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

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Abstract

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

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1. Introduction

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

In this context, we have recently reported ionic dendrimer porphyrin as an efficient photosensitizer for photodynamic therapy [6–9]. To obtain high quantum yields and effective energy absorption, photosensitizers must generally have large π-conjugation domains. Therefore, most of photosensitizers easily form aggregates, which provide a self-quenching effect of the excited state in aqueous medium due to their π−π interaction and hydrophobic characteristics [10,11]. To overcome these problems, the structure of ionic dendrimer porphyrin is promising, because the substitution of large dendritic wedges
sufficiently prevents the formation of aggregates and provides high solubility in the aqueous medium. Furthermore, a charged ion surface can form polyion complex micelles by means of electrostatic interaction with an oppositely charged block copolymer. These types of polyion complex micelles [12] with a PEG shell were demonstrated to accumulate effectively and specifically in solid tumor tissue due to the hyperpermeability of tumor capillaries. However, the dendrimer porphyrin has a relatively short wavelength absorption, where the absorption maximum is 430 nm, which is a limitation to improvement for practical PDT application. In relation to this fact, several phthalocyanine molecules, including the one with a dendritic architecture, are of interest as a potential photosensitizer with appropriate wavelength absorption for practical PDT application [13–16]. Herein, we report the first example of dendritic phthalocyanine-incorporated polyion complex micelle formation and demonstrate an order-of-magnitude enhancement in photodynamic efficacy of the phthalocyanine dendrimer through the micelle encapsulation at an excitation wavelength with clinical relevance (~600 nm).

2. Materials and methods

2.1. Materials

N^\text{ω}-Z-L-lysine and bis(trichloromethyl) carbonate (triphosgene), for the synthesis of polyethylene glycol-block-poly-L-lysine (PEG-b-PLL), were purchased from Tokyo Kasei Co., Ltd. α-Methoxy-ω-amino-poly(ethylene glycol) (MeO-PEG-NH₂) was a kind gift from Nippon Oil and Fats Co., Ltd. Chemicals for dendrimer synthesis were purchased from Tokyo Kasei Co., Ltd. or Aldrich Chemical Co., Ltd. Tetrahydrofuran and hexane, used as a solvent for the synthetic reactions, were distilled from sodium benzophenone ketyl under Ar just before use. n-Pentanol and 1,8-diazabicyclo-(5,4,0)-undec-7-ene (DBU) for phthalocyanine dendrimer synthesis, were used as received without further purification.

2.2. Synthesis of block copolymer

MeO-PEG-NH₂ was precipitated in diethylether from chloroform, dried under reduced pressure and subsequently freeze-dried from benzene prior to use in the block copolymer synthesis. PEG-b-PLL was synthesized by a previously reported procedure [17]. Briefly, the N-carboxy anhydride of N^\text{ω}-Z-L-lysine was polymerized by initiation with CH₂O-PEG-NH₂ (12,000 g/mol) in DMF under Ar, followed by deprotection of the Z group.GPC measurement of PEG-b-PLL exhibited single sharp peak at Mw of 16,600 and Mn of 16,300 based on PEG standards. From the ^1H NMR measurement in D₂O, the polymerization degree of the PLL segment was determined to be 39.

2.3. Synthesis of dendrimer phthalocyanine

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

Polyion complex micelles were made from charged DPcZn with PEG-b-PLL. In a typical procedure, the PEG-b-PLL was dissolved in an aqueous NaH2PO4 solution and added to an aqueous solution of DPcZn in Na2HPO4 to give a solution containing polyion complex micelles. The ratio of positive charge to negative charge was fixed at 1:1.

2.5. Measurements

The DLS measurements were performed using a Photal dynamic laser scattering DLS-7000 spectrometer (Otsuka Electronics Co., Ltd., Osaka, Japan) equipped with GLG3050 488 nm Ar laser (NEC Co., Ltd., Japan) and/or Zetasizer Nano ZS-90 (Malvern Co., Ltd., USA) with 532 nm laser irradiation. The UV–Vis and fluorescence spectra were measured using a V-550 spectrophotometer (JASCO, Tokyo, Japan) and Type 850 spectrofluorometer (Hitachi, Tokyo, Japan), respectively.

MALDI-TOF-MS was performed on a Bruker model Protein TOF mass spectrometer with dithranol as the matrix. 1H NMR spectroscopy was performed in CDC13 or D2O on a JEOL GSX-270 spectrometer operating at 270 MHz. GPC was performed with TOSOH HLC-8220 equipped with TSK-gel G4000HHR and G3000HHR column (eluent: DMF + 10 mM LiCl, temperature: 40 °C, detector: RI).

2.6. Oxygen consuming measurement

The oxygen consumption amount was measured using a Clark-type oxygen microelectrode with a tip diameter of 200 μm (PO2-100DW, Eikou Kagaku Co., Ltd., Tokyo, Japan). The microelectrode was inserted into the PBS, which contained 3.13 μM of DPcZn or DPcZn/m and 10% FBS as a singlet oxygen acceptor, so that the tip was 100 μm above the bottom of the solution. Semiconductor laser light (660 nm; FWHM 6 nm, 25 mW/cm²) was used for light irradiation. The solution was static and exposed to the atmosphere. Before each measurement, the system was calibrated in saline bubbled with air, in which the partial oxygen pressure was assumed to be 150 mm Hg.

2.7. Cell culture

HeLa cells were used in the cell culture studies. In the cytotoxicity assay, different concentration of DPcZn or DPcZn/m in Dulbecco’s modified Eagle’s medium (DMEM +10% FBS) were added to cells in 96-well culture plates (n=4). After a 24 h incubation at 37 °C, the photosensitizers were removed, and then plates were photoirradiated for 15–60 min with broad-band visible light using a halogen lamp (150 W) equipped with a filter passing light of 400–700 nm (fluence energy: 27–107 kJ/m²). The viability of the cells was evaluated using mitochondrial respiration via the 3-(4,5-dimethyl thiazole-2-yl)-2,5-diphenyltetrazolium bromide cleavage assay (MTT assay) following incubation for 48 h after photoirradiation or removing the photosensitizers by washing in the case of the dark toxicity investigation.

2.8. Cellular uptake amount

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

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

The synthesis of the ionic dendrimer phthalocyanine was accomplished by the method of Ng’s group [15]. The second generation of dendritic phenol was reacted with 4-nitrophthalonitrile by an alkali-mediated coupling reaction to obtain the corresponding dendritic phthalonitrile, which was then treated with Zn(OAc)$_2$ and DBU in n-pentanol to give dendrimer phthalocyanine. Each step of synthesis was characterized by MALDI-TOF-MS and $^1$H NMR measurement, and reaction yields were almost comparable to the literature. The dendrimer phthalocyanine thus obtained was treated with a THF/H$_2$O mixture solution of NaOH to obtain ionic dendrimer phthalocyanine (DPcZn; Fig. 1). DPcZn exhibited significantly high solubility at various pHs of the aqueous medium (over pH 4.3).

A cationic block copolymer (poly(ethyleneglycol)-block-poly-l-lysine: PEG-b-PLL; Fig. 1) was synthesized by the polymerization of the N-carboxy anhydride of N$^\epsilon$-Z-L-lysine, initiated by ω-aminated poly(ethyleneglycol) (CH$_3$O-PEG-NH$_2$:12,000 g/mol) in DMF, followed by deprotection of the Z group according to a previously reported method [17]. The degree of polymerization was determined to be 39, which was confirmed by $^1$H NMR. GPC measurement exhibited single sharp peak and relatively small molecular weight value compare to $^1$H NMR result because of the interaction between PLL segment and GPC column.

Polyion complex micelles were prepared from negatively charged DPcZn with oppositely charged PEG-b-PLL. In a typical procedure, the PEG-b-PLL (14.2 mg) was dissolved in an aqueous NaH$_2$PO$_4$ (10 mM, 6.15 mL) and added to a solution of DPcZn (5 mg) in aqueous Na$_2$HPO$_4$ (10 mM, 13.85 mL) to give a solution containing polyion complex micelles encapsulating ionic DPcZn (Fig. 2). The ratio of positive charge to negative charge was fixed at 1:1. After mixing the two solutions, the pH of the solution becomes 7.3 (10 mM PBS). The resulting micelle has a diameter of ca. 50 nm with a narrow size distribution (unimodal, $\mu^2/\Gamma^2=0.12$), determined by a dynamic light scattering measurement (Zetasizer Nano ZS-90, Malvern Co., Ltd., USA) (Fig. 2). Furthermore, the diffusion coefficient of the resulting micelle was independent of the detection angle of the DLS measurement, suggesting that the polyion complex micelle of DPcZn with PEG-b-PLL is a narrowly dispersed spherical assembly.

3.2. Electronic absorption of dendrimer and micelle

Electronic absorption spectra of the dendrimer and micelle were measured (Fig. 3). DPcZn exhibits B band absorption at 
350 nm and strong Q band absorption at 685 nm, indicating successful dispersion as a monomeric species in the aqueous solution [16]. According to the formation of the polyion complex micelle, the absorption maximum of Q band absorption was slightly changed to 630 nm, indicating the possibility of slight aggregate formation of the core phthalocyanine units. Also, fluorescent intensity of DPcZn was drastically decreased by inclusion into the micelle (data not shown). The relatively small dendritic wedges may not perfectly prevent the aggregate formation of the phthalocyanine core units especially in the densely packed micellar core. Note that DPcZn (Mw=4901) is smaller than that of previously reported ionic dendrimer porphyrin (Mw=8029).

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

3.3. Oxygen consumption ability of the dendrimer phthalocyanine and micelle

The oxygen consumption amount was measured to evaluate ROS generation under photoirradiation [18]. Note that the DPcZn/m was sufficiently stable in the 10 mM PBS with 10% FBS, where the size and polydispersity of DPcZn/m in the 10 mM PBS with 10% FBS were almost comparable to those without FBS (data not shown). The oxygen partial pressure (PO2) of DPcZn solution is significantly reduced by the irradiation of laser light (660 nm; FWHM 6 nm, 25 mW/cm2) (Fig. 4). Although the consumption ability of DPcZn/m was lower than that of DPcZn, the PO2 of DPcZn/m solution was also effectively reduced, indicating that either DPcZn or DPcZn/m can take part in the photochemical reaction to generate ROS. On the other hand, either DPcZn or DPcZn/m solution without 10%
FBS shows almost negligible change in the PO2 upon the photoirradiation, indicating that the proteins in FBS act as sacrificial acceptors of ROS. In other words, if proteins do not exist in the medium, once generated, ROS promptly revert to oxygen molecules because of their short lifetimes.

3.4. Cytotoxicity of dendrimer phthalocyanine and micelle

The cytotoxicity of phthalocyanine dendrimer was assessed against HeLa cells (Fig. 5). The viability of cells upon photoirradiation was evaluated by MTT assay and determined to be a function of concentration and photoirradiation time with DPCZn and its micelle. DPCZn and DPCZn/m were incubated with the cells for 24 h and then fully washed with PBS to remove non-associated photosensitizers prior to photoirradiation. However, either DPCZn or DPCZn/m exhibited photoinduced cytotoxicity upon the photoirradiation, where the cells were photoirradiated for 15–60 min with broad-band visible light using a halogen lamp (150 W) equipped with a filter passing light of 400–700 nm (fluence energy: 27–107 kJ/m2). According to the exposure time increase, either DPCZn or DPCZn/m exhibited an increase in photocytotoxicity (Fig. 5).

Very interestingly, the aspect of the photocytotoxicity increase is significantly different between DPCZn and DPCZn/m. As shown in Fig. 6, DPCZn exhibits a relatively small time-dependency, whereas DPCZn/m exhibits a remarkable change in the cell viability depending on the photoirradiation time. Typically at 60-min photoirradiation, DPCZn/m exhibited almost 100 times higher photocytotoxicity than free DPCZn. Although electronic absorption and oxygen consumption behaviours exhibited quenching signature, DPCZn/m have significantly high PDT efficacy compare to DPCZn alone. On the other hand, the cell viability exhibits abnormal increase with increase in the concentration of DPCZn/m at the 15-min light irradiation. There is various reasons can be considerable such as compositional change of micellar structure or microenvironment change around photosensitizers. To understand this phenomenon, we need further investigation.

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

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

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

4. Conclusions

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

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References


