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Woo-Dong Jang[a]; Nobuhiro Nishiyama[bc]; Kazunori Kataoka[d]
[a] Department of Chemistry, College of Science, Yonsei University, Seoul, South Korea [b] Center for Disease Biology and Integrative Medicine, The University of Tokyo, Tokyo, Japan [c] Center for NanoBio Integration, The University of Tokyo, Tokyo, Japan [d] Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Agency (JST), Tsukuba, Japan

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Supramolecular Assembly of Photofunctional Dendrimers for Biomedical Nano-Devices

WOO-DONG JANG[a,*], NOBUHIRO NISHIYAMA[b,c] and KAZUNORI KATAOKA[b,c,d,e]

[a]*Department of Chemistry, College of Science, Yonsei University, 134 Sinchondong, Seodaemun-gu, Seoul 120-749, South Korea;* [b]*Center for Disease Biology and Integrative Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan;* [c]*Center for NanoBio Integration, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan;* [d]*Department of Materials Engineering, Graduate School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan;* [e]*Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Agency (JST), Tsukuba 305-8562, Japan*

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Recently, dendrimers are attracting much attention for their development as biomedical nano-devices. In the present paper, we introduce dendrimer photosensitizers, composed of a focal photosensitizing unit surrounded by poly(benzyl ether) dendritic wedges, for photodynamic therapy (PDT). Ionic dendritic porphyrins (DPs) formed supramolecular polyion complex (PIC) micelles through the electrostatic interaction with oppositely charged block copolymers, and showed no self-quenching effect of the focal photosensitizing unit due to the large dendritic wedges. The DP-loaded PIC micelles showed selective accumulation to the ophthalmic neovascular lesion and effective photodynamic effect. For the effective light delivery, dendrimer phthalocyanine (DPc) has also been developed and examined for its photodynamic effect.

Keywords: Dendrimer; Porphyrin; Photodynamic therapy; Polyion complex micelle

INTRODUCTION

Dendrimers are attracting increasing attention as one of the representative materials for the organic nano-object [1–4]. Dendrimers have regularly branched chemical structures with a well predictable three-dimensional architecture. Unlike linear polymers, dendrimers have no molecular weight distribution due to their highly designed stepwise synthetic procedure. Various functions can be realized by a modification of their branching units, surface functionalities, building blocks, or focal core. Generally, dendrimers show unique functions that are unrealizable by using linear polymers. Recently, we have developed ionic poly(benzyl ether) dendrimers with a photosensitizing unit in the core, which showed a significantly distinguishable aspect compared to conventional photosensitizers [5,6]. The charged surface of an ionic dendrimer can interact with an oppositely charged block copolymer via an electrostatic interaction to result in the formation of supramolecular polyion complex (PIC) micelle [7]. We have researched the photodynamic effect of the dendrimer-loaded PIC micelles against several disease models. In the present paper, we would like to review recent advances in the photofunctional dendrimer assemblies for biomedical applications.

PHOTODYNAMIC EFFECT OF DENDRIMER PORPHYRIN

Together with recent advances in laser technology, photodynamic therapy (PDT) is attracting attention as a promising technology for cancer treatment [8,9]. PDT involves the systemic administration of a photosensitizer, which is followed by laser light irradiation with an appropriate wavelength to the target tissue. When the photosensitizers absorbed the laser light, the photosensitizers are excited to the singlet state and then transformed to long-lived triplet state via an intersystem crossing. The triplet state of photosensitizers can transfer their excited energy or electron to oxygen molecules to generate

*Corresponding author. E-mail: wdjang@yonsei.ac.kr

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reactive oxygen species (ROS), such as singlet oxygen or peroxide radicals (Fig. 1). The ROS thus generated have a high cytotoxicity and destroy target tumor tissue. PDT has several distinguishable advantages compared with other malignant disease treatments. In many cases, chemotherapy results in side effects or sequelae, because therapeutic agents influence on the normal tissue as well as therapeutic target. In the case of PDT, selective photoirradition to the target tissue possibly minimize such adverse effects, because photosensitizers are harmless under light-free conditions.

However, most photosensitizers have poor solubility in an aqueous medium due to their large π-conjugation domain and hydrophobic characteristics. Therefore, photosensitizers easily form aggregates in the aqueous medium, which might result in a decrease of PDT efficacy through a self-quenching of the excited state [10].

For these reasons dendrimer porphyrins (DPZns, Fig. 2) are one of the most promising materials, because the substitution of large dendritic wedges sufficiently prevents the formation of aggregates and provides high solubility to the aqueous medium [11]. Figure 3 shows cell viability curves of Lewis Lung Carcinoma (LLC) against the concentration of photosensitizers after photoirradiation. Compared to protoporphyrin IX (PIX), a major conventional photosensitizer, DPZns show 10–100 times higher photocytotoxicity. Typically, cationic dendrimer porphyrin (32(+)DPZn) shows 10 times higher photocytotoxicity than anionic dendrimer porphyrin (32(−)DPZn). Higher photocytotoxicity of 32(+)DPZn can be explained by the effective association against the cellular membrane, because the cellular membrane is negatively charged. Very importantly, 32(+)DPZn and 32(−)DPZn respectively showed 157- and 113-fold lower dark toxicity compared to PIX, indicating that DPZns have high photodynamic efficacy with minimum side effect.

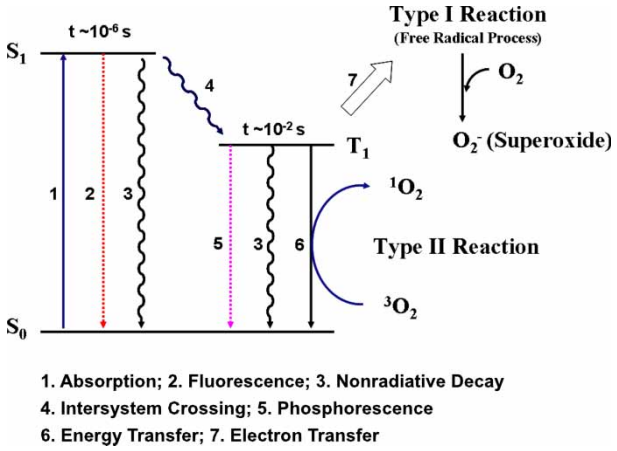

FIGURE 1 Photodynamic process.

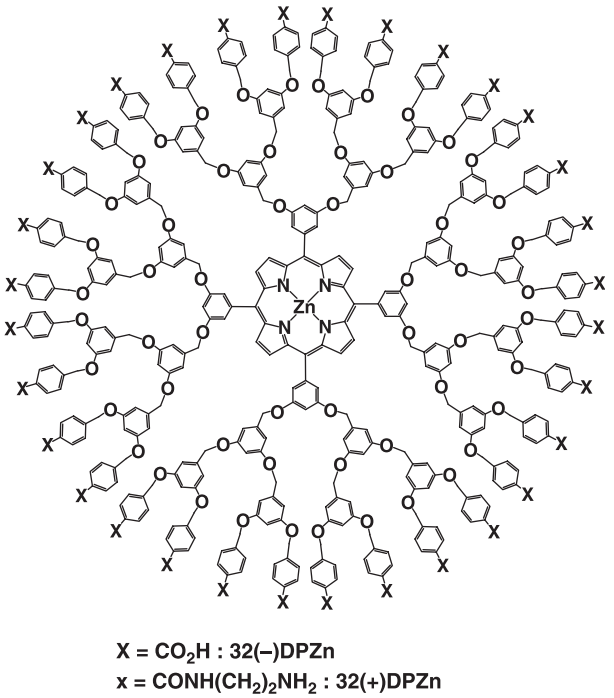

FIGURE 2 Structure of dendrimer porphyrins.

DP-LOADED POLYION COMPLEX MICELLE

PIC micelle can be formed via the electrostatic interaction between a pair of charged block copolymers and oppositely charged macromolecules [12,13]. The 32(−)DPZn and 32(+)DPZn can form a polyion complex micelle by mixing with poly(ethylene glycol)-*block*-polylysine (PEG-*b*-PLys) and poly (ethylene glycol)-*block*-poly(aspartic acid) (PEG-*b*-PAsp), respectively, in an aqueous medium (Fig. 4), and they are expected as nanocarriers for dendrimer photosensitizers. The dynamic and static light

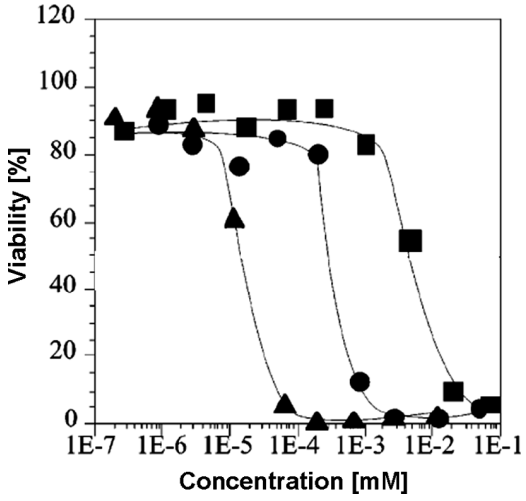

FIGURE 3 Viability of LLC cells treated with PIX(●), 32(+)DPZn(▲), and 32(−)DPZn(■) after photoirradiation at incubation time of 8 h.

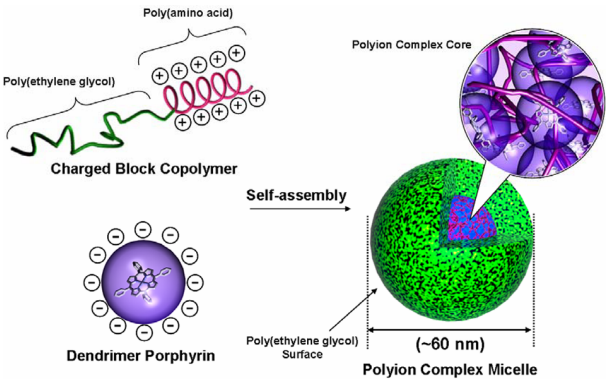

FIGURE 4 Formation of polyion complex micelle.

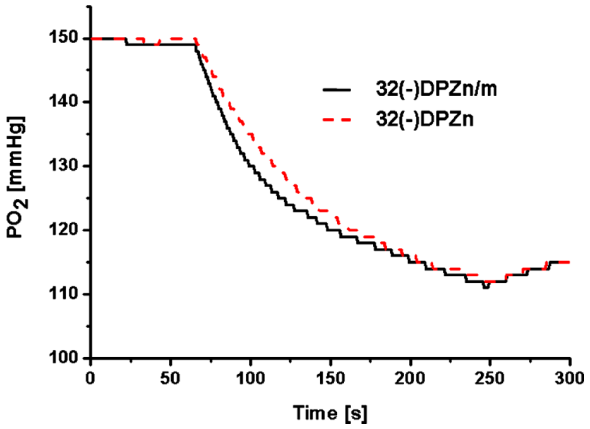

FIGURE 5 Photoinduced oxygen consumption of the 32(−)DPZn and 32(−)DPZn/m. Light was irradiated from 60 s to 240 s.

scattering (DLS and SLS) measurements revealed the formation of uniform-sized micelles with a diameter of approximately 50 nm. Both PIC micelles showed a high stability upon dilution at physiological salt concentration and wide range of pH. The ζ-potential values of PIC micelles were almost neutral, coinciding with the charge neutralization between the DPZns and charged segment of block copolymers as well as encapsulation of the PICs within the PEG layer. These features are very important from a therapeutic point of view. For target delivery of the therapeutic agent, the nanocarriers should have a tolerance against dilution and maintain long circulation times in the blood compartment. The PEG layer of PIC micelles provides a high biocompatibility and tolerability against non-selective adhesion of biomolecules. Also, the PIC micelles might show selective accumulation in the tumor tissue due to the so-called enhanced permeability and retention (EPR) effect [14], which accounts for vascular hyperpermeability and impaired lymphatic drainage in solid tumors. Such enhancement of tumor accumulation of PIC micelle incorporating DPZn might improve the PDT efficacy of solid tumors.

PDT EFFECT OF DP-LOADED PIC MICELLE

As mentioned above, low molecular weight conventional photosensitizers generally show collisional quenching effect, whereas dendritic substitution of photosensitizers prevents collisional quenching of focal photosensitizing unit even in much high concentration within the micellar core. In fact, large dendritic photosensitizers showed almost comparable fluorescence intensity before and after the formation of PIC micelle. In relation to this fact, the photoinduced oxygen consumption of the 32(−)DPZn and 32(−)DPZn-loaded PIC micelle (32(−)DPZn/m) was measured in order to estimate the efficiency of photochemical reactions (Fig. 5). Very interestingly, it was revealed that the oxygen consumption rate of 32(−)DPZn/m was almost identical to that of the free 32(−)DPZn in an aqueous medium, indicating that the singlet oxygen molecules can successfully escape the micellar structure without a decrease of the photodynamic efficacy [15]. From the standpoint of the application for PDT, the DPZn ensures an effective photochemical reaction of the porphyrin core regardless of the local concentrations. It is possible that 32(−)DPZn/m achieves an elevated concentration of local singlet oxygen, which cannot be achieved by other formulations containing conventional photosensitizers.

On the other hand, the cellular uptake amount of free 32(−)DPZn and 32(−)DPZn/m increased with the incubation time, and 32(−)DPZn/m showed 6–8-fold higher uptake amounts than free 32(−)DPZn. Considering the negatively charged surface of mammalian cells, charge neutralization of 32(−)DPZn by PEG-*b*-PLL could improve the cellular uptake of 32(−)DPZn/m. Although the photosensitizers are assumed to be extremely concentrated in the subcellular organelles such as the endosomes and lysosomes, no fluorescent quenching was observed for the cells incubated with 32(−)DPZn/m. This observation contrasts to the fact that the conventional mesochlorin e_6-conjugated *N*-(2-hydroxypropyl) methacrylamide (HPMA) copolymers exhibit fluorescence quenching when the cells are incubated with HPMA at an extremely high concentration [16]. Notably, the photocytotoxicity of 32(−)DPZn/m was remarkably enhanced compared to free 32(−)DPZn. Incorporation of 32(−)DPZn into the micelles resulted in approximately a 130–280 fold increased photocytotoxicity. The remarkably enhanced photocytotoxicity of 32(−)DPZn/m may not be fully explained by the above-mentioned 6–8-fold increases in their cellular uptake amount. The 32(−)DPZn/m is assumed to produce a significantly high concentration of ROS due to effective separation of the center porphyrin units by the dendritic envelope. The concrete mechanism of efficient ROS generation within the

micellar structure remains to be answered. However, a dendritic structure preventing aggregation of the center porphyrin should be essential for the enhanced photocytotoxicity.

IN VIVO PDT EFFECT OF 32(−)DPZN-LOADED MICELLE

The exclusive age-related macular degeneration (AMD), a condition caused by choroidal neovascularization (CNV), is a major cause of legal blindness in developed countries. Recently, Visudyne®, a liposomal formulation of verteporfin, was approved for clinical use, and large randomized control studies demonstrated to prevent severe visual loss by means of PDT [17,18]. However, because of the intractableness of AMD, most patients require repeated treatments every three months, still suffering from a reduced quality of life [19]. For effective PDT against AMD, the selective delivery of photosensitizer to the CNV lesions and an effective photochemical reaction at the CNV sites might be necessary. In regard to the efficient delivery of photosensitizers, low-density lipoproteins and antibodies have been used as a carrier molecule; however, photosensitizers also distribute to normal vessels to some extent because normal vessels also express such markers. In addition, the increased loading of photosensitizers to the drug carriers possibly decreases PDT efficacy due to the aggregation of photosensitizers. Thus, there is a strong impetus to develop novel photosensitizer formulations from the standpoint of both the efficiency of the delivery and photochemical reactions of the photosensitizers themselves.

Based on the above information, we applied PDT using 32(−)DPZn/m for the treatment of AMD model [20]. In this study, experimental CNV was delivered to the rats by laser photocoagulations to each eye. And then, 400 μL of 32(−)DPZn/m or free 32(−)DPZn, including 1.5 mg/mL of 32(−)DPZn, was administered by tail vein injection into rats 7 days after the photocoagulation. The rats were sacrificed, and the eyes were immediately enucleated to observe the accumulation of 32(−)DPZn in the CNV lesions. The results showed an effective and selective accumulation of 32(−)DPZn/m in the CNV lesions, indicating that the CNV lesions may have characteristic features similar to solid tumor vasculatures, such as hyperpermeability and impaired lymphatic drainage. The enhanced accumulation of 32(−)DPZn/m into CNV lesions resulted in a significantly pronounced PDT effect, which was evaluated using fluorescein angiography. When the PDT laser was applied 15 minutes after the injection of a 32(−)DPZn/m, 78% of the CNV lesions showed no fluorescein leakage at day 1. At day 7, the hypofluorescence persisted, suggesting that the leakage from the CNV lesions was still reduced. Moreover, skin phototoxicity was not macroscopically observable when the rats were exposed to broadband visible light (Xenon lamp equipped with a filter passing light of 377–700 nm, incident light irradiance; approximately 30 mW/cm^2) 4 hours after the injection of the 32(−)DPZn/m, which is in sharp contrast to the results observed after the injection of a clinically used photosensitizer formulation, Photofrin® (Fig. 6).

Alternatively, corneal neovascularization is one of the major causes of visual loss. The normal cornea is avascular tissue, but under certain pathological conditions, capillaries from the limbal plexus invade the corneal tissue [21]. A wide range of inflammatory, infectious, degenerative, and traumatic disorders may induce corneal neovascularization. In the case of corneal neovascularization, similar to the model of AMD, 32(−)DPZn/m was also selectively accumulated in the neovascular tissue [22]. PDT with 10 J/cm^2 of energy after i.v. administration of 32(−)DPZn/m exhibited complete regression of all neovascular lesions at day 7. From these *in vivo* results, we can conclude that DPZn may have a great potential as a photosensitizer for the treatment of several ophthalmologic diseases.

DENDRIMER PHTHALOCYANINE

In vitro and *in vivo* results indicated that DPZns have a great potential to use as photosensitizers for PDT. Especially, 32(−)DPZn/m showed selective accumulation to the target tissue of ophthalmologic vascular diseases, resulting in enhanced PDT efficacy. However, the DPZn has relatively short excitation wavelengths, where the absorption maximum is 430 (soret band) and 560 nm (Q band), which might be a limitation for PDT except for the transparent tissues such as ophthalmologic organs. Skin tissue has melanin dyes, which absorb short wavelength light to prevent photochemical genetic disorder, and also heme proteins account for most of the absorption of light in the visible region.

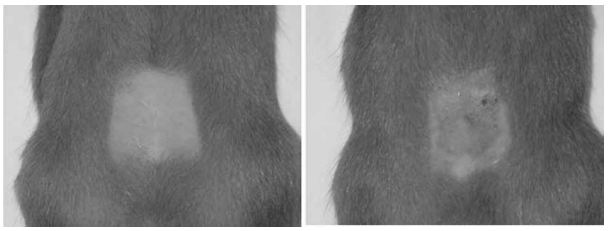

FIGURE 6 Macroscopic observation of skin phototoxicity. When the rats were exposed to broadband visible light 4 hours after the injection of 32(−)DPZn/m (left), skin phototoxicity was not macroscopically observable. Severe phototoxicity was observed after the injection of Photofrin® (right).

Such light absorption by human bodies prevents the photochemical reactions of photosensitizers. Therefore, photosensitizers should have long wavelength absorption to improve availability for deeper lesions such as solid tumors.

In this regard, several phthalocyanine molecules have been widely researched as potential photosensitizers with appropriate absorption wavelength for practical PDT application [23–26]. Also, we have developed ionic dendrimer phthalocyanine (DPcZn; Fig. 7), which has absorption maximum at 685 nm. DPcZn also nicely formed PIC micelle (ca. 50 nm, unimodal) through the electrostatic interaction with PEG-*b*-PLL [27]. DPcZn-loaded polyion complex micelle (DPcZn/m) was stable in a phosphate buffered solution containing 10% FBS, maintaining the size and polydispersity of DPcZn/m. According to the formation of the PIC micelle, the absorption maximum was slightly changed to 630 nm, indicating the possibility of slight aggregate formation of the core phthalocyanine units. 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 32(−)DPZn (MW = 8029). Consistently, DPcZn/m showed a lower oxygen consumption rate than DPcZn alone. Nevertheless, either DPcZn or DPcZn/m can take part in the photochemical reaction to generate ROS.

The light-induced cytotoxicity (photocytotoxicity) of DPcZn was assessed against cultured tumor cells. Under dark conditions, the cytotoxicities of DPcZn and DPcZn/m were negligible. However, either DPcZn or DPcZn/m exhibited photocytotoxicity upon the photoirradiation (400–700 nm). As the exposure time increased, either DPcZn or DPcZn/m exhibited an increase in photocytotoxicity. Very interestingly, the aspect of the photocytotoxicity increase is significantly different between DPcZn and DPcZn/m. As shown in Fig. 8, 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 behaviors exhibited a quenching signature, DPcZn/m have a significantly higher PDT efficacy compared to DPcZn alone. On the other hand, the cell viability exhibits an abnormal increase with increase in the concentration of DPcZn/m at the 15 min light irradiation. To understand this phenomenon, we need further investigation.

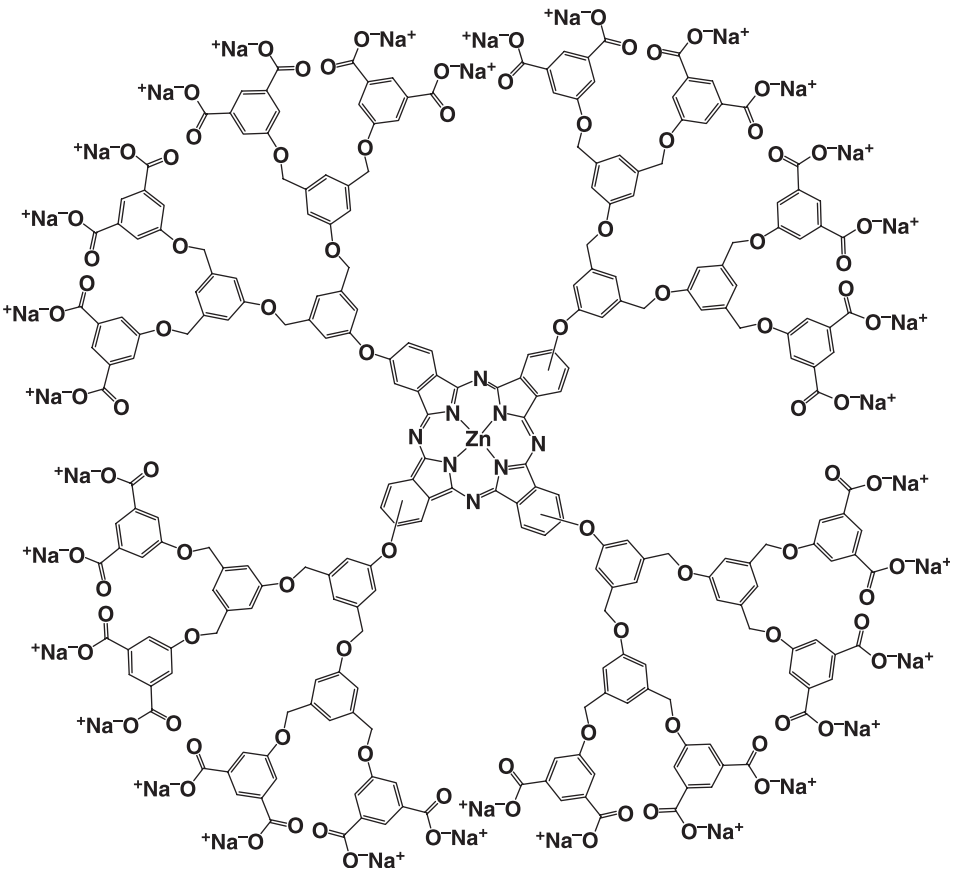

FIGURE 7 Structure of dendrimer phthalocyanine.

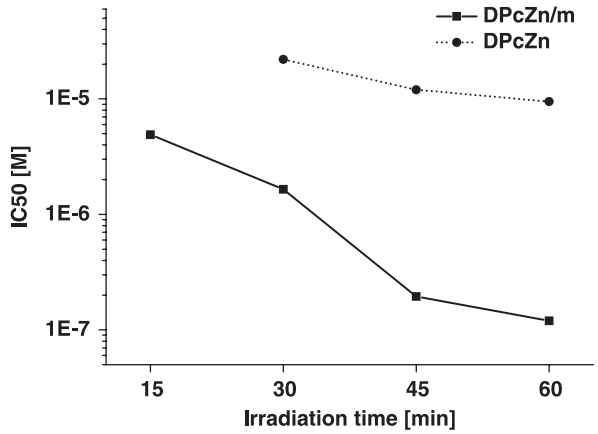

FIGURE 8 Photoirradiation-time-dependent IC50 changes of DPcZn/m and DPcZn against HeLa cells.

CONCLUSION

In this paper, we reviewed photophysical properties of the DPs and DP-loaded PIC micelles. The DP-loaded PIC micelles have unique photochemical properties and show remarkable photodynamic effect. The PDT with DP-loaded micelles succeeded in the treatment of neovascular disease models without any sign of side effects. Our nanocarriers integrated with dendrimer photosensitizers are expected to be a clinically useful photosensitizer formulation for PDT. Although many problems can still be improved, the dendrimers have a very high possibility for use as nanobio tools. As well as photofunctional dendrimers, several dendrimers have already been developed for practical use in various medical fields. Because dendrimers have unique characteristics that cannot be realized in linear polymers, we are expecting that further development of dendrimers will be carried out as a biomedical nano device having high-performance and high efficiency.

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