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Preparation of a water-soluble fluorinated zinc phthalocyanine and its effect for photodynamic therapy

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Abstract

An amphiphilic fluorinated phthalocyanine, zinc tetracarboxyoctafluorophthalocyanine (ZnC_4F_8Pc) was synthesized and characterized. Its photodynamic efficiency for HeLa cells was compared with hydrophilic zinc octacarboxyphthalocyanine (ZnC_8Pc) and hydrophobic zinc hexadecafluorophthalocyanine ($ZnF_{16}Pc$). ZnC_4F_8Pc had a remarkable photodynamic effect among the phthalocyanines used. The effect is apparently caused by the fact that ZnC_4F_8Pc is mainly accumulated in the hydrophobic lipid membrane and is in the photoactive monomer form in HeLa cells.

Keywords: Phthalocyanine; Photodynamic therapy

1. Introduction

For clinical photodynamic therapy (PDT), the photosensitizers such as hematoporphyrin derivatives (HpD) and Photofrin have been used exclusively to date [1]. However, as these compounds absorb light of relatively short wavelength, and the penetration depth of short-wavelength light in tissue is relatively shallow, the benefits of current PDT are limited to shallow tissues only [2,3]. Therefore, compounds with absorption bands of longer wavelength are more adequate for PDT. Since phthalocyanines exhibit advantageous photophysical properties for PDT such as photostability, long lifetimes of the photoexcited triplet state, and high molar absorption in the red region of the visible spectrum, they may be quite advantageous as photosensitizers [4,5].

Zinc hexadecafluorophthalocyanine ($ZnF_{16}Pc$) is a hydrophobic compound and it selectively accumulates in tumors [6]. However, its photodynamic effect is quite low [6]. Although the reason for its low photodynamic effect is not clarified yet, it is known that aggregated compounds decrease photodynamic activities suggesting that the phthalocyanines may be aggregated in cells. With the aim of developing a compound that has the beneficial characteristics of phthalocyanines as well as adequate photodynamic effect, zinc tetracarboxyoctafluorophthalocyanine (ZnC_4F_8Pc, see Scheme 1) was synthesized in this study. To prevent the potential aggregation condition, the number of fluoro groups is limited, and to increase hydrophilicity carboxyl groups are bonded. Uptake of the compound in tumor cells was measured and the relationship of its photodynamic efficiency and hydrophobicity are discussed.

2. Materials and methods

2.1. Materials

Tetrafluorophthalonitrile (TFP) was from Tokyo Kasei Kogyo (Tokyo, Japan). Minimal essential medium (MEM) was from Gibco-BRL (Burlington, Canada), 1,3-diphenylisobenzofuran (DPBF) was from Aldrich (Milwaukee, WI, USA), fetal bovine serum (FBS) from J.R.H. Biosciences (Lenexa, KS, USA), and 3-(4,5-dimethyl-2-thiazolyl)-2-5-diphenyl-2H-tetrazolium bromide (MTT) was purchased from Dojindo Lab. (Kumamoto, Japan). Other materials were of the highest grade available.

ZnC_4F_8Pc was synthesized as described previously [7] with some modifications. The synthesis route is shown in Scheme 1. Briefly, a mixture of dicyanophthalimide (300 mg, synthesized according to the literature [8]), TFP (1.0 g) and zinc chloride (410 mg) was refluxed in 1-chloronaphthalene (24 ml) for 5 h at 250°C. The black precipitate

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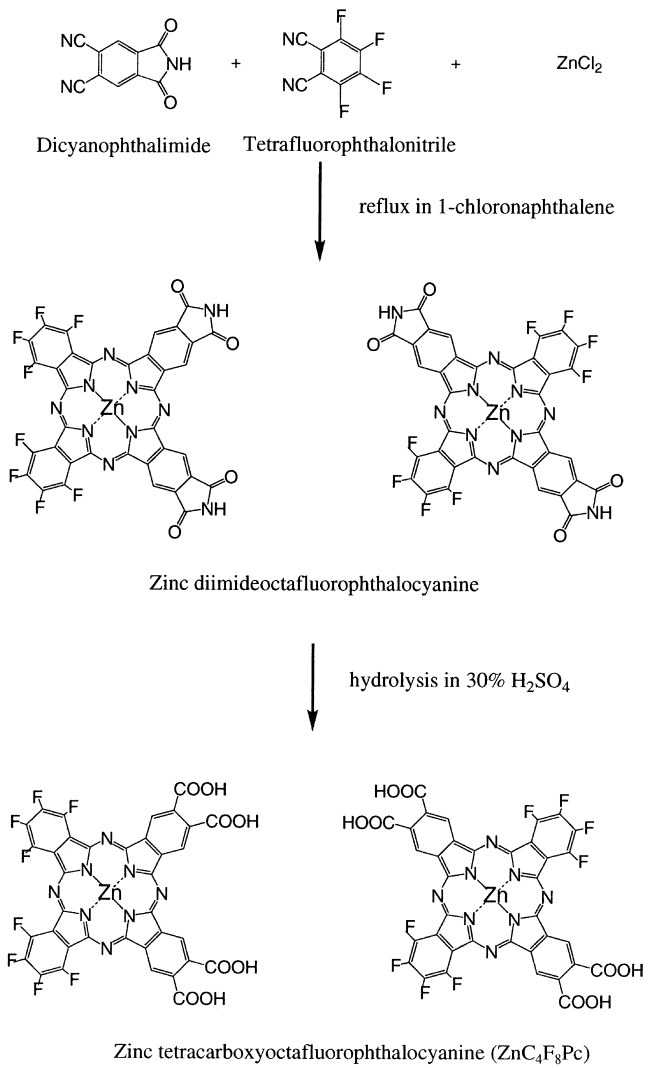

Scheme 1. Synthesis route of zinc tetracarboxyoctafluorophthalocyanine (ZnC_4F_8Pc).

product was washed with hexane, benzene and water and then dried. By-products were removed using a silica column. ZnC_4F_8Pc was obtained by hydrolysis of zinc diimideoctafluorophthalocyanine. Zinc diimideoctafluorophthalocyanine was hydrolyzed with 30% sulfuric acid at 100°C for 1 h, washed with water, dissolved in 0.1 mol/l NaOH, and then filtered. ZnC_4F_8Pc in the supernatant was precipitated by the addition of hydrochloric acid, and the precipitate was washed with water and then dried. The synthesized ZnC_4F_8Pc was characterized by ^{1}H-nuclear magnetic resonance (NMR) (VXR-300, Varian) and fast atom bombardment mass spectrometry (FAB-MS) (JEOC JMS-700 mass spectrometer equipped with FAB ionization). Other phthalocyanines: zinc hexadecafluorophthalocyanine ($ZnF_{16}Pc$), zinc octacarboxyphthalocyanine (ZnC_8Pc) and zinc tetrasulfophthalocyanine (ZnS_4Pc), were synthesized according to the literature [9–11].

2.2. Cells

HeLa cells were kindly provided by Professor S. Sato of Jikei University Hospital, Japan. They were grown in MEM supplemented with 10% FBS and antibiotics.

2.3. Measurement of singlet oxygen

Singlet oxygen measurement was carried out by a DPBF decomposition reaction [12]. Phthalocyanines (1.0×10^{-7} mol dm^{-3}) and DPBF (5.0×10^{-5} mol dm^{-3}) were dissolved in pyridine, transferred to a glass tube in the dark, and then irradiated. As the light source a halogen lamp (Toshiba 425WN-EH, 500 W) was used. Light of wavelength less than 500 nm was excluded by a HOYA Y-50 filter. A decrease of DPBF concentration was followed by an absorbance at 415 nm.

2.4. Measurement of lipid affinity of phthalocyanines

The lipids were extracted from mouse liver with chloroform–methanol (1:2, v/v). Quantification of the lipid was carried out according to the literature [13]. Lipid affinity of the phthalocyanines was estimated as the accumulated amount of phthalocyanines in the lipids. Phthalocyanines in methanol (1.0×10^{-6} mol, 1 μl) was added to lipid (0.6 mg) in 1 ml of phosphate-buffered saline (PBS) for 2 h. Then hexane (3.0 ml) was added to extract the lipid and the corrected hexane layer. After the evaporation of the solvent, the remaining solid was dissolved in a chloroform–pyridine (1:10, v/v) solvent mixture for fluorescence measurement. To quantify the amount of phthalocyanines the following excitation and detection wavelengths were selected

$ZnF_{16}Pc$	Excitation: 614 nm, detection: 687 nm
ZnC_4F_8Pc	Excitation: 620 nm, detection: 700 nm
ZnC_8Pc	Excitation: 681 nm, detection: 720 nm

2.5. Phthalocyanine uptake in HeLa cells

Cellular phthalocyanine uptake was determined by fluorescence spectroscopy. Cells were first seeded (6.0×10^5) onto 6-cm tissue culture dishes in 4 ml of MEM containing 10% FBS, then incubated at 37°C overnight. After washing twice with PBS, the cells were incubated in the dark at 37°C for 2 h with 2 ml of MEM containing 1.0×10^{-6} mol/l phthalocyanine. The cells were washed with cold PBS, and harvested using a cell scraper. After centrifugation, cells were sonicated in pyridine to extract the phthalocyanine. The concentration of phthalocyanine in the extract was determined by fluorescence measurement using a fluorescence spectrometer (F-4010; Hitachi, Japan). The excitation and emission wavelengths are described

above. Protein concentrations were determined by the method of Lowry et al. [14].

2.6. Detection of lipid peroxide

The lipid peroxide formed in HeLa cells by lipid photooxidation with the accumulated phthalocyanine was analyzed by the TBA method [15]. 1,1,3,3-Tetraethoxypropane was used for standard. Fluorescence intensity of thiobarbituric acid reactive substance (TBARS) was monitored at 545 nm for detection and 515 nm for excitation.

2.7. Fluorescence of phthalocyanine in HeLa cells

As aggregated phthalocyanine has a very low fluorescence quantum yield, fluorescence intensity of the cell may relate to the phthalocyanine monomer concentration. HeLa cells (9.0×10^5) were incubated with 2 ml MEM containing 1.0×10^{-5} mol/l phthalocyanine for 2 h at 37°C. After incubating and washing the cells with saline, they were harvested using a cell scraper and suspended in MEM. To measure fluorescence spectra, excitation wavelengths of 614 nm for $ZnF_{16}Pc$, and 620 nm for ZnC_4F_8Pc were used. There was no absorption from cellular components in the 600–620 nm range.

2.8. Photocytotoxicity

HeLa cells (5×10^3 cells per well) were plated in 200 μl MEM containing 10% FBS, and incubated overnight (37°C, 5% CO_2) in a 96-well plate. The medium was then removed and the cells were incubated for 2 h with 100 μl of various concentrations of phthalocyanines in MEM. The cells were then washed with PBS, and 200 μl of MEM containing 10% FBS was added, and exposed to light from 500-W halogen lamps (Toshiba 425WN-EH, equipped with a water filter and a Y-50 cut-off filter) for 8 min. The total fluorescence was 31 J/cm^2. Cell survival was assessed by measurement of MTT reduction at 20 h after irradiation. The MTT assay was carried out as described by Carmichal et al. [16].

3. Results and discussion

3.1. Characterization of synthesized phthalocyanines

The molecular structure of the synthesized ZnC_4F_8Pc was characterized by ^{1}H-NMR (data not shown). The analysis indicated that the compound exists as a mixture of two isomers (see Scheme 1). FAB-MS measurement showed the molecular mass of the compound to be 828, which is attributed to demetallated ZnC_4F_8Pc. Atomic absorption spectroscopic measurement confirmed that ZnC_4F_8Pc contains one zinc atom per molecule.

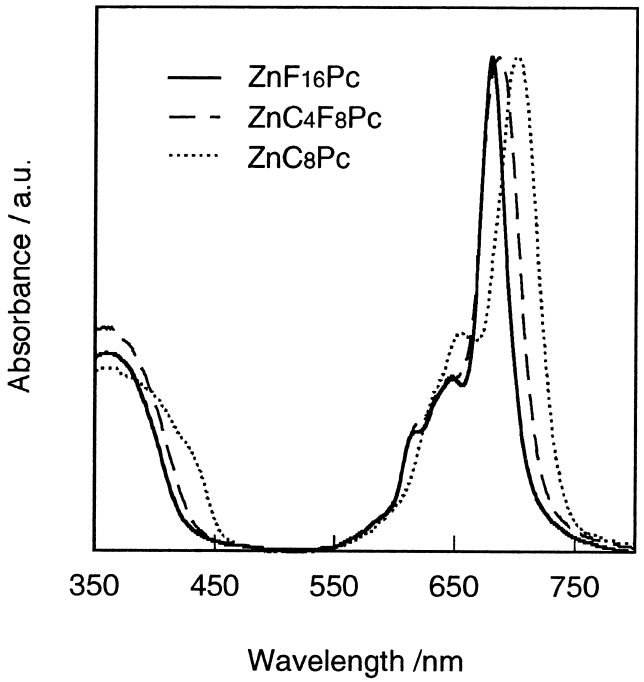

Fig. 1. Absorption spectra of $ZnF_{16}Pc$, ZnC_4F_8Pc and ZnC_8Pc in pyridine. The concentration of zinc phthalocyanine was 2.3×10^{-6} mol/l.

The absorption spectra and the fluorescence spectra of the phthalocyanines are shown in Fig. 1.

Singlet oxygen production is significantly related to the photodynamic activity. Photosensitizers that form singlet oxygens are fundamentally important for photoinduced reaction processes. Therefore, singlet oxygen production was estimated by measuring the decomposition of DPBF. Time dependence of DPBF decomposition is shown in Fig. 2. ZnC_4F_8Pc was more active than ZnC_8Pc and ZnS_4Pc, but less active than $ZnF_{16}Pc$, showing that fluorinated compounds have higher activity for photoinduced singlet oxygen production.

Lipid affinity was estimated by the accumulation of phthalocyanines in the lipid. Hydrophilicity of the photosensitizers is also important for cellular uptake. As shown in Table 1, fluorinated phthalocyanines, $ZnF_{16}Pc$ and ZnC_4F_8Pc, accumulated in the lipid, whereas nonfluorinated ZnC_8Pc did not, showing that the fluorinated phthalocyanines contain hydrophobic regions.

3.2. Effects of organic solvents on the aggregation of phthalocyanines

Phthalocyanines easily aggregate in water, while aggregated phthalocyanine dissociates in hydrophobic surroundings [17,18]. The aggregation degree of phthalocyanines is highly affected by the surrounding conditions, especially organic solvents. Fig. 3 shows the absorption spectra of zinc phthalocyanines in various organic solvents. The concentration of zinc phthalocyanine was 2.3×10^{-6} mol/l in all solvents. The absorption spectra of ZnC_4F_8Pc and

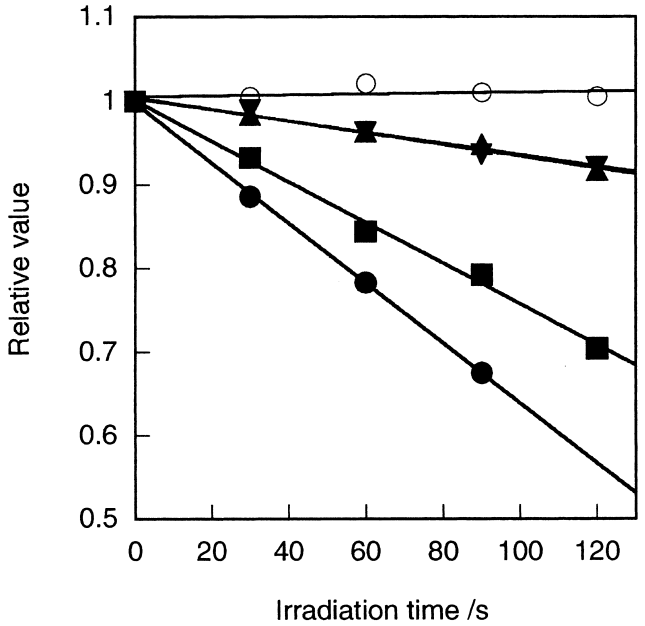

Fig. 2. Degradation of DPBF by singlet oxygen generated from ZnC_4F_8Pc (■), $ZnF_{16}Pc$ (●), ZnC_8Pc (▲), ZnS_4Pc (▼) and control (○). Phthalocyanine (1.0×10^{-7} mol/l) and DPBF (5.0×10^{-5} mol/l) were irradiated in pyridine.

$ZnF_{16}Pc$ in hydrophilic solvent such as methanol shows a strong absorption peak around 630 nm, which indicates phthalocyanine aggregation [19]. In hydrophobic surroundings, absorption at 670 nm of monomeric phthalocyanines increased. In both phthalocyanines absorption by monomeric phthalocyanines increased with hydrophobicity. The aggregation degree of $ZnF_{16}Pc$ was larger than that of ZnC_4F_8Pc. From the absorption spectra results, it seems that aggregation of ZnC_4F_8Pc can be prevented by controlling the number of fluoro groups in the molecule and adding hydrophilic carboxyl groups.

3.3. Uptake and dissociation of phthalocyanines in HeLa cells

Fig. 4 shows phthalocyanine uptake in HeLa cells. HeLa cells were incubated with various concentrations of phthalocyanines at 37°C. After 2 h of incubation, phthalocyanine concentrations were measured. Cellular phthalocyanine uptake increased with phthalocyanine concentration. Fluorinated phthalocyanines such as ZnC_4F_8Pc and $ZnF_{16}Pc$ accumulated in HeLa cells more than the non-fluorinated phthalocyanine, ZnC_8Pc. As fluorinated phthalocyanines are hydrophobic compounds, they may favor the hydrophobic environment [20].

Table 1
Affinity of zinc phthalocyanines against lipid[a]

Phthalocyanine	Phthalocyanine concentration (10^{-10} mol/mg lipid)
$ZnF_{16}Pc$	1.9±0.32
ZnC_4F_8Pc	0.64±0.05
ZnC_8Pc	~0

[a] 1×10^{-6} mol of phthalocyanines were contacted with 0.6 mg of lipid.

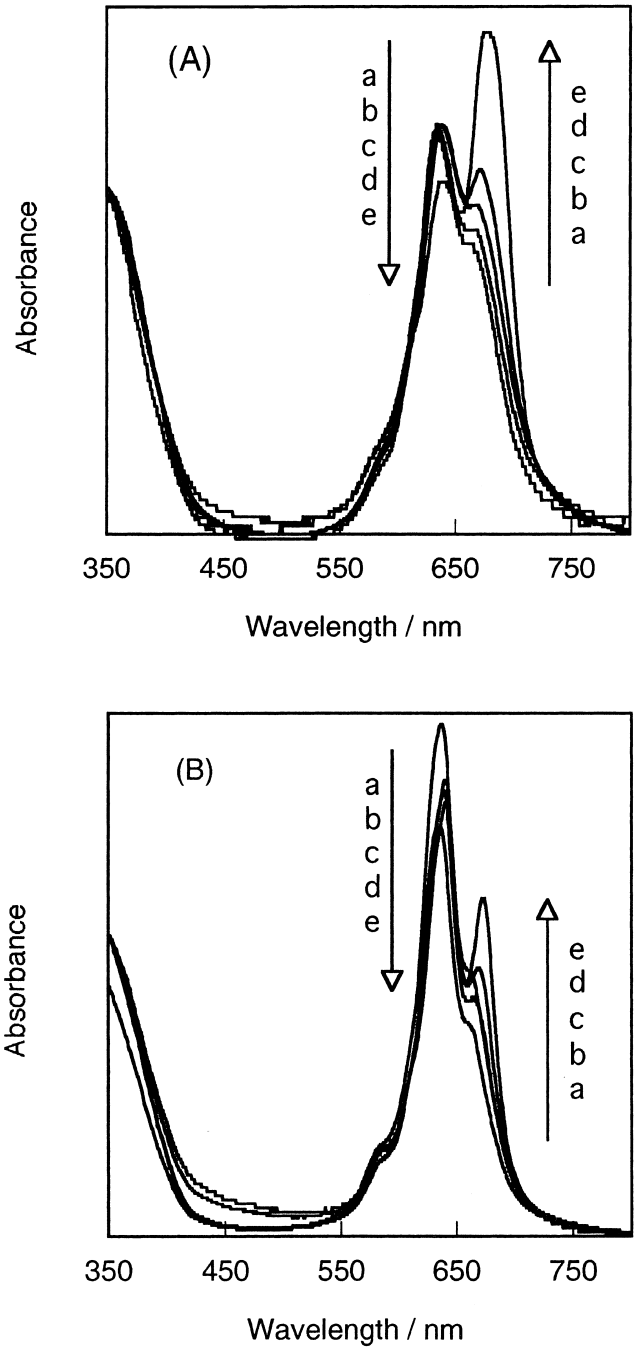

Fig. 3. Absorption spectra of ZnC_4F_8Pc (A) and $ZnF_{16}Pc$ (B) in methanol (a), ethanol (b), butanol (c), hexanol (d) and octanol (e). The concentration of zinc phthalocyanine was 2.3×10^{-6} mol/l in all solvents.

Phthalocyanines easily aggregate, and aggregated phthalocyanine does not serve as a photosensitizer [17,18]. Although aggregated phthalocyanines do not fluoresce, phthalocyanines in their monomeric forms do. Despite the fact that $ZnF_{16}Pc$ in HeLa cells more than ZnC_4F_8Pc, ZnC_4F_8Pc emitted fluorescence in the cells, whereas

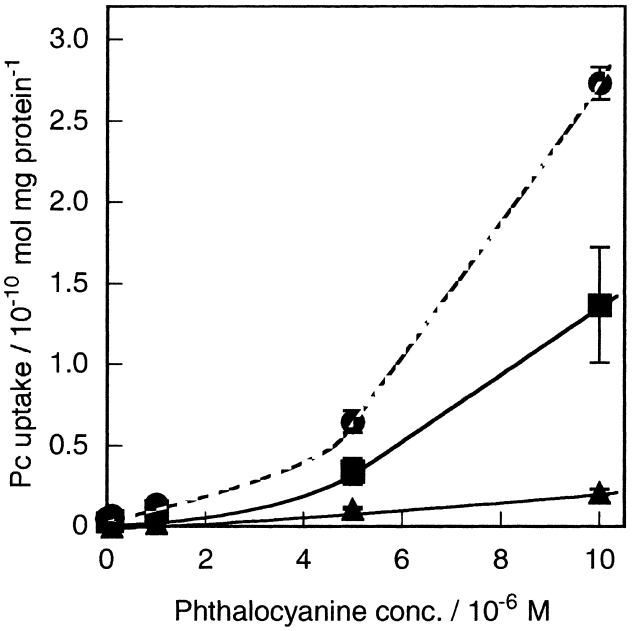

Fig. 4. Cellular uptake of phthalocyanines. Cells were incubated with ZnC_4F_8Pc (■), ZnF16Pc (●) and ZnC_8Pc (▲) for 2 h. Bars represent S.D.s of the means of three replicate experiments.

$ZnF_{16}Pc$ did not (Fig. 5), showing that ZnC_4F_8Pc was in the monomer form and $ZnF_{16}Pc$ was aggregated.

3.4. Formation of lipid peroxide

The formation of lipid peroxide is one of the important factors indicating cell membrane damage. As shown in Fig. 6, ZnC_4F_8Pc displayed remarkably high lipid peroxide formation, suggesting that this hydrophobic phthalocyanine accumulated in the hydrophobic cell membrane.

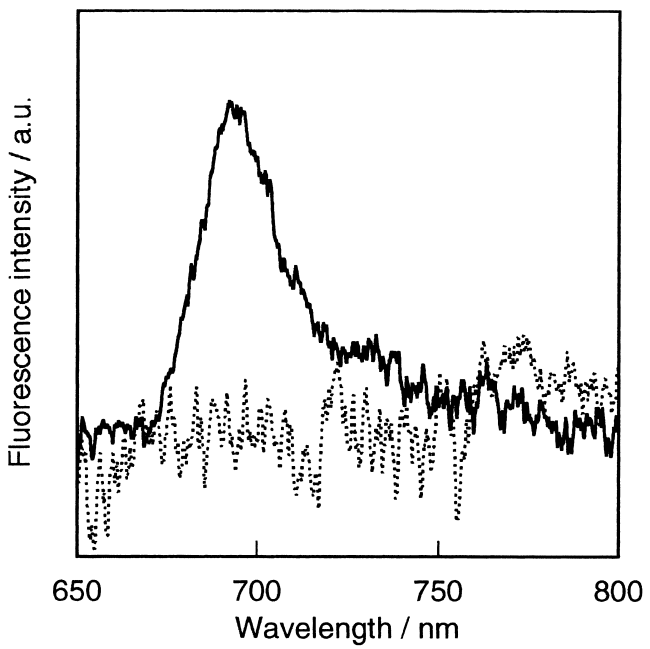

Fig. 5. Fluorescence spectra of ZnC_4F_8Pc (solid line) and $ZnF_{16}Pc$ (broken line) in HeLa cells. Cells were incubated with 1.0×10^{-5} mol/l of each phthalocyanine for 2 h. Excitation wavelengths were 620 nm for ZnC_4F_8Pc and 614 nm for $ZnF_{16}Pc$.

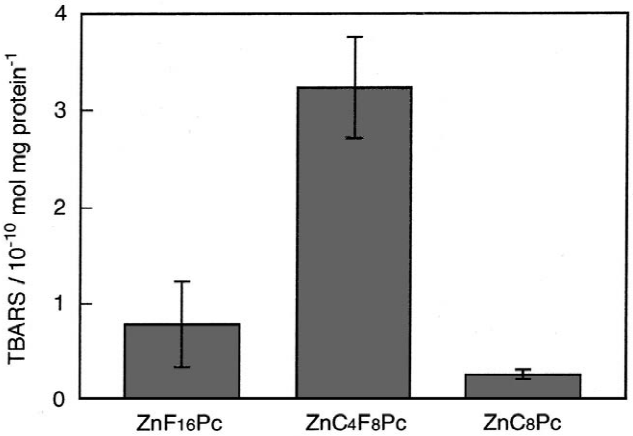

Fig. 6. Measurement of lipid peroxidation by phthalocyanines. Cells were incubated with 1.0×10^{-6} mol/l of phthalocyanines and irradiated. The TBARS were normalized to the total cellular protein content. Bars represent S.D.s of the means of three replicate experiments.

3.5. Photodynamic effect of phthalocyanines

In the presence of each phthalocyanine, cell survival decreased following irradiation and remained the same without irradiation (data not shown). Among the phthalocyanines used, ZnC_4F_8Pc showed the highest photodynamic effect (Fig. 7). As photodynamic damage occurs near the site where the photosensitizer is uptaken by the cell, ZnC_4F_8Pc may locate in the hydrophobic site in tumor cell. Thus, the difference in photodynamic efficiency of phthalocyanines was apparently influenced by the dissociation degree of the sensitizer, and the phthalocyanine localization site in the cells.

4. Abbreviations

DPBF	1,3-Diphenylisobenzofuran
FAB-MS	Fast atom bombardment mass spectrometry
FBS	Fetal bovine serum
HpD	Hematoporphyrin derivative
MEM	Minimal essential medium
MTT	3-(4,5-Dimethyl-2-thiazolyl)-2-5-diphenyl-2H-tetrazolium bromide
NMR	Nuclear magnetic resonance
PBS	Phosphate-buffered saline
PDT	Photodynamic therapy
TBARS	Thiobarbituric acid reactive substance
TFP	Tetrafluorophthalonitrile
$ZnF_{16}Pc$	Zinc hexadecafluorophthalocyanine
ZnC_8Pc	Zinc octacarboxyphthalocyanine
ZnC_4F_8Pc	Zinc tetracarboxyoctafluorophthalocyanine

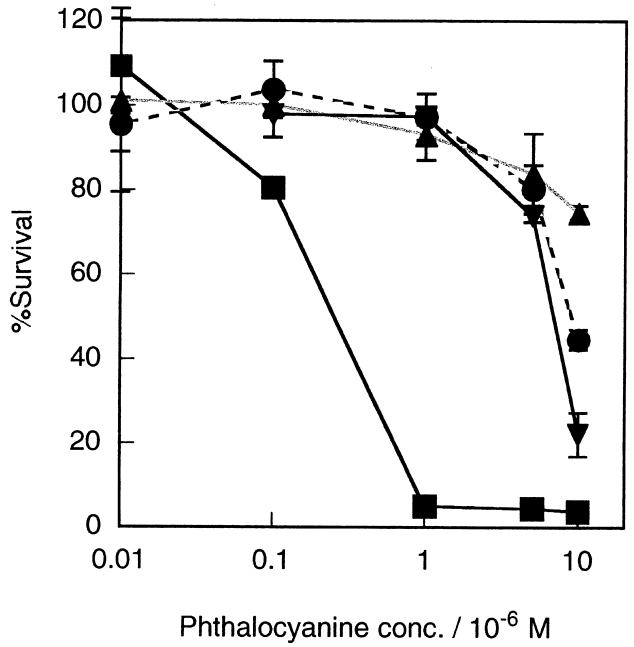

Fig. 7. Photocytotoxicity of phthalocyanines. Cells were incubated with ZnC_4F_8Pc (■), $ZnF_{16}Pc$ (●), ZnC_8Pc (▲), ZnS_4Pc (▼) for 2 h and irradiated (>500 nm, 31 J/cm^2). Bars represent S.D.s of the means of three replicate experiments.

ZnS_4Pc Zinc tetrasulfophthalocyanine

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