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Size and Surface Charge Effect of 5-Aminolevulinic Acid-Containing Liposomes on Photodynamic Therapy for Cultivated Cancer Cells

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ABSTRACT 5-Aminolevulinic acid (ALA)-containing liposomes having various average diameters and/or positive surface charges were prepared, and their photodynamic therapy (PDT) efficacy for murine thymic lymphoma cells, EL-4 cells, cultivated in vitro was investigated. The PDT efficacy for EL-4 cells and the accumulation of ALA-induced protoporphyrin IX (PpIX) in the cells increased with a decrease in the average diameter of liposomes. In particular, the ALA-containing liposomes smaller than 63.5 nm in diameter promoted the PDT efficacy in comparison with that of ALA alone. We also found no significant changes in PDT efficacy and PpIX accumulation with increasing positive surface charges of liposomes.

KEYWORDS Photodynamic therapy, 5-Aminolevulinic acid, Liposome, Cancer cell, PDT efficacy, Accumulation of protoporphyrin IX

INTRODUCTION

Photodynamic therapy (PDT) involves the administration and localization of a photosensitizing agent to cancer tissues, followed by the activation of the agent by light with a specific wavelength. The agent then converts the energy to molecular oxygen, which results in the formation of cytotoxic singlet oxygen. This therapy leads to a sequence of photochemical and photobiological processes that cause irreversible photodamage to cancer tissues. PDT is one of the most useful cancer therapies because of its few side effects and minimal invasiveness, thus leading to an improvement in the "quality of life (QOL)" of patients (Dougherty et al., 1998; Kurwa & Barlow, 1999).

Recently, clinical PDT treatment for cancer tissues using 5-aminolevulinic acid (ALA) has attracted much attention, and many studies have been reported on ALA-based PDT (ALA-PDT) (Ahmadi et al., 2004; Nadeau et al., 2004; Pollock et al., 2004; Uzdensky et al., 2004; van den Akker et al., 2003; Wu et al., 2003; Zhang & Zhang, 2004; Ziolkowski et al., 2004). Although ALA itself has no photosensitizing action, it is useful for PDT. When an excess

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amount of ALA is administered to cells, an endogenous photosensitizer, protoporphyrin IX (PpIX), is formed and temporarily accumulated in the cells (Lopez et al., 2004). However, preliminary clinical studies point out that oral administration of ALA over 20 mg/kg B.W. induces nausea and an elevation of blood liver enzyme level within 24 hours because of temporary liver dysfunction (Tope et al., 1998). Therefore, a method to enhance PDT efficacy without increasing ALA dosage is needed.

Liposomes are defined as structures composed of lipid bilayer membranes with an inner core of water phase. They can incorporate water-insoluble compounds in the lipid bilayers and water-soluble compounds in the inner core (Abe et al., 1995). Liposomes are widely used as a drug carrier, a model for biomembranes, etc., and have been studied pharmaceutically, biochemically, and physicochemically. Liposomes are of particular interest as a nanoparticle carrier to deliver pharmacologically active agents, for example, drugs, genes, enzymes, etc., to cells, because they can improve the toxicity and stability of pharmacologically active agents. It is well known that polyethyleneglycol-coated liposomes (PEG-liposomes) are not taken up by macrophages in the reticuloendothelial system (RES) and hence stay in circulation for a relatively long period of time (Kaneda, 2000). To enhance tissue targeting, in addition, liposome surfaces have been modified with those antibodies and/or ligands that are recognized by specific cell characteristics (Kaneda, 2000). Thus, we predict that the uptake of ALA into cancer tissues can be promoted by using ALA-containing liposomes.

The size and surface charge of liposomes play an important role in promoting the association and interaction of liposomes with cells, leading to the subsequent liposome uptake (Cullis, 2000; Dan, 2002; Kawahara et al., 2003). In the present study, we prepared ALA-containing liposomes with various particle sizes and/or positive surface charges and examined their effectiveness in ALA-PDT for murine thymic lymphoma cells, EL-4 cells, cultivated in vitro.

EXPERIMENTAL

Materials

L-α-dipalmitoylphosphatidylcholine (DPPC, 99.7%), cholesterol (99%), octadecylamine (ODA, 90.0%), and 5-aminolevulinic acid (ALA, 98%) were purchased from NOF. Co., SIGMA, Kanto Chemical. Co., and Cosmo Bio Co., Ltd., respectively. They were used without further purification. Pyrene (98%) used as a fluorescence probe was purchased from WAKO Pure Chemical Industries, Ltd., and was used after triple extraction with ethanol.

Preparation of Liposomes Containing ALA

ALA-containing liposomes were prepared using Bangham's method (Bangham et al., 1965). A 1.83×10^{-2} g of DPPC and 3.8×10^{-3} g of cholesterol were dissolved in chloroform in a test tube. The molar mixing ratio of DPPC to cholesterol was 7:3. ODA was also added at various molar mixing ratios. The solvent was evaporated under N_2 gas, and a thin lipid film was formed on the wall of the test tube. To remove the residual solvent completely, the test tube was allowed to stand in a desiccator under reduced pressure. After 5 mL of 0.28 mol/L aqueous ALA solution was added into the test tube (concentration of ALA: 5.0×10^{-3} mol/L), the test tube was kept for 5 min at 60°C, then agitated on a vortex mixer for a few minutes to give ALA-containing liposomes. The liposome dispersions were extruded through Millipore filters (Whatman Co.) with a constant pore size (pore diameter of Millipore filters: 100, 160, 200, 400, and 800 nm) with an extruder (Lipex Biomembranes, Inc.) to give homogeneously sized liposome dispersions with different average diameters. Finally, each of the liposome dispersions was dialyzed against physiological saline for 3 hours at 10°C, with physiological saline changed every hour. After 3-hour dialysis, the concentration of free ALA in the outer water phase of the liposome dispersions was measured with the method reported by Okayama (Okayama et al., 1990) to confirm no presence of free ALA in the outer water phase.

Trapping Efficiency of Liposomes for ALA

After ALA-containing liposome dispersions were dialyzed, the liposomes were broken by adding ethanol, and the concentration of ALA was determined with Okayama's method (Okayama et al., 1990).

Trapping efficiency of the liposomes for ALA was estimated by the following equation (see equation below). Here, the concentration of the aqueous ALA solution used for preparing liposome dispersions was 0.28 mol/L as mentioned above.

$$\text{Trapping efficiency} = \frac{\text{Concentration of ALA after dialysis}}{\text{Concentration of the aqueous ALA solution for preparing liposome dispersions}} \times 100(\%)$$

Particle Size Distribution and ζ-Potential of ALA-Containing Liposomes

The particle size distribution and the ζ-potential of the liposomes were measured with a dynamic light scattering measuring apparatus (NICOMP 380ZLS, Particle Sizing Systems, Inc.) and a ζ-potential analyzer (DELSA 440sx, Beckman Coulter, Inc.), respectively.

Microfluidity of Bilayer of ALA-Containing Liposomes

ALA-containing liposomes, the bilayer membranes of which contained 6.0×10^{-3} mol/L of pyrene, were prepared by the same method as that described above. The fluorescence spectra of pyrene in liposome bilayer membranes were measured using a fluorescence spectrophotometer (V-530, JASCO Co., excitation wavelength: 335 nm, emission wavelength: 350–650 nm). Pyrene monomer generally forms excimer by the face-to-face collision above a certain concentration of the probe. Fluorescence peaks at 395 and 473 nm are assigned to pyrene monomer and its excimer, respectively. The excimer/monomer ratio of emission intensity (I_E/I_M) indicates the microfluidity in the surroundings of pyrene. The larger is the I_E/I_M of pyrene in liposome bilayer membranes, the higher the microfluidity of the membranes (Thomas, 1980; Yokouchi et al., 2001).

PDT Efficacy

Murine thymic lymphoma cells, EL-4 cells, were diluted with RPMI 1640 medium containing 10% FBS to give a concentration of 5×10^5 cells/mL. Five milliliters of the diluted EL-4 dispersion was poured into a cell culture dish. The cell dispersion was incubated with a given amount of liposome dispersion and/or ALA alone for an hour at 37°C. Here, the total dose of ALA in any liposome dispersion and ALA systems was constant, 1.2×10^{-2} mol/L. After the incubation, the cell dispersion was irradiated with polychromatic visible light for an hour using a metal halide lamp (wavelength: 550–750 nm, fluence rate: 40 mW/cm^2). The cell dispersion was then incubated with a trypan blue solution, and the number of survival cells was counted. Survival rate was expressed as the percentage of the number of surviving cells against the blank, which is defined as the number of cells survived after being incubated with chemicals, but not exposed to the light. The lower the survival rate, the greater the PDT efficacy becomes.

Accumulation of PpIX in Cells

We also prepared EL-4 cell dispersion with ALA-containing liposomes by the same method as that described above. After the removal of RPMI 1640 medium from the cell dispersion with a centrifugal separator at 1200 rpm for 5 min, Hanks' solution was poured into it, and the fluorescence intensity of PpIX in the resultant cell dispersion was measured with a fluorescence spectrophotometer (V-530, JASCO Co., excitation wavelength: 410 nm, emission wavelength: 635±5 nm). The fluorescence intensity of PpIX accumulated in the cells was estimated from the difference in the fluorescence intensity at 635 nm between samples and the blank with no added chemicals.

RESULTS AND DISCUSSION

PDT Efficacy of ALA-Containing Liposomes with Various Diameters

Figures 1 (a) to (e) show the particle size distributions based on number % (solid line) and volume % (dashed line) of ALA-containing liposomes extruded through Millipore filters with 100, 160, 200, 400, and 800 nm pore diameters, respectively. Using particle size distribution based on number % and

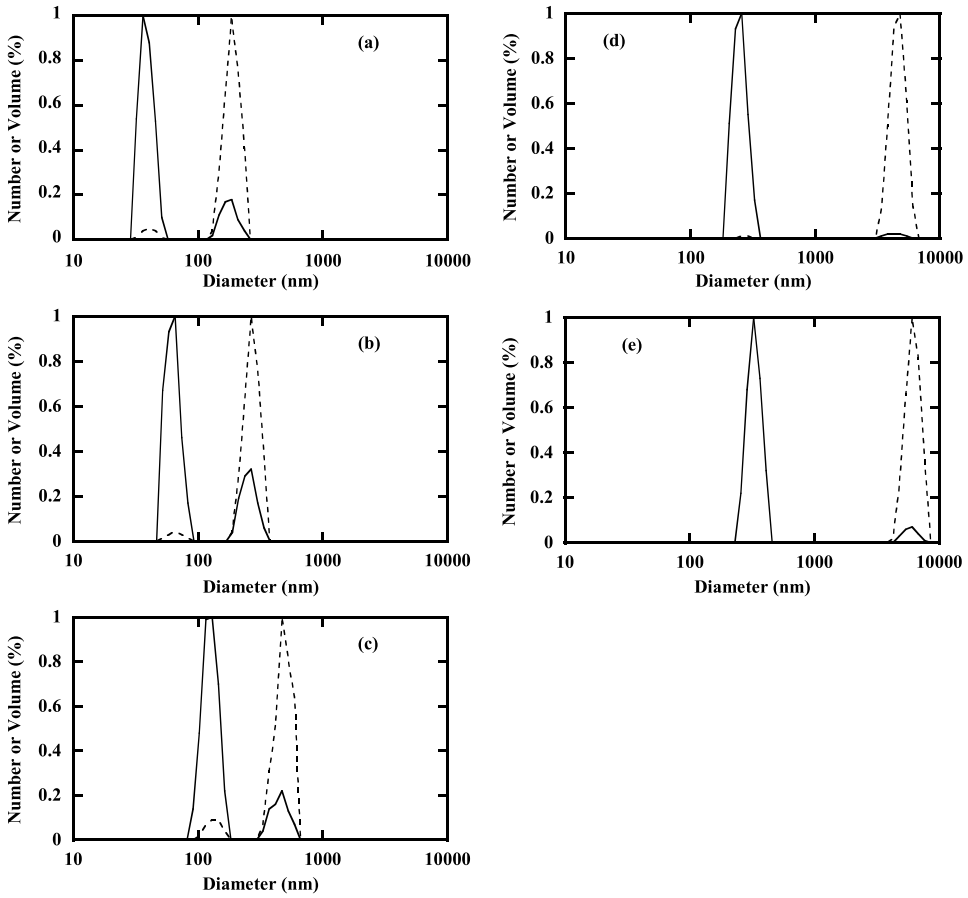

FIGURE 1 **Average Diameter Distributions of ALA-Containing Liposomes Based on Number % (Solid Line) and Volume % (Dashed Line). The Pore Diameters of Millipore Filters: (a) 100, (b) 160, (c) 200, (d) 400, and (e) 800 nm.**

volume %, we can obviously distinguish smaller and larger particle size distributions. As can be seen from these figures, there were two major particle size distributions of the liposomes. The larger particle size distribution would be caused by aggregation and/or coalescence of the smaller particle size liposomes. As a result of the number % based particle size distribution, moreover, the average diameters of the liposomes were (a) 36.8, (b) 63.5, (c) 127.7, (d) 249.7, and (e) 331.4 nm.

Figure 2 depicts changes in the trapping efficiency of liposomes for ALA with their average diameter. The trapping efficiency of liposomes was in the range of 4.5–5.5%. In general, Bangham's method employed in this study yields a multilamellar vesicle type of liposomes, and their trapping efficiency is comparably smaller (a few percentage points) (Szoka & Papahadjopoulos, 1978). The results on trapping efficiency then indicates that we can prepare the liposomes trapping ALA in their inner core of the water phase by Bangham's method.

Figure 3 exhibits changes in the survival rate of EL-4 cells and the fluorescence intensity of PpIX accumulated in the cells with the average diameter of liposomes. The survival rate increased with increasing average diameter of liposomes. Moreover, the survival rate of liposomes having average diameters less than 63.7 nm became significantly lower than that of ALA alone, $90.7 \pm 0.35\%$ (mean$\pm$SD), as shown by the dotted line in the figure ($p<0.05$ by Student's *t*-test). When the average diameter was ver 127.7 nm, however, the survival rate of liposomes was almost equal to that of ALA alone. Thus, PDT efficacy is promoted by the liposomes smaller than 63.5 nm in diameter compared with that of ALA alone. On the other hand, the fluorescence intensity of PpIX accumulated in the cells decreased with increasing average diameter of liposomes. In particular, the accumulation of PpIX in liposomes with diameters less than 63.5 nm was comparably larger than that for ALA alone, shown by the dashed line in the figure. The enhancement of PDT efficacy by liposomes of

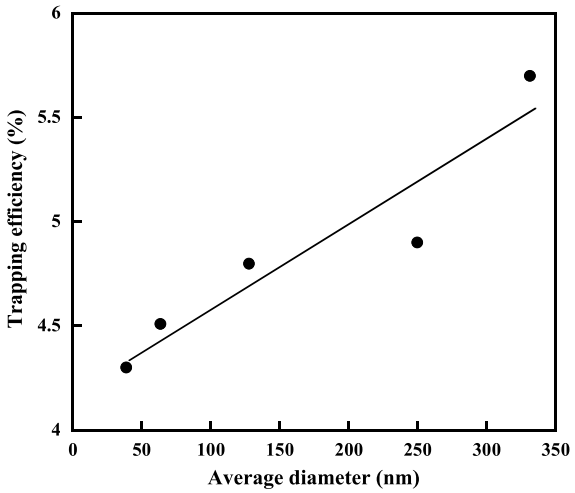

FIGURE 2 **Changes in Trapping Efficacy of ALA-Containing Liposomes with Average Liposome Diameters.**

smaller sizes is therefore caused by the increment in PpIX accumulation in the cells.

In general, since liposome adsorption on the cell surface seems to be the rate-determining step for the uptake (Schwendener et al., 1984), liposomes adsorbed more firmly on the cell surface are more susceptible to subsequent uptake into the cells. The interaction of liposomes with cells is affected by the surface characteristics of liposomes, for example, microfluidity and surface charge of their bilayer membranes. We then measured the microfluidity and the ζ-potential of bilayer membranes of ALA-containing liposomes with various diameters. As a result, the microfluidity and the ζ-potential of ALA-containing liposomes were independent of their average diameters. This suggests that the interaction between liposomes and the cells is almost constant independently of liposome size.

When liposomes are incorporated into cells, fusion, lipid exchange, and their combination can occur. In particular, endocytosis works as a main mechanism for uptake of liposomes. It is well known that the endocytotic uptake of liposomes into cells occurs more easily for smaller liposomes (Charrois & Allen, 2003). Thus, smaller ALA-containing liposomes were readily incorporated into EL-4 cells and promoted PpIX accumulation in the cells. In addition, ALA is a highly water-soluble agent and is impossible to pass through cell membranes. ALA may be incorporated through GABA amino acid transporter (Rud et al., 2000). The fact that the accumulation of PpIX in ALA-containing liposomes with diameters less than 63.5 nm was greater than ALA alone would therefore be caused by the difference in the uptake process between ALA-containing liposomes and ALA.

PDT Efficacy of ALA-Containing Liposomes with Various Surface Charges

Figure 4 shows changes in the ζ-potential of ALA-containing liposomes, the bilayer membranes of which contain ODA at various molar mixing ratios. The average diameter and trapping efficiency of the liposomes were around 60 nm and 5%, respectively. The ζ-potential of the liposome without ODA was almost zero. Addition of ODA made the ζ-potential positive, and the ζ-potential increased with increasing molar ratio of ODA. This increment in the ζ-potential of liposomes is attributable to a positive charge of ODA in the liposome bilayer membranes.

The ζ-potential of EL-4 cells was about −15 mV. We can then predict that the interaction of the liposomes with the cells, followed by liposome uptake into the cells, is enhanced by positive surface charges on the liposome surface. However, the survival rate, or PDT efficacy, for EL-4 cells and the PpIX accumulation of positively charged liposomes in the cells were almost identical with those of electrically neutral liposomes (without ODA), shown in Fig. 5.

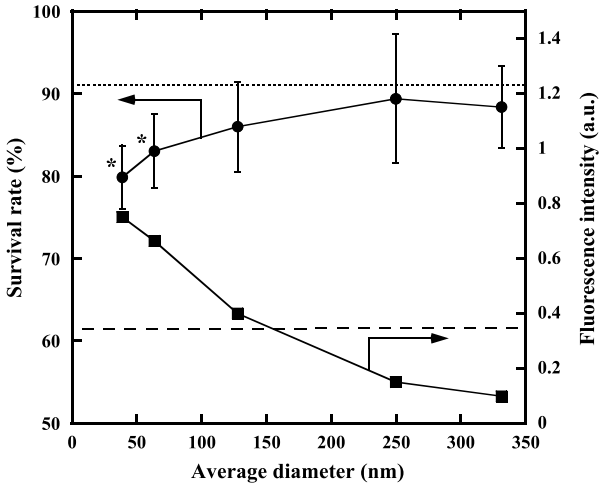

FIGURE 3 **Changes in Survival Rate for EL-4 Cells and Fluorescence Intensity of PpIX Accumulated in the Cells with Average Liposome Diameters. Survival Rate and Fluorescence Intensity of PpIX in Administration of ALA Alone are Shown by Botted and Dashed Lines, Respectively. Values of Survival Rate are Mean ± SD. *: $p < 0.05$ Compared With Survival Rate in Administration of ALA Alone by Student's *t*-Test.**

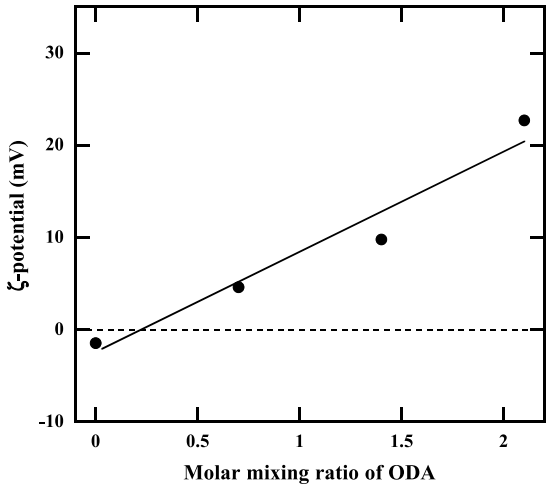

FIGURE 4 **Changes in ζ-Potential of ALA-Containing Liposomes with Molar Mixing Ratio of ODA to DPPC.**

We measured the microfluidity of bilayer membranes of ALA-containing liposomes with various ζ-potentials. As a result, no changes were caused in the microfluidity of liposome bilayer membranes by the addition of ODA. We can then ignore the influence of the microfluidity of bilayer membranes on the interaction between the liposomes and the cells.

Webb et al. (1995) investigated the permeation of cationic drugs through liposome membranes with positively charged ODA. They verified that ODA was effective in depressing the leakage of the drugs from the inner water phase (Webb et al., 1995), because of the electrical repulsion between the drug and the liposome membranes. The pK_a values of ALA in an aqueous buffer solution (pH=7.4) at 21°C are 4.1 and

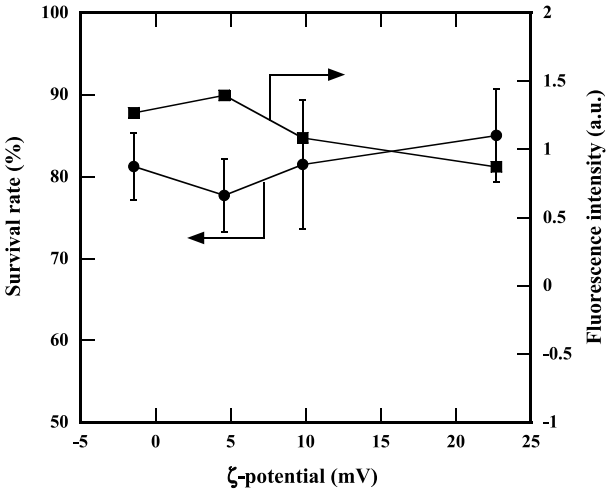

FIGURE 5 **Changes in Survival Rate for EL-4 Cells and Fluorescence Intensity of PpIX Accumulated in the Cells with ζ-Potential of ALA-Containing Liposomes. Values of Survival Rate are Mean ± SD.**

8.7 (Uehlinger et al., 2000). Since the pH value of 0.28 mol/L aqueous ALA solution used in this study was 2.4, the amino group of ALA molecule was ionized in the aqueous solution. Thus, even if ALA-containing liposomes with a highly positive surface charge are incorporated into the cells, the release of ALA from the liposomes into the cytoplasm would be extremely lower, which results in a lower PDT efficacy and PpIX accumulation.

In conclusion, the PDT efficacy and the accumulation of PpIX for EL-4 cells incubated in vitro increased with a decrease in average liposome diameters. In particular, ALA-containing liposomes smaller than 63.5 nm in diameter promoted the PDT efficacy in comparison with that of ALA alone. Moreover, we found no significant changes in PDT efficacy and PpIX accumulation in the cells, independently of liposome surface charges.

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