administration of low-molecular-weight PSs results in diffused localization to the surrounding tissues, which may decrease the efficiency of PCI and cause phototoxicity to the surrounding tissues. In the case of the combination of the pDNA- and DPc-loaded micelles, both micelles have similar properties characterized by the core–shell structure having the size of sub-100 nm, and thus are expected to have similar tissue distribution and intracellular localization after systemic administration. Recently, we developed a ternary complex composed of a pDNA/cationic peptide complex enveloped with DPc, which includes all the necessary components in one nanocarrier system [76]. This ternary complex also exhibited more than 100-fold light-induced enhancement of the in vitro transgene expression without compromising cell viability. Importantly, the ternary complex achieved in vivo light-induced, site-directed transfection in the rat eye after subconjunctival injection followed by laser irradiation. Thus, nanocarriers integrated with photosensitizing units might allow spatial and temporal control of the function of the loaded nucleic acids or proteins.

The concept of PCI is also useful for designing light-responsive nanocarriers for chemotherapeutic agents [79–82]. Recently, the photoactivated drug carriers have received increasing attention [83,84]; however, the existing systems, such as the systems based on the photoisomerization of azobenzene derivatives, are mainly triggered by UV light, which cannot penetrate the target tissues and may potentially damage healthy tissues. In this regard, light-responsive nanocarriers based on the PCI concept can be activated by red or far-red light, and thus are more realistic systems for in vivo applications. Berg and Høgset were the first to combine PCI and chemotherapy, and reported that the in vitro and in vivo efficacy of cell membrane-impermeable bleomycin was considerably enhanced by the PCI [75]. However, most chemotherapeutic agents permeate the cell membranes, exerting their potent cytotoxicity without the PCI. Therefore, we designed cytosolically activated polymeric micelles in which comptothecin (CPT) is covalently conjugated to block copolymers via a disulfide bond that is selectively cleaved at the cytosol due to 100–1000 times higher concentration of glutathione than that in the extracellular environment [82]. The CPT-loaded micelle was prepared by conjugating a thiolated CPT derivative (CPT-DP) to thiolated PEG-poly(glutamic acid) [PEG-P(Glu-DP)] block copolymers via a disulfide bond as schematized in Fig. 11. In this formulation, CPT was modified via an ester bond to stabilize the active lactone ring under physiological conditions, thereby protecting its antitumor activity during the circulation. We evaluated the in vitro cytotoxicity of the CPT-loaded micelle in combination with the non-toxic concentration of the DPc-loaded micelle with and without photoirradiation against HeLa cells. The results showed that the cytotoxicity of the CPT-loaded micelle was significantly enhanced by the PCI using the DPc-loaded micelle [82]. Importantly, our system is highly

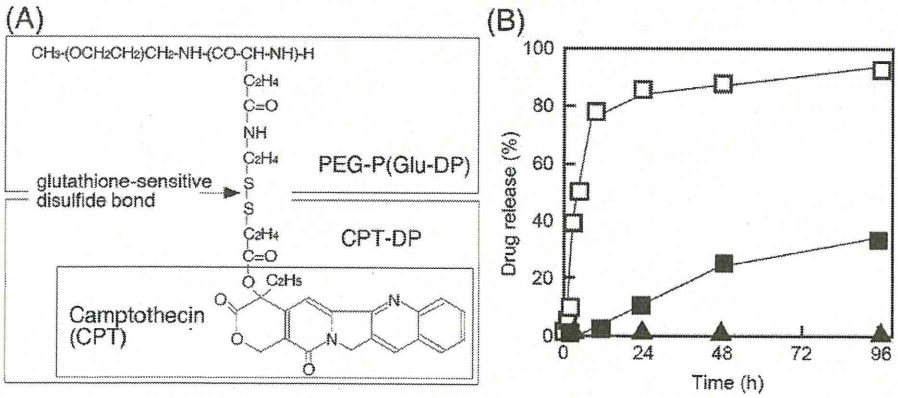

Fig. 11. (A) Chemical structures of glutathione-sensitive camptothecin (CPT)-conjugated block copolymers, which form cytosolically activated polymeric micelles. (B) Release of thiolated CPT (CPT-DP) from the glutathione-sensitive CPT-loaded micelle under different conditions (filled triangles: phosphate buffered saline (pH 5.5, 150 mM NaCl); filled squares: phosphate buffered saline (pH 7.4); open squares: phosphate buffered saline (pH 7.4) containing 3 mM dithiothreitol). (Reprinted with permission from Ref. [82]. Copyright 2008 by Springer Science.)

selective to light irradiation, in contrast to previous results by PCI-mediated chemotherapy using chemotherapeutic agents and low-molecular-weight PSs, in which the enhanced cytotoxicity of chemotherapeutic agents was observed only when the PS alone showed significant photocytotoxicity [79,80]. The highly light-selective activation of chemotherapeutic agents by our system may be attributed to (i) the above-mentioned selective photodamage to the endo-/lysosomal membranes by the DPc-loaded micelle and (ii) cytoplasmic condition-selective CPT release after the endosomal escape of the CPT-loaded micelle by the PCI. Therefore, our system can minimize the drug leakage from the micelle during the circulation but exert cytotoxicity selectively at the photoirradiated site. In the PCI-mediated chemotherapy, the drug released from the nanocarriers after the PCI can diffuse in the tumor tissue, allowing the treatment of thicker and hypoxic tumors, which are known to be intractable by PDT alone due to the limited light penetration and low oxygen concentration, respectively. Another benefit of the PCI-mediated chemotherapy is that PDT has been shown to overcome multidrug resistance in many in vivo tumor models. It has been reported that PCI has the ability to circumvent the multidrug resistance in adriamycin-resistant breast cancer MCF-7 cells through the release of adriamycin localized at the endo-/lysosomes after the PCI [79,81]. These results indicate the great potential of PCI-mediated chemotherapy using cytosolically activated nanocarriers.

6. Conclusion

Although the use of nanocarriers for PS delivery is promising, a development strategy to effectively incorporate PSs into nanocarriers but minimize the loss of the singlet oxygen quantum yield is very important. We demonstrated that the dendrimer photosensitizer-loaded micelles showed remarkably enhanced photocytotoxicity, which could not be explained solely by the elevated intracellular PS concentration. Although the mechanism remains to be clarified, it is conceivable that alterations in intracellular PS localization and photodamaged sites and/or generation of a high concentration of ROS at a local site by nanocarriers may contribute to the enhanced photocytotoxicity. The in vivo PDT efficacy and emergence of the skin phototoxicity might be related to the tissue distribution of PSs. In this regard, the nanocarrier-encapsulated PSs might show higher tumor accumulation and tumor-specificity compared with PSs alone due to the EPR effect. We revealed that the dendrimer photosensitizer-loaded micelles showed a higher antitumor effect than clinically used Photofrin without any sign of the phototoxicity to the normal tissues even at a high dose. Furthermore, the use of nanocarriers provides an opportunity to further optimize the PDT efficacy, such as by multiple light irradiation. The nanocarriers with integrated PDD function might have great potential for clinical use. For example, the PDD-guided PDT is expected to have utility for the treatment of microcarcinoma, which are difficult to completely remove by endoscopic mucosal resection (EMR). Finally, the development of nanocarriers should also be importance for PCI, an emerging technology for the light-selective delivery of cell membrane-impermeable compounds. We demonstrated that in vivo PCI-mediated transfection can be achieved by nanocarriers optimized for PCI. Thus, the development of nanocarriers with integrated smart functions should be a key to further improvement and clinical applications of PDT and related technologies.

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Enhanced photodynamic cancer treatment by supramolecular nanocarriers charged with dendrimer phthalocyanine

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ABSTRACT

Photodynamic therapy (PDT) is a promising method for the localized treatment of solid tumors. In order to enhance the efficacy of PDT, we have recently developed a novel class of photosensitizer formulation, i.e., the dendrimer phthalocyanine (DPc)-encapsulated polymeric micelle (DPc/m). The DPc/m induced efficient and unprecedentedly rapid cell death accompanied by characteristic morphological changes such as blebbing of cell membranes, when the cells were photoirradiated using a low power halogen lamp or a high power diode laser. The fluorescent microscopic observation using organelle-specific dyes demonstrated that DPc/m might accumulate in the endo-/lysosomes; however, upon photoirradiation, DPc/m might be promptly released into the cytoplasm and photodamage the mitochondria, which may account for the enhanced photocytotoxicity of DPc/m. This study also demonstrated that DPc/m showed significantly higher *in vivo* PDT efficacy than clinically used Photofrin® (polyhematoporphyrin esters, PHE) in mice bearing human lung adenocarcinoma A549 cells. Furthermore, the DPc/m-treated mice did not show skin phototoxiciy, which was apparently observed for the PHE-treated mice, under the tested conditions. These results strongly suggest the usefulness of DPc/m in clinical PDT.

1. Introduction

Photodynamic therapy (PDT), an effective modality for treating localized tumors, involves the systemic administration of porphyrin- or phthalocyanine-based photosensitizers (PSs), followed by local photoirradiation of solid tumors with the light of a specific wavelength [1–3]. Upon photoirradiation, PSs generate reactive oxygen species (ROS) such as singlet oxygen, leading to photochemical destruction of tumor vessels and tumor tissues. PDT shows a high clinical complete response to early-stage superficial tumors, including lung, esophageal, gastric, and cervical cancers. Also, PDT is quite useful as an initial treatment for malignant tumors because organ functions are maintained, thus saving the patient's stamina for further treatments. However, PDT is known to be accompanied by skin hyperphotosensitivity, so that the patient needs to stay in a darkened room for at least 2 weeks. This effect is attributable to the lack of tumor selectivity by currently approved PSs, such as Photofrin® [3]. Tumor-selective PSs and their formulations are expected to restrain unfavorable side effects and improve the efficacy of PDT against intractable tumors. In this context, the use of long-circulating nanocarriers such as liposomes [4], water-soluble polymers [5–7] and polymeric micelles [8–10] is a promising way to improve the tumor selectivity of PSs. It has been demonstrated that such nanocarriers can preferentially and effectively accumulate in solid tumors, since the tumor tissues are characterized by the enhanced permeability and retention (EPR) effect, which consists of microvascular hyperpermeability to circulating macromolecules and impaired lymphatic drainage [11].

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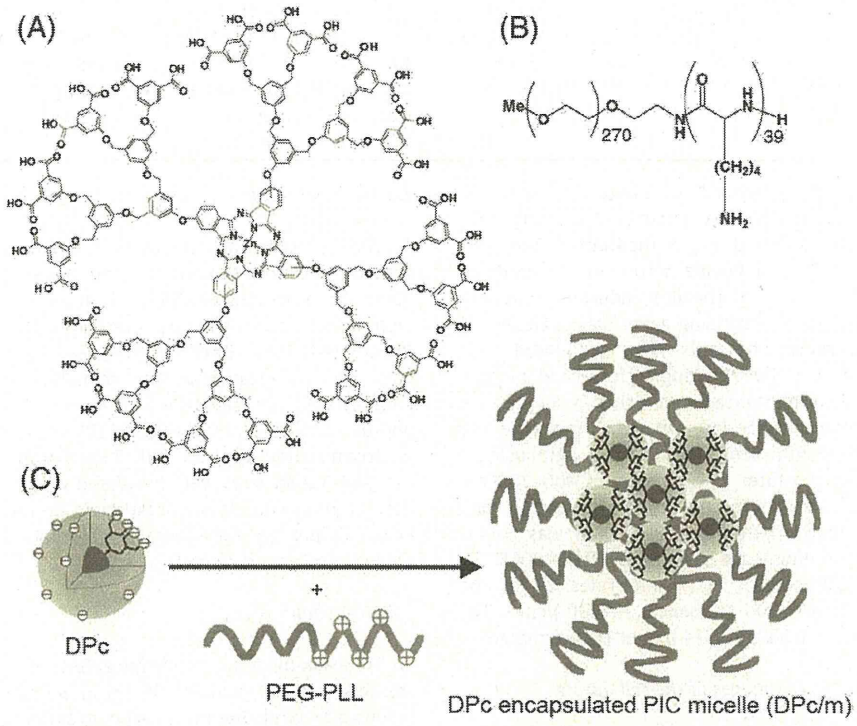

Fig. 1. Chemical structures of anionic dendrimer phthalocyanine (DPc) (A) and poly(ethylene glycol)–poly(L-lysine) (PEG–PLL) block copolymer (B). The DPc-encapsulated polyion complex (PIC) micelle (DPc/m) was formed by mixing DPc and PEG–PLL at a charge stoichiometric ratio (C).

Regarding the development of nanocarriers encapsulating PSs, most conventional PSs may have several serious drawbacks. Since potent PSs generally have large π-conjugation domains, they easily form aggregates due to their π–π and hydrophobic interactions. Such aggregate formation severely decreases the ROS formation essential to the PDT effect [12,13]. Also, this propensity may hamper the encapsulation of PSs into nanocarriers such as liposomes and polymeric micelles. To solve such problems, we have developed ionic dendrimer photosensitizers where the core of porphyrin or phthalocyanine is surrounded by large dendritic wedges [14,15]. Unlike conventional PSs, dendrimer photosensitizers exhibit effective ROS production even at extremely high concentrations, because the dendritic wedges sterically prevent or weaken aggregation of the center dye molecules [13,16]. In addition, ionic groups on the peripheries of dendrimer photosensitizers allow their stable incorporation into polyion complex (PIC) micelles through the electrostatic interaction with oppositely charged poly(ethylene glycol)(PEG)–polyelectrolyte block copolymers [10,13,15,16]. Polymeric micelles, which are characterized by a size of several tens of nanometers and a core–shell architecture, are potent nanocarriers for site-specific drug delivery, as several formulations encapsulating antitumor agents have already progressed to clinical studies [17–19]. We have already demonstrated that PIC micelles encapsulating third-generation dendrimer porphyrin showed remarkably high photocytotoxicity against cancer cells and successfully treated experimental disease models of choroidal neovascularization (CNV) in rats [20], indicating that dendrimer photosensitizer-encapsulated PIC micelles show great promise for use in clinical PDT.

Recently, we prepared PIC micelles through the electrostatic interaction between the anionic dendrimer phthalocyanine (DPc) and the PEG–poly(L-lysine) block copolymer (PEG–PLL) (Fig. 1) [15]. Since DPc has strong Q-band absorption at 685 nm, at which the light deeply penetrates tissues, the DPc-encapsulated PIC micelle (DPc/m) is assumed to be effective in PDT of solid tumors. In our previous paper, DPc/m showed approximately three- to four-fold decrease in the oxygen consumption rate compared with free DPc, indicating a decrease in quantum yield of singlet oxygen formation [15]. Compared with aforementioned dendrimer porphyrin, a relatively small dendritic wedge of DPc may not entirely prevent collisional quenching in the micellar core. Nevertheless, DPc/m displayed approximately 100-fold higher *in vitro* photocytotoxicity against human cervical cancer HeLa cells compared with free DPc when the cells were photoirradiated for 60 min with broad-band light (400–700 nm) using a low power halogen lamp [15]. However, the underlying mechanisms of the enhanced photocytotoxicity of DPc/m remain to be clarified yet, because DPc/m showed only 4 times higher cellular uptake compared with free DPc. In the present paper, we have studied *in vitro* photocytotoxicity of DPc/m when human lung adenocarcinoma A549 cells were photoirradiated by a diode laser (670 nm), and found that DPc/m showed unique photochemical processes inside of the cells to induce cell death in an unprecedentedly fast manner. Also, we have studied *in vivo* antitumor activity of DPc/m against subcutaneous tumor models in mice.

2. Materials and Methods

2.1. Preparation of DPc and DPc/m

The synthesis of anionic dendrimer phthalocyanine (DPc) has been reported previously [15,21]. The synthesized DPc is composed of the 2nd generation aryl ether dendrimer with a Zn(II)-phthalocyanine center and 32 carboxylic groups on its periphery (Fig. 1A). Poly(ethylene glycol)–poly(L-lysine) block copolymer (PEG–PLL) (Fig. 1B) was synthesized by the polymerization of the *N*-carboxy anhydride of N^{ε}-Z-L-lysine initiated by CH_3O-PEG-NH_2 (12,000 g/mol), followed by deprotection of the Z group according to a previously reported method [22]. The polymerization degree of PLL segment was determined to be 39 by the ^1H NMR measurement. The DPc-encapsulated PIC micelle (DPc/m) was prepared at a charge stoichiometric ratio of negatively-charged DPc and positively-charged PEG–

PLL (Fig. 1C). The resulting DPc/m had a diameter of ca. 50 nm with a narrow size distribution (unimodal, $\mu_2/\Gamma^2=0.12$) and zeta-potential value of −0.56±0.56 mV, which were measured by a Zetasizer nanoseries (Malvern Instruments Ltd., UK).

2.2. Cell culture and cytotoxicity assay

Human lung adenocarcinoma A549 cells were obtained from Riken Bioresource Center Cell Bank (Tsukuba, Japan). A549 cells were maintained in Dulbecco's modified Eagle medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum in a humidified atmosphere containing 5% CO_2 at 37 °C. The light-induced cytotoxicity (photocytotoxicity) of each photosensitizing agent was evaluated as follows: In a darkened room, the cells were incubated with photosensitizing agents for 24 h. After washing with PBS and medium replacement, the cells were photoirradiated using a low power halogen lamp or a high power diode laser. In the former case, the culture plate was irradiated with broad-band visible light using a halogen lamp equipped with a filter passing light of 400–700 nm (fluence rate: 3.0 mW/cm^2; irradiation time: 15, 30, 45, and 60 min; fluence: 2.7–10.8 J/cm^2). In the latter case, each well was photoirradiated by a 670 nm, continuous wave diode laser (HLD2000MT 7A, High Power Devices, North Brunswick, NJ) (fluence rates: 25, 50, and 100 mW/cm^2; irradiation time: 1000 s; fluence: 25–100 J/cm^2). The cell viability was evaluated by MTT assay 24 h after photoirradiation.

2.3. Observation of morphological changes of the cell during photoirradiation

To investigate the modality of light-induced cell death, we examined the morphological changes occurring in DPc- and DPc/m-treated cells during photoirradiation in a time-lapse manner. In this experiment, A549 cells were incubated with DPc or DPc/m for 24 h at the 99% cell growth-inhibitory concentration (IC_{99}). After the medium was replaced, the morphological changes in the cells during photoirradiation by the light source of a time-lapse sectioning fluorescent microscope (ApoTome/Axiovert 200 M, Carl Zeiss, Oberkochen, Germany; fluence rate: ~120 mW/cm^2) were continuously monitored using the microscope's differential interference contrast (DIC) mode. In addition, the fluorescent images from DPc and Rhodamine 123 (Rh123) (Molecular Probes, Eugene, OR), a specific dye to the mitochondria, were simultaneously observed.

2.4. Intracellular localization of DPc/m

To evaluate the intracellular localization of DPc/m, PEG–PLL was labeled with Alexa fluor 488 carboxylic acid, succinimidyl ester (5.0 mg, 7.8 μmol) (Invitrogen) according to the manufacturer's protocol. After removal of unbound dye by dialysis and lyophilization, the ratio of the dye to PEG–PLL was estimated to be 3.8 by measuring UV–Vis spectra. The Alexa 488-labeled DPc/m was prepared as above-mentioned.

Intracellular localization of Alexa 488-labeled DPc/m in A549 cells was observed by confocal laser scanning microscopy (CLSM) (LSM510META, Carl Zeiss). After 24-h incubation with Alexa 488-labeled DPc/m and subsequent washing, the cells were treated with LysoTracker Red DND-99, MitoTracker Red 580, and ER Tracker Red (Molecular Probes) for the staining of endo-/lysosomes, mitochondria, and endoplasmic reticulum, respectively. The fluorescent images of the cells without photoirradiation were observed by CLSM.

2.5. Measurement of ROS production in the mitochondria

The ROS production in the mitochondria in A549 cells was detected by MitoSOX Red reagent (Invitrogen), which rapidly accumulates in the mitochondria and exhibits fluorescence upon oxidation by superoxide and other ROS. After 24-h incubation with DPc or DPc/m, the cells were incubated with MitoSOX Red (5 μM) for 10 min at 37 °C. Then, the fluorescent image of MitoSOX Red in living cells during photoirradiation using the light source of the microscope was observed by CLSM (TCS SP2 Spectral Confocal System, Leica, Nussloch, Germany).

2.6. In vivo antitumor effect

The antitumor activity of DPc, DPc/m or Photofrin® (porfimer sodium, PHE from Wyeth, Madison, NJ) was tested against subcutaneous tumor models of A549 cells in mice ($n=6$). A549 cells (3×10^6) were subcutaneously transplanted into the left back of 6-week-old female nude mice (BALB/c *nu/nu*) (Clea Japan, Tokyo, Japan). Fifteen days after transplantation, one of the photosensitizing agents was administered intravenously at a dose of 1.85 mg/kg (0.37 μmol/kg photosensitizing unit) for DPc and DPc/m and 1.65 mg/kg (2.7 μmol/kg photosensitizing unit) for PHE. Twenty-four hours after the administration, tumor sites were irradiated with a diode laser with a light dose of 100 J/cm^2. This animal study protocol was approved by the Ethics Committee for Laboratory Animals of the National Defense Medical College, Tokorozawa, Japan.

2.7. Skin phototoxicity

Six-week-old female nude mice (BALB/c *nu/nu*) were intravenously administered 4.2 μmol/kg DPc/m or 8.1 μmol/kg Photofrin® (PHE), followed by white light irradiation to abdominal skin using a halogen lamp at the fluence of 60 J/cm^2 at 0.5, 1, 2 or 4 days after administration. Four days after irradiation, macroscopic changes in the skin as well as in the organs were observed.

3. Results

3.1. In vitro PDT using halogen lamp

Table 1 summarizes the 50% cell growth inhibitory concentrations (IC_{50}) of DPc, DPc/m and Photofrin® (PHE) against A549 cells. Note that none of the photosensitizing agents showed dark toxicity. As shown in Table 1, DPc/m exhibited an irradiation time-dependent increase in cytotoxicity, which is consistent with our previous results of the photocytotoxicity against HeLa cells [14]. Eventually, DPc/m achieved 78-fold higher photocytotoxicity than free DPc at 10.8 J/cm^2. It is worth mentioning that DPc/m was 3.9 times more effective than even clinically used PHE on a molar basis of photosensitizing units.

3.2. In vitro PDT using diode laser

The photocytotoxicity of DPc and DPc/m against A549 cells are summarized in Table 2 and Fig. S1 in Supporting Information. As shown in Table 2, DPc/m exhibited 44-fold higher photocytotoxicity than DPc at 25 J/cm^2. It is noteworthy that DPc displayed a fluence-rate-dependent increase in photocytotoxicity, whereas DPc/m was

Table 1
In vitro cytotoxicity of photosensitizing agents after photoirradiation using halogen lamp

Photoirradiate condition		IC_{50} (μM)[a] of photosensitizing agent		
Time (min)	Fluence (J/cm^2)	DPc	DPc/m	PHE
0	0	N.D.[b]	N.D.	N.D.
15	2.7	N.D.	1.0	1.0
30	5.4	N.D.	0.20	0.60
45	8.1	N.D.	0.20	0.35
60	10.8	7.0	0.090	0.35

[a] 50% cell growth-inhibitory concentration.
[b] IC_{50} was higher than the highest examined concentration (20 μM).

Table 2
In vitro cytotoxicity of photosensitizing agents after photoirradiation using diode laser

Photoirradiate condition	IC_{50} (μM) of photosensitizing agent	
Fluence (J/cm^2)	DPc	DPc/m
25	20	0.45
50	4.8	0.16
100	0.86	0.11

less influenced by the fluence rate. Eventually, DPc/m showed only 7.9-fold higher photocytotoxicity than DPc at 100 J/cm^2.

3.3. Observation of morphological changes of the cell during photoirradiation

The morphological changes occurring in the DPc- and DPc/m-treated cells during photoirradiation were monitored in a time-lapse manner. The observations revealed marked differences between DPc and DPc/m (Fig. 2). The DPc/m-treated cells swelled rapidly, accompanied by membrane blebbing, resulting in the disappearance of the initial shape of the cells within a few minutes of photoirradiation. In contrast, the DPc-treated cells showed gradual shrinkage while maintaining their pseudopodial structures during photoirradiation.

In addition, the fluorescent images from DPc and Rh123, a specific dye to the mitochondria, were simultaneously monitored and are also shown in Fig. 2. Regarding the fluorescence from DPc (red), the DPc-treated cells exhibited a transient burst in the fluorescence: the fluoresce of DPc increased appreciably 60–180 s after the initiation of photoirradiation and became obscure within 400 s. Such unique behavior was also seen in the DPc/m-treated cells; however, the fluorescent burst occurred faster than DPc. On the other hand, there were characteristic differences in the Rh123 fluorescence (green). The Rh123 fluorescence in the DPc/m-treated cells disappeared immediately after the initiation of light irradiation. In contrast, the fluorescence of Rh123 in the DPc-treated cells remained even after 450 s photoirradiation. These results suggest that DPc/m might affect mitochondrial functions during photoirradiation, leading to rapid cell death with characteristic morphological changes.

3.4. Intracellular localization of DPc/m

The intracellular localization of DPc/m in A549 cells before photoirradiation was observed by CLSM. The fluorescence image in Fig. 3A reveals that Alexa 488-labeled DPc/m showed punctate fluorescence co-localized with LysoTracker Red DND-99. In contrast, the fluorescence pattern of Alexa 488-labeled DPc/m was apparently different from those of MitoTracker Red 580 and ER Tracker Red, as shown in Fig. 3B and C, respectively. We also observed the intracellular localization of DPc and DPc/m after 24-h incubation by utilizing DPc's fluorescence, and found that both of them were co-localized with LysoTracker Green DND-26 (Fig. S2 in Supporting Information). These results indicate that DPc and DPc/m may have been internalized through the endocytic pathway and may have localized in the endo-/lysosomes before photoirradiation.

3.5. Measurement of ROS production in the mitochondria

Since DPc/m is assumed to affect the mitochondria, as suggested in Fig. 2, we evaluated ROS production in the mitochondria of A549 cells using MitoSOX Red. As shown in Fig. 4, MitoSOX Red exhibited apparent fluorescence in the DPc/m-treated cells after 1 min photoirradiation, whereas no appreciable fluorescence appeared in the DPc-treated cells even after prolonged photoirradiation. Thus, DPc/m might induce photodamage to the mitochondria and thus affect their functions.

3.6. In vivo antitumor effect and skin phototoxicity

The mice bearing subcutaneous A549 tumors were treated with the PDT using DPc, DPc/m or Photofrin® (PHE), and the relative tumor volumes after photoirradiation are shown in Fig. 5. As a result, the tumors in the DPc/m-treated mice grew significantly slower than those in the DPc-treated mice. It is worth mentioning that the PDT effect of DPc/m was superior to that of PHE, although the injected dose of DPc/m was 7.3-fold lower than that of PHE on the basis of photosensitizing units. On the other hand, we also evaluated *in vivo* phototoxicity after PDT. As shown in Fig. 6B, severe damage to the skin and liver was observed in PHE-administered mice (8.1 μmol/kg photosensitizing unit). In contrast, DPc/m did not induce such damage, although a relatively high dose of DPc/m (4.2 μmol/kg photosensitizing unit) was administered in this study (Fig. 6A). Thus, DPc/m was demonstrated to be a safe but effective photosensitizer formulation over clinically used PHE.

4. Discussion

The targeted delivery of PSs using nanocarriers such as liposomes and polymeric micelles have been studied to improve the therapeutic efficacy and restrain side effects in PDT [4–10,13–16]. The use of nanocarriers is expected to increase the concentration of PSs in the tumor tissue based on the EPR effect [10]; however, incorporation of

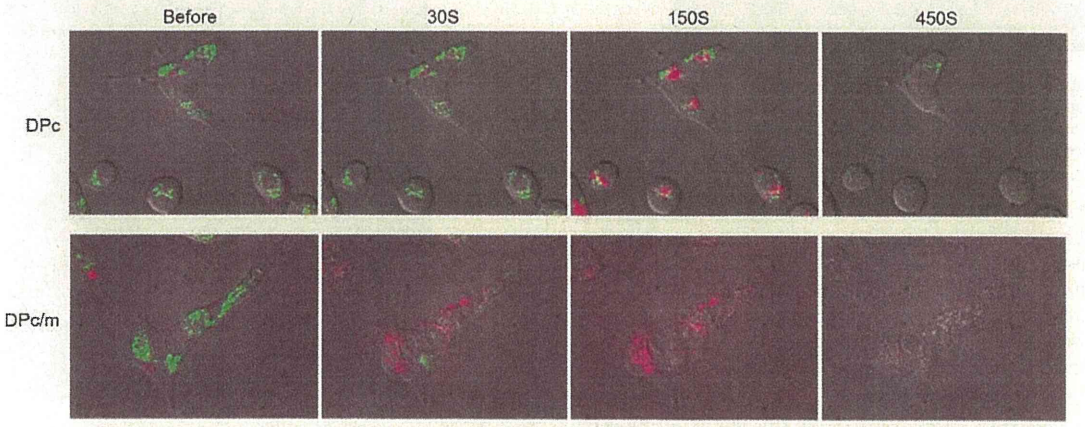

Fig. 2. Time-dependent morphological changes of the DPc- and DPc/m-treated A549 cells during photoirradiation. The fluorescences from DPc (red) and Rhodamine 123 (Rh123) (green) were simultaneously monitored.

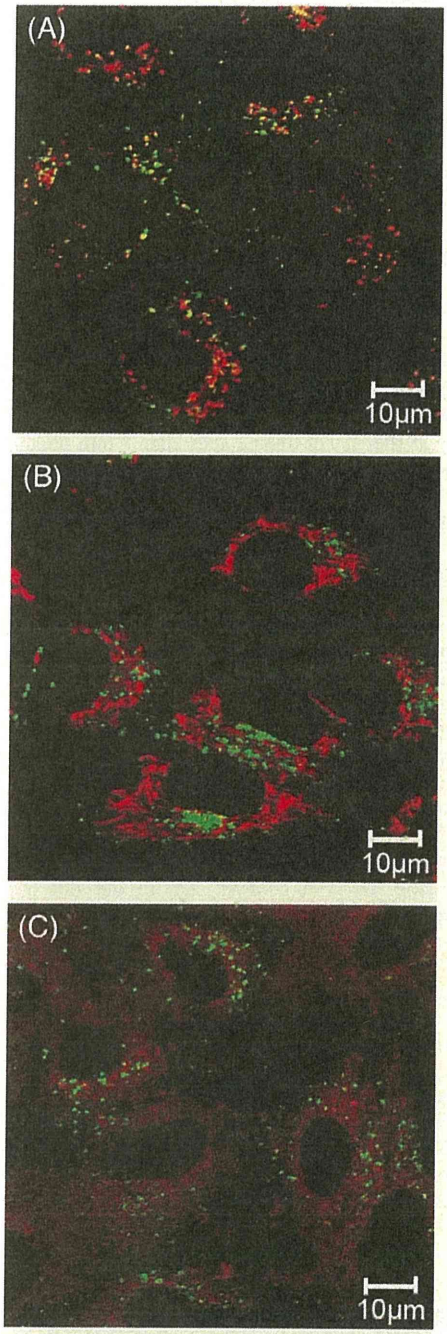

Fig. 3. Intracellular localization of Alexa488-labeled DPc/m (green) in A549 cells without photoirradiation. The cells were stained with LysoTracker Red DND-99 (red) for staining the lysosomes (A), MitoTracker Red 580 (red) for staining the mitochondria (B) and ER Tracker Red for staining endoplasmic reticulum (ER) (C).

PSs into nanocarriers generally decreases the singlet oxygen quantum yield due to the concentration quenching of PSs, leading to considerable reduction of photocytotoxicity [12,13]. In this study, we demonstrated that our unique photosensitizer–nanocarrier combination, i.e., dendrimer phthalocyanine (DPc)-encapsulated polymeric micelles (DPc/m), showed remarkably enhanced photocytotoxicity over free DPc, and achieved significantly higher *in vitro* and *in vivo* PDT effect compared with clinically used Photofrin® (PHE). Therefore, we also focused on the underlying mechanisms of the efficient photo-induced cell death by DPc/m.

In Table 1, DPc/m showed 78-fold higher photocytotoxicity than free DPc. Such remarkable enhancement of DPc/m's photocytotoxicity cannot be explained by the intracellular concentration of DPc: DPc/m exhibited only 7.6-fold higher cellular uptake than free DPc in A549 cells (data not shown). In addition, we previously demonstrated that DPc/m showed an approximately three- to fourfold slower oxygen consumption rate compared with free DPc [15]. We therefore assume that different mechanisms may underlie photo-induced cell death between DPc/m and free DPc. In the present paper, we also studied the effect of photoirradiation by a high-power diode laser on photocytotoxicity. As a result, there were considerable differences in the fluence-rate-dependency of photocytotoxicity between DPc and DP/m (Table 2). Meanwhile, we have also studied the time-dependent morphological changes of the cells during PDT, and found marked differences between DPc and DPc/m (Fig. 2). The DPc/m induced unprecedentedly rapid cell death accompanied by characteristic morphological changes including swelling and membrane blebbing. Note that such characteristic morphological changes in the DPc/m-treated cells appear to be similar to the characteristics of *oncosis*, which is reported to be induced by several pathological conditions, such as hypoxia, inhibition of ATP production, and increased permeability of the plasma membrane [23].

To further study unique light-induced cell death by DPc/m, we have studied intracellular localization of DPc/m. We observed that both DPc and DPc/m selectively accumulated in the endo-/lysosomes, suggesting their cellular internalization through the endocytosis (Fig. 3 and Fig. S2). However, once the cells were photoirradiated, the DPc and DPc/m may have been translocated from the endo-/lysosomes to the

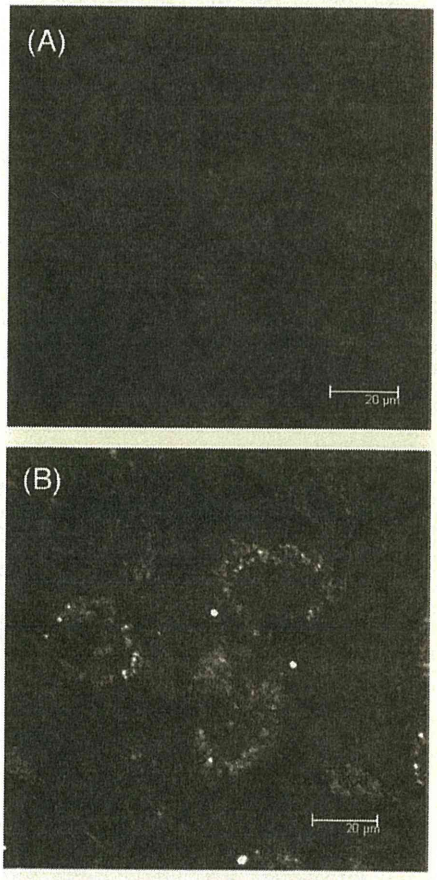

Fig. 4. Fluorescence of MitoSOX Red in the DPc- (A) and DPc/m (B)-treated A549 cells after 1 min photoirradiation.

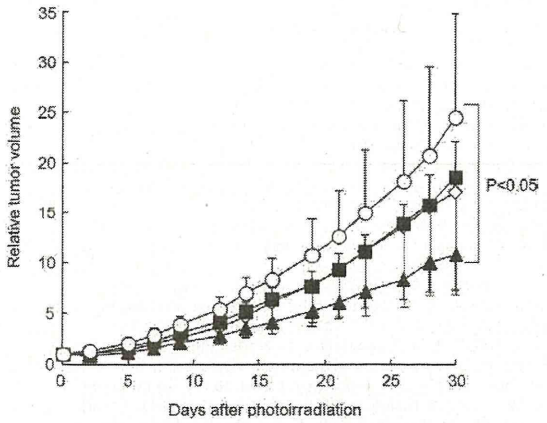

Fig. 5. Growth curves of subcutaneous A549 tumors in control mice (open circle) and mice administered with 0.37 μmol/kg DPc (closed square), 0.37 μmol/kg DPc/m (closed triangle) and 2.7 μmol/kg Photofrin® (PHE) (open diamond) (n=6). Twenty-four hours after administration of photosensitizing agents, the tumors were photoirradiated using a diode laser (fluence: 100 J/cm^2).

cytoplasm, because DPc fluorescence became apparent in a diffusive manner in both the DPc- and DPc/m-treated cells (Fig. 2). The mechanism of this translocation is the photochemical disruption of the endo-/lysosomal membranes: this is called photochemical internalization (PCI), a new concept for light-induced cytoplasmic delivery of cell-membrane-impermeable low-molecular-weight drugs as well as macromolecular compounds, such as nucleic acids and proteins [24,25]. In our previous papers, we indeed demonstrated that DPc and DPc/m allowed the efficient endosomal escape of non-viral gene vectors, achieving light-induced and site-directed transfection *in vitro* and *in vivo* [27,28]. Possibly, the increased fluorescence of DPc/m in Fig. 2 may reflect the destabilization of the micellar structure due to photochemical reactions: this destabilization may be essential to the interaction of DPc/m with the endo-/lysosomal membranes.

It is known that the efficiency of light-induced cell death in PDT depends on which sites in the cell are photodamaged [2,3]. In this regard, photodamage to the mitochondria could induce efficient cell death, whereas endo-/lysosomes are less susceptible to photocytotoxicity [3]. Therefore, we focused on the effects of PDT using DPc/m on the mitochondria. In Fig. 2, we evaluated the time-dependent changes in the fluorescence of Rh123, a specific dye to the mitochondria, during photoirradiation in PDT using DPc or DPc/m. The fluorescence of Rh123 in the DPc/m-treated cells immediately disappeared after the initiation of photoirradiation, whereas that in the DPc-treated cells remained even after 450 s photoirradiation. Note that the fluorescent intensity of Rh123 is correlated with the amount of ATP in the cells [28]. Therefore, it is assumed that DPc/m might directly or indirectly clip the ATP in the cell. To clarify whether the effects of DPc/m on the mitochondrial functions are attributed to its direct photodamage to the mitochondria or not, we detected the ROS production in the mitochondria by using MitoSOX Red (Fig. 4). As a result, only DPc/m showed appreciable ROS production in the mitochondria upon photoirradiation. These results suggest that DPc/m might induce photodamage to the mitochondria and thus affect their functions.

Based on all the aforementioned results, DPc and DPc/m are assumed to undergo the following steps in the photo-induced cell death. (i) DPc and DPc/m are internalized through the endocytic pathway and accumulate in the endo-/lysosomes. In this step, DPc/m showed 7.6-fold higher cellular uptake than free DPc. (ii) Upon photoirradiation, DPc and DPc/m are translocated from the endo-/lysosomes to the cytoplasm through the photochemical disruption of the endo-/lysosomal membranes. We previously reported that DPc/m is more efficient than DPc in the PCI-mediated gene transfection [26]. (iii) Only DPc/m might accumulate in the mitochondria and produce the ROS, resulting in exhaustion of ATP in the cell. In contrast, DPc showed no ROS production in the mitochondria. These steps may account for DPc/m's much higher photocytotoxicity compared to DPc. Also, PDT using DPc/m induced unique cell death, similar to a characteristic of *oncosis*. The photodamage to the mitochondria and/or the exhaustion of intracellular ATP may be attributed to this unique cell death by DPc/m. In addition to the subcellular localization of DPc/m and the photodamaged sites in the cell, the characteristic structure of DPc/m, in which several tens DPc molecules are concentrated in a 50 nm nanocontainer, may also contribute to the remarkably high photocytotoxicity. Note that DPc/m from PEG–PLL with the polymerization degree of PLL of 43 contained 77 DPc molecules in the core (Fig. S3 in Supporting Information). The high local concentration of DPc within the micellar core is assumed to generate a high concentration of ROS at a local site, achieving a high photochemical oxidation level that exceeds the threshold of cell death. Note that, in the case of other nanocarriers containing conventional PSs, it may be difficult to achieve such a high local concentration of ROS due to the concentration quenching of PSs [13]. Furthermore, although ROS, especially singlet oxygen, have very short half-lives (~2 μs) in aqueous media [3], a high local concentration of ROS achieved by DPc/m may allow the oxidization of the greater regions in the cell. Such effects of spatially regulated incorporation of PSs into nanocarriers on PDT are quite intriguing, and further studies should be performed.

In this study, we also demonstrated that DPc/m showed significantly higher *in vivo* antitumor activity against subcutaneous A549 tumors compared with clinically used PHE, although the injected dose of DPc/m was 7.3-fold lower than that of PHE on the basis of photosensitizing units (Fig. 5). This superior effect may be explained by the effective accumulation of DPc/m based on the EPR effect and its enhanced photocytotoxicity, as mentioned above. In general, *in vivo* PDT effect is known to be very complicated and can be affected by several parameters, including accumulation and penetrability of

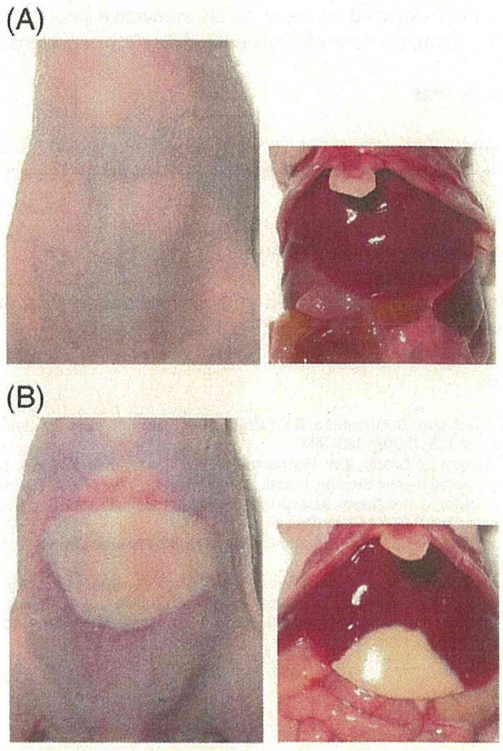

Fig. 6. Macroscopic observation of the skin and organs in the mice treated with 4.2 μmol/kg DPc/m (A) and 8.1 μmol/kg Photofrin® (PHE) (B) at 4 days after light irradiation to abdominal skin using a halogen lamp (fluence: 60 J/cm^2).

photosensitizers (PSs) in the tumor tissue, possible targets (i.e., tumor vasculature and cancer cells), and tissue penetration of light [1]; therefore, there may be a room for further improvement in the *in vivo* efficacy. In this regard, we are going to study the mechanism of *in vivo* PDT effect by DPc/m, optimizing several factors to maximize the therapeutic effect in the near future. Importantly, the PHE-administered mice showed severe damages to the skin and liver after exposure to white light; however, the DPc/m-administered mice did not show such side effects regardless of the injected dose (Fig. 6). These results may be attributed to the reduced accumulation of DPc/m in the skin and normal organs. In general, the skin phototoxicity might result from non-specific accumulation of conventional PSs in the skin, because they could penetrate the endothelium and eventually accumulate in various organs and tissues. In contrast, nanocarriers or macromolecular photosensitizers are assumed not to pass through the tight junctions of the vasculature in the tissues except for the liver, spleen, and tumor, which possess leaky vasculatures. Therefore, it is assumed that nanocarriers or macromolecular photosensitizers may not accumulate in the skin, thus preventing skin phototoxicity. Furthermore, no skin damage was found after photoirradiation even at 1 h after DPc/m administration (data not shown). Possibly, DPc/m in the bloodstream may not be photoactivated by light irradiation, owing to strong absorption by a heme in red blood cells.

5. Conclusion

The DPc/m elicited remarkably effective and rapid light-induced cell death. Following internalization by endocytosis, DPc/m seemed to be translocated from the endo-/lysosomes to the cytoplasm during photoirradiation and subsequently induce photodamage to the mitochondria. Such unique intracellular localization of DPc/m may be responsible for the enhanced photocytotoxicity mentioned above. In animal experiments, DPc/m showed significantly higher antitumor activity than clinically used PHE. Furthermore, unlike the PHE-treated mice, the DPc/m-treated mice showed no sign of skin phototoxicity. Thus, DPc/m is expected to serve as an innovative photosensitizer formulation to improve the effectiveness and safety of current PDT.

Acknowledgment

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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.2008.10.010.

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Research Paper

A Photo-Activated Targeting Chemotherapy Using Glutathione Sensitive Camptothecin-Loaded Polymeric Micelles

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Purpose. A novel photo-activated targeted chemotherapy was developed by photochemical internalization (PCI) of glutathione-sensitive polymeric micelles incorporating camptothecin (CPT) prepared from thiolated CPT (CPT-DP) and thiolated poly(ethylene glycol)-*b*-poly(glutamic acid) (PEG-*b*-P(Glu-DP))
Methods. PEG-b-P(Glu-DP) and CPT-DP were synthesized and characterized by ^{1}H-NMR and gel permeation chromatography, and then mixed to prepare CPT-loaded polymeric micelles (CPT/m). The CPT release from the micelle was studied by reverse phase liquid chromatography. The PCI-activated cytotoxicity of CPT/m against HeLa cells was studied in combination with a non-toxic concentration of dendrimer phthalocyanine-loaded micelles (DPc/m) as the photosensitizer.
Results. The diameter of CPT/m was 96 nm and the drug loading was 20% (*w/w*). CPT was slowly released under the conditions reproducing the extracellular or endosomal environments. However, under the reductive conditions mimicking the cytosol, CPT was rapidly released achieving approximately 90% of the drug release after 24 h. The cytotoxicity of CPT/m was drastically increased on photoirradiation, whereas the CPT/m were not cytotoxic without PCI.
Conclusions. The CPT/m released the drug responding to reductive conditions. The PCI-induced endosomal escape exposed CPT/m to the cytosol triggering the drug release. Thus, CPT/m in combination with DPc/m will behave as smart nanocarriers activated only at photoirradiated tissues.

KEY WORDS: camptothecin; chemotherapy; environment sensitive-polymeric micelles; photochemical internalization.

INTRODUCTION

The site-specific drug delivery has become a key issue in cancer therapy, as the use of chemotherapeutic agents is often limited due to severe side effects, and the development of stimuli-responsive drug delivery systems that allow selective activation of the drugs at the target site will be crucial for successful therapies. In this way, the use of light for the activation of drug carriers is an attractive strategy because it is a safe energy source and it offers a high level of control in terms of wavelength, duration, intensity, and site of the photoirradiation, which can modulate the quantity of drug released, the timing of the release event and its location. The existing photoactivated drug delivery systems are mainly triggered by UV light, such as the systems based on the photoisomerization of azobenzene derivatives (1–5). Nevertheless, these systems are not suitable for biological applications because of the high absorbance of UV light by many biomolecules and the potential damage healthy tissues. Thus, it is necessary to develop realistic drug delivery systems activated by light with a longer wavelength in order to minimize photoinduced damage and to increase the depth of light penetration into tissues.

Recently, several macromolecules and other molecules that do not readily penetrate the plasma membrane have been used in combination with photochemical internalization (PCI) for the site-specific enhancement of their therapeutic efficacy by selective photochemical rupture of endocytic vesicles and consequent release of endocytosed macromolecules into the cytosol (6–13). The PCI concept is based on the

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ABBREVIATIONS: CPT, camptothecin; CPT-DP, thiolated CPT; PEG-*b*-P(Glu-DP), thiolated poly(ethylene glycol)-*b*-poly(glutamic acid); CPT/m, CPT-loaded polymeric micelles; DLS, dynamic light scattering; DPc/m, dendrimer phthalocyanine-loaded micelles; PCI, photochemical internalization; PDT, photodynamic therapy; PS, photosensitizer; SPTDP, disulfide amine linker; RPLC, reverse phase liquid chromatography.

use of photosensitizers, which induce the formation of reactive oxygen upon exposure to light of appropriate wavelengths (14). These photochemical reactions are the basis for photodynamic therapy (PDT), a treatment modality where light exposure leads to photosensitizer-induced killing of cancer cells used in various types of cancer (15,16). The PCI allows macromolecules located in the vesicles to reach the cytosol and to exert their biological activity instead of being degraded by lysosomal hydrolases. Furthermore, this PCI-based relocalization and activation of the macromolecules has the advantage of minimal side effects because the effect is localized to the irradiated area. The PCI employs light with clinically relevant wavelengths even in the near IR region, allowing a therapeutic effect in deeper lesions of the target tissues and a negligible damage to healthy tissue. In addition, after PCI, the macromolecules are exposed to the extremely reductive environment of the cytosol. Since the concentration of glutathione at the cytosol is 100–1,000 times higher than that in blood (17), it can be used as an efficient stimulus for the specific drug carrier activation. Consequently, the combination of PCI and drug carriers responsive to reductive environment will present the spatial and temporal triggering of drug action by photoirradiation.

The PCI-induced chemotherapy should be performed utilizing drug carriers that selectively accumulate in tumor tissues and enter cells *via* endocytosis. In this way, the polymeric micelles have shown reduced non-specific accumulation in normal tissues and preferential accumulation in solid tumors by the enhanced permeability and retention effect (18–22). Previously, we have reported polymeric micelles for the delivery of chemotherapeutics and photosensitizing agents with enhanced activity both *in vitro* and *in vivo* (11–13,18,20,23,24). Moreover, we have utilized dendrimer phthalocyanine (DPc)-loaded polymeric micelles (DPc/m), as photosensitizers, to increase the transfection efficiency of polyion complex micelles incorporating plasmid DNA by PCI (11–13). Thus, in the present study, we will utilize DPc/m to induce PCI for the specific activation of glutathione-sensitive camptothecin (CPT)-loaded polymeric micelles (CPT/m). Since both the CPT/m and the DPc/m are expected to exhibit prolonged blood circulation, selectively accumulate in tumor tissues and be taken up by cancer cells *via* endocytosis as previously reported for similar systems (18–21; Fig. 1), the combination will behave as a photoactivated drug delivery system for *in vivo* application.

CPT and its derivatives are very potent antitumor agents (25), though their full clinical potentials have yet to be realized because of the lack of water-solubility, the instability of the pharmacologically active lactone ring and the fast non-specific distribution to the whole body (25,26). Thus, the development of adequate carriers for the delivery of CPT is imperative. Herein, the CPT/m were prepared by conjugating a thiolated-camptothecin derivative (CPT-DP) to thiolated poly(ethylene glycol)-poly(glutamic acid) [PEG-*b*-P(Glu-DP)] block copolymer *via* a disulfide bond given that this bond is stable in blood and specifically cleaved in a reductive environment (17). Accordingly, the *in vitro* release profiles were studied under different conditions. Moreover, in order to prove the concept of a photoactivated drug delivery system, we tested the ability of CPT/m combined with a non-toxic concentration of DPc/m to specifically eradicate cancer cells *in vitro* after photoirradiation.

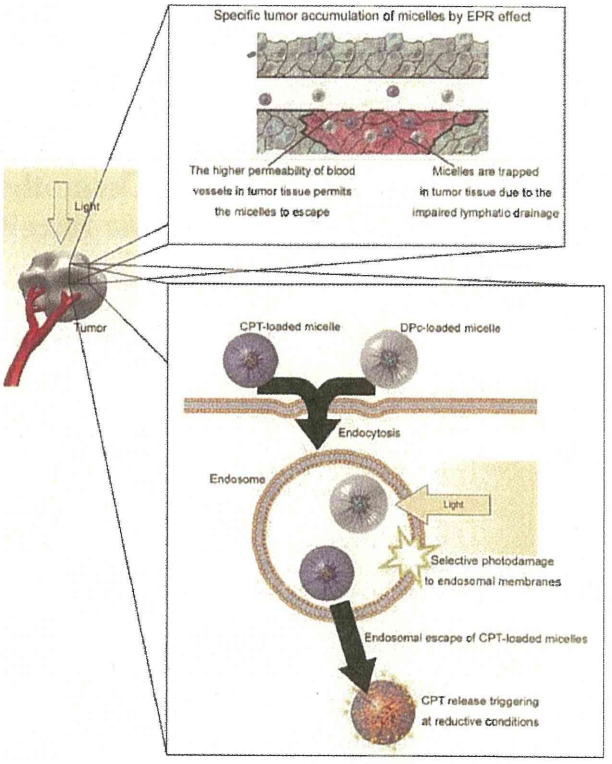

Fig. 1. Photochemical internalization-activated targeting chemotherapy. The polymeric micelles encapsulating camptothecin (*CPT*) and dendrimer phthalocyanine (*DPc*) accumulate in tumor tissue by the enhanced permeability and retention effect. The tumor is irradiated at the relevant wavelength. Both micelles are considered to be taken up by the cell through the endocytic pathway. The localization of the *DPc-loaded micelles* in the endosome allows the selective photodamage of the endosomal membrane upon photoirradiation, thereby inducing the endosomal escape of *CPT-loaded micelles* and the selective release of CPT under reductive conditions.

MATERIALS AND METHODS

Materials

γ-Benzyl L-glutamate and *N*-z-L-lysine were bought from Sigma Chemical Co., Inc. (St. Louis, MO, USA). Bis(trichloromethyl)carbonate (triphosgene) was purchased from Tokyo Kasei Kogyo Co., Inc. (Tokyo, Japan). Dichloromethane, 4-dimethylaminopyridine (DMAP), dicyclohexylcarbodiimide (DCC), camptothecin, *N,N*-Dimethylformamide (DMF), dimethyl sulfoxide (DMSO), dithiothreitol (DTT) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Wako Pure Chemical Co., Inc. (Osaka, Japan). Methoxycarbonylsulfenyl chloride, 2-mercaptopyridine and 3-mercaptopropionic acid were purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI, USA). α-Methoxy-ω-aminopoly(ethylene glycol) (CH_3O-PEG-NH_2; Mw=5,000 and 12,000) was purchased from Nippon Oil and Fats Co., Inc. (Tokyo, Japan). All solvents for the polymer syntheses were distilled just before use.