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7-Acetoxycoumarin Dimer-Incorporated and Folate-Decorated Liposomes: Photoresponsive Release and *in Vitro* Targeting and Efficacy

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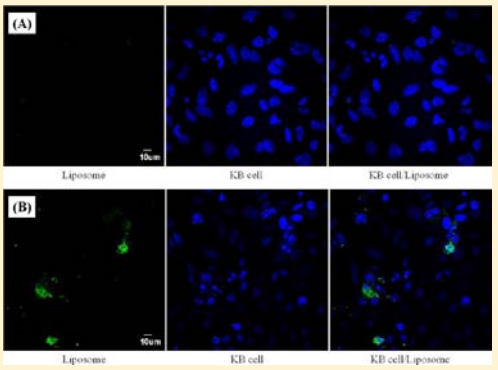
ABSTRACT: Photoresponsive and cancer cell (KB cell)-targetable liposome was developed by incorporating 7-acetoxycoumarin dimer (ACD) in the liposomal membrane and modifying the surface of liposome with folate. The liposomes were prepared from the dry mixed thin film of egg phosphatidylcholine (EPC), ACD, and folate conjugate (DSPE-PEG_{2000}-folate) by a film hydration method, where the molar ratios of EPC/ACD/DSPE-PEG_{2000}-folate were 10/0/0, 9/1/0, 9/1/0.05, and 9/0/0.05. The liposomal membranes were multilamellar and the diameter was on the order of hundreds of nanometers. The release degrees in 60 min of 5(6)-carboxyfluorescein (CF) from EPC/ACD/DSPE-PEG_{2000}-folate liposome were less than 4% without the irradiation of UV light (λ = 254 nm, 6 W), but more than 20% under the irradiation of UV light (λ = 254 nm, 6W), possibly due to the phototriggered de-dimerization of ACD. Under confocal laser scanning microscopy, KB cells treated with EPC/ACD/DSPE-PEG_{2000}-folate liposomes exhibited CF fluorescence of liposomes at the positions where 4′,6-diamidino-2-phenylindole (DAPI) fluorescence of the cell nucleus was shown, indicating the liposomes targeted the cancer cells. In flow cytometric analysis, the cancer cells treated with EPC/ACD/DSPE-PEG_{2000}-folate liposomes exhibited much higher fluorescence than the untreated cells did, indicating that there was a specific interaction between the liposome and the cancer cell. With the irradiation of UV light, EPC/ACD/DSPE-PEG_{2000}-folate liposomes markedly promoted the *in vitro* anti-cancer efficacy of DOX without causing acute *in vitro* toxicity.



INTRODUCTION

Liposomes are defined as phospholipid bilayer vesicles.[1−3] They have been studied for a few decades for their application in drug delivery due to their versatility. For example, they are easily prepared by various methods, are nonimmunogenic to human body, and are capable of encapsulating both hydrophilic therapeutic compounds and lipophilic ones.[3] The biodistribution can be controlled by altering the composition and the size, and the localization at a specific site (so-called active targeting) can be achieved by decorating the surface with some ligands.[3] In addition, liposomes can be designed to release their payload in response to stimuli such as temperature change, pH change, specific targets, and photo irradiation by including stimuli-responsive compounds in the liposomal membrane or by modifying the surface with the smart compounds. Among them, photoresponsive liposomes are one of drug carriers which have attracted much attention of scientists in the field of drug delivery. They release their contents by various mechanisms, namely, photo cross-linkage,[4] photo isomerization,[5] photo cleavage,[6] and photo oxidization.[7] Recently, photoresponsive liposomes were prepared by incorporating coumarin in the liposomal membrane[8] or by immobilizing coumarin-grafted polymer onto the surface.[9,10] Coumarins undergo photo dimerization and photo de-dimerization under the irradiation of adequate UV light.[11−13] Due to the photoreaction, the liposomal membrane can be fluctuated or defected by mechanical stress, and a phototriggered release can take place. In order to enhance the therapeutic efficacy of a drug while decreasing the toxicity, the drug carriers are required to be localized and release their contents at target sites.

In this study, a cancer cell-targetable and photoresponsive liposome was prepared by incorporating 7-acetoxycoumarin dimer (ACD, a photoresponsive compound) in the liposomal membrane and decorating the surface with folate (a ligand for a receptor on cancer cells). ACD-incorporated and folate-decorated liposomes (ACD-Fol-Lip) can be localized at cancer cells by the specific interaction of the liposomal folate and the receptor on cancer cells. Subsequently, ACD-Fol-Lip can release an anti-cancer drug (e.g, doxorubicin) at the cancer cells in response to the irradiation of a UV light (λ < 254 nm), because ACD is photocleaved and it can disturb the packing of liposomal membrane (Scheme 1). The *in vitro* photoresponsive release from ACD-Fol-Lip was investigated under the irradiation of a UV light (e.g., λ = 254 nm). The *in vitro* localization of ACD-Fol-Lip at cancer cell (e.g., KB cell) was observed by confocal laser scanning microscopy and flow

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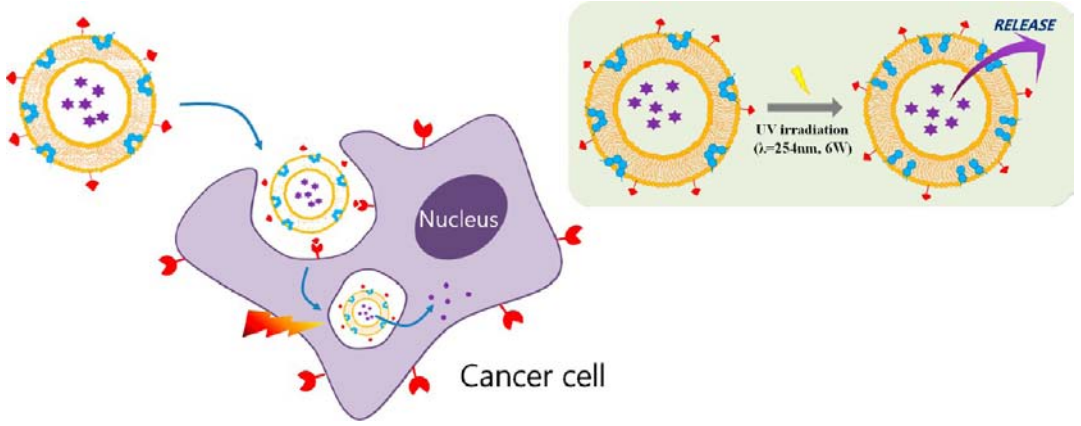
Scheme 1. ACD-Incorporated and Folate-Decorated Liposomes Can Target Cancer Cells and Release Their Payload by UV Irradiation



cytometric analysis, and the *in vitro* anti-cancer efficacy and *in vitro* toxicity of ACD-Fol-Lip were evaluated using cancer cells (e.g., KB cell) and normal cells (e.g., Raw264.7 cell) with the irradiation of UV light.

RESULTS AND DISCUSSION

^{1}H NMR Spectroscopy. Figure 1A shows the ^{1}H NMR spectrum of AC monomer. The signals of aromatic protons appeared in the range of 6.47 ppm to 8.09 ppm. The signal of acetoxy protons was found around 2.32 ppm. The area of aromatic ring signals was 5.06 and that of acetoxy group signals was 3.15. Thus, the molar ratio of aromatic ring to acetoxy group was calculated to be 1:1.04, indicating that all the hydroxyl groups of AC were replaced by acetoxy groups. Figure 1B shows the ^{1}H NMR spectrum of AC dimer. The signals of aromatic protons appeared in the range of 6.68 ppm to 6.79 ppm, the signal of cyclobutane bridge protons was found at 4.07 ppm and 4.18 ppm, and the signal of acetoxy protons was found at 2.26 ppm. The area of acetoxy group signals was 3.41, that of cyclobutane signals was 2.31, and that of aromatic ring signals was 3.34. Therefore, the molar ratio of acetoxy group/aromatic ring/cyclobutane bridge was calculated to be 1.00:1.01:0.98, indicating that AC dimer was successfully prepared. Figure 1C shows the ^{1}H NMR spectrum of DSPE-PEG_{2000}-folate. The signals of aromatic protons of folate were found at 6.62 ppm, 6.92 ppm, 7.66 ppm, and 8.64 ppm, and the signal of methyl protons of DSPE were found at 0.85 ppm. The area of aromatic ring signals was 2.47, and that of methyl group signal was 3. Thus, the molar ratio of folate to DSPE was calculated to be 0.99:1, indicating that almost 100% of DSPE-EPG_{2000}-amine was conjugated to folate.

Transmission Electron Microscopy. Figure 2 shows the TEM photos of EPC liposomes, EPC/ACD liposomes, and EPC/ACD/DSPE-PEG_{2000}-folate liposomes. The membranes of EPC liposomes were multilamellar and the size was hundreds of nanometers (Figure 2A). The film hydration method adopted in the present work is known to produce multilamellar vesicles.[18] Inclusion of ACD in the liposome had little effect on the structure and the size (Figure 2B). ACD is sparingly soluble in aqueous phase because of its low polarity, so it may be mostly intercalated into the liposomal membrane. The intercalation of ACD can disturb the packing of liposomal membrane, but the effect would be little because of its small amount (the molar ratio of EPC/ACD was 9/1), nor did the incorporation of DSPE-PEG_{2000}-folate in the liposomal membrane have an effect on the structure and the size (Figure 2C). DSPE-PEG_{2000}-folate is an amphiphilic molecule because two acyl chains of DSPE are hydrophobic and PEG_{2000}-folate moiety is hydrophilic. Accordingly, the acyl chains will be incorporated into the liposomal membrane and PEG_{2000}-folate will face toward aqueous phase. Since DSPE is a phospholipid and its packing parameter insignificantly deviates from that of EPC, its incorporation into the EPC liposomal membrane will hardly affect the bilayer structure. Furthermore, the amount of PEG_{2000}-folate seems to be too small to disturb the packing of EPC bilayer.

Fluorescence Quenching of CF. The fluorescence quenching degree of CF entrapped in liposomes was summarized in Table 1. The quenching degree in EPC liposomes was fairly high, 89.3%. The high value indicates that the liposomes were formed with a high efficiency because the quenching degree can be used as a measure of liposome formation degree. The packing parameter of EPC is around 1 so the phospholipid is known to form stable bilayer vesicles.[19,20] The quenching degree in EPC/ACD liposome was 88.3%, and it was close to the quenching degree in EPC liposome. That is, the inclusion of ACD in EPC liposome had little effect on the fluorescence quenching degree. This means that ACD could be intercalated into the liposomal membrane without disrupting the membrane packing. On the other hand, the inclusion of DSPE-PEG_{2000}-amine and DSPE-PEG_{2000}-folate in the liposome resulted in a significant reduction in the fluorescence quenching degree. EPC/ACD/DSPE-PEG_{2000}-folate liposome, EPC/ACD/DSPE-PEG_{2000}-amine liposome, EPC/DSPE-PEG_{2000}-folate liposome, and EPC/DSPE-PEG_{2000}-amine liposome exhibited 66.7%, 63.4%, 50.1%, and 61.6% in the fluorescence quenching, and the values were quite a bit lower than the fluorescence quenching of liposomes without DSPE-PEG_{2000}-amine and DSPE-PEG_{2000}-folate (e.g., EPC liposome and EPC/ACD liposome). DSPE-PEG_{2000}-amine and DSPE-PEG_{2000}-folate are kinds of surfactants because they have two acyl chains as a hydrophobic part and have DSPE-PEG_{2000}-amine or DSPE-PEG_{2000}-folate as a hydrophilic one. Accordingly, some EPC can be assembled into mixed micelles with DSPE-PEG_{2000}-amine and DSPE-PEG_{2000}-folate, leading to a lower degree of liposome formation and a lower fluorescence quenching.

Zeta Potentials and Sizes. The zeta potentials of liposomes were summarized in Table 1. The zeta potential of EPC liposomes was −28.4 mV. It is known that EPC liposomes

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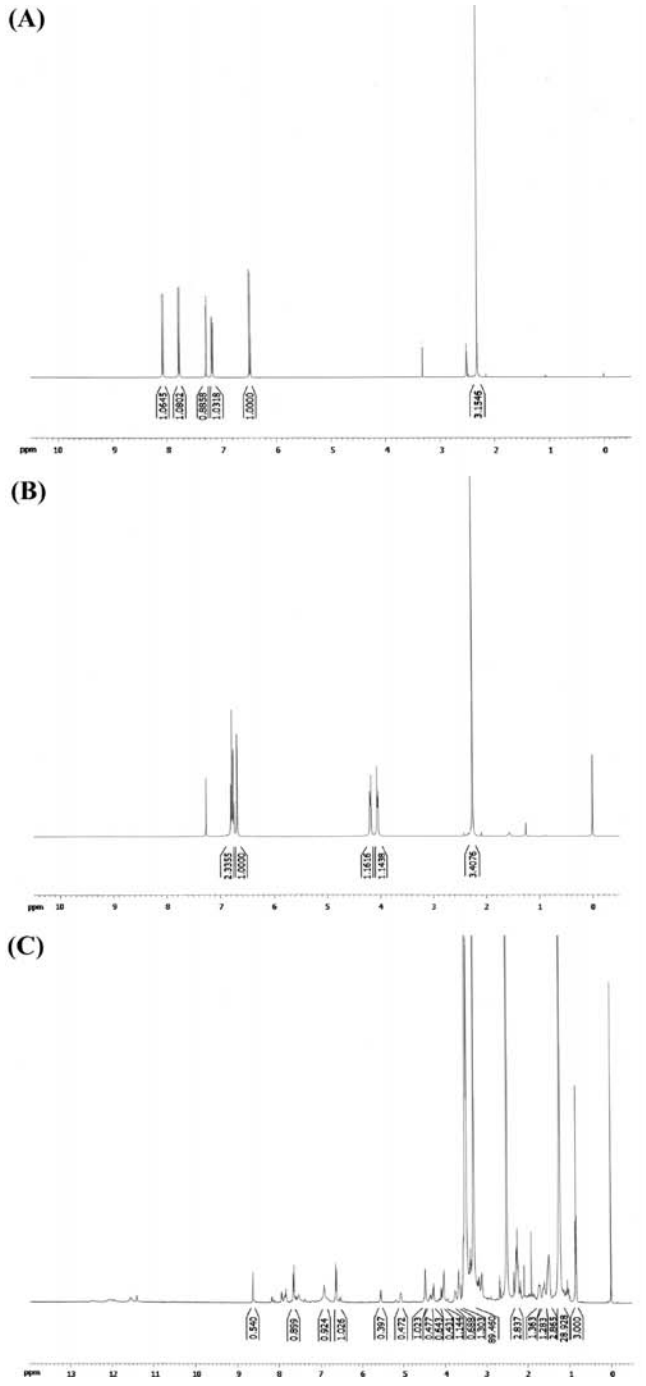

Figure 1. ^{1}H NMR spectra of AC monomer (A), AC dimer (B), and DSPE-PEG$_{2000}$-folate (C).

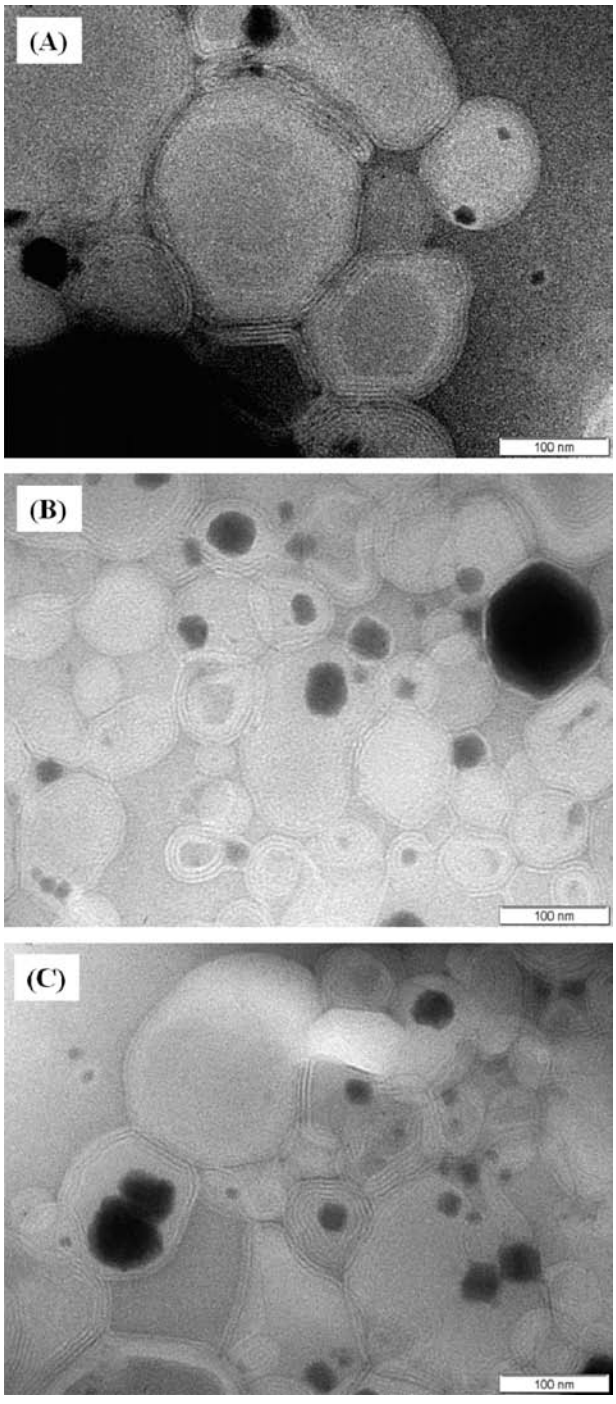

Figure 2. TEM photos of EPC liposomes (A), EPC/ACD liposomes (B), and EPC/ACD/DSPE-PEG$_{2000}$-folate liposomes (C). Magnification was 40 000× and bar represents 100 nm.

have a negative zeta potential due to the phosphate group.[21] The zeta potential of EPC/ACD liposome was −36.2 mV and the value was lower than that of EPC liposome. Inclusion of ACD in liposomal membrane can contribute to the negative zeta potential because ACD has a carbonyl group. The zeta potential of EPC/ACD/DSPE-PEG$_{2000}$-folate liposomes, −10.2 mV, was much higher than that of EPC/ACD liposomes, −36.2 mV, possibly because the amino groups of folate can reduce the negative zeta potential. The zeta potential of EPC/ACD/DSPE-PEG$_{2000}$-amine liposomes, −1.8 mV, was also much higher than that of EPC/ACD liposomes, possibly due to the neutralization effect of amino group-terminated PEG. The zeta potential of EPC/DSPE-PEG$_{2000}$-folate liposome and that of EPC/DSPE-PEG$_{2000}$-amine liposomes were −4.9 mV and 3.3 mV, and the values were higher than the value of EPC liposomes, −28.4. This may be ascribed to the amino groups of folate and amino group-terminated PEG. Besides the charge neutralization effect described above, the surface grafted PEG-chains can shift away from the lipid surface the hydrodynamic plane at which zeta potential is measured. This could be one of the major reasons the zeta potential has become less negative upon including PEG on the liposomal surface. It was reported that the zeta potential of PEGylated liposome was quite

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Table 1. Fluorescence Quenching Degree of CF Entrapped in Liposomes, the Zeta Potential and the Mean Diameter of Liposomes, and Specific Loading of DOX in Liposomes (Mean ± SD, $n = 3$, $p < 0.05$)

	EPC	EPC/ACD	EPC/ACD/DSPE-PEG_{2000}-folate	EPC/ACD/DSPE-PEG_{2000}-amine	EPC/DSPE-PEG_{2000}-folate	EPC/DSPE-PEG_{2000}-amine
Quenching (%)	89.3 ± 3.96	88.3 ± 0.47	66.7 ± 3.88	63.4 ± 6.06	50.1 ± 2.48	61.7 ± 3.25
Size (nm)	198.1 ± 10.47	223.0 ± 6.79	361.4 ± 39.24	240.2 ± 8.70	626.6 ± 18.95	611.3 ± 24.75
Zeta potential (mV)	−28.4 ± 2.19	−36.2 ± 0.64	−10.2 ± 0.57	−1.8 ± 0.42	−4.9 ± 0.52	3.3 ± 0.04
Specific loading (mg/mg)	0.026 ± 0.0025	0.023 ± 0.0025	0.024 ± 0.0046	0.023 ± 0.0035	0.021 ± 0.0025	0.023 ± 0.0021

different from that of the bare liposome due to the steric effect of PEG.[22] The mean diameter of EPC liposome was 198.1 nm, and that of EPC/ACD liposomes was 223.0 nm, greater than the mean diameter of EPC liposomes. This is possibly because the inclusion of ACD in the liposomal membrane can decrease the curvature of liposomes. When either DSPE-PEG_{2000}-amine or DSPE-PEG_{2000}-folate was included in the EPC liposome and EPC/ACD liposome, the sizes of the liposomes markedly increased. For example, the mean diameters of EPC/ACD/DSPE-PEG_{2000}-folate liposome, EPC/ACD/DSPE-PEG_{2000}-amine liposome, EPC/DSPE-PEG_{2000}-folate liposome, and EPC/DSPE-PEG_{2000}-amine liposome were 361.4 nm, 240.2 nm, 626.6 nm, and 611.3 nm, respectively, and the sizes were greater than those of liposomes without DSPE-PEG_{2000}-amine or DSPE-PEG_{2000}-folate (e.g., EPC liposome and EPC/ACD liposome). DSPE will be anchored into the liposomal membrane, and PEG or PEG-folate will face toward aqueous phase. So, the hydrophilic surface modifier (PEG_{2000}-amine or PEG_{2000}-folate) would contribute to the size increase. On the other hand, the absolute zeta potentials of liposomes bearing PEG_{2000}-amine or PEG_{2000}-folate were much lower than those of liposomes without them. Accordingly, the former liposomes will agglomerate more readily than the latter liposomes. It is known that colloidal particles agglomerate due to the lack of electrostatic repulsion when the absolute of zeta potential is less than 25 mV.[23] According to the TEM photo, the size of liposome bearing DSPE-PEG_{2000}-folate was not markedly different from that of liposome without the surface modifier. Thus, a main reason for the increased size could be the agglomeration caused by the lower absolute value of zeta potential. The modification of the liposomal surface with PEG can prevent the agglomeration of liposomal particles because PEG is hydrated and flexible in an aqueous phase and acts as a molecular spring.[24] However, PEG grafted to the liposomal surface in the present study has either amine group or folate moiety at the end of the chains. Since the amine group and the folate could electrostatically interact with the negatively charged liposomal surface, the mobility of the PEG chains would be suppressed due to the electrostatic interaction. In this circumstance, the surface potential rather than the steric effect of PEG would be a major factor affecting the colloidal stability of the liposomes.

Specific Loading of DOX in Liposomes. The specific loadings of DOX in liposomes were summarized in Table 1. The calibration curve of DOX was $Y = 16246X + 21.4$ ($R^2 = 0.9980$), where X is the concentration of DOX in mg/mL and Y is the fluorescence intensity of DOX solution. The specific loadings fell within 0.021 mg/mg to 0.026 mg/mg and they were not significantly different from each other. The major factors determining the specific loading of a water-soluble ingredient will be the size and the structure of liposome.[27] Neither the inclusion of ACD nor the incorporation of DSPE-PEG_{2000}-folate had a substantial effect on the size of the structure of liposome on the TEM photos (Figure 2). This may explain why the specific loading was insignificantly different lipid.

de-dimerizations of ACD. Figure 3 shows the de-dimerization degree of ACD. The de-dimerization degree

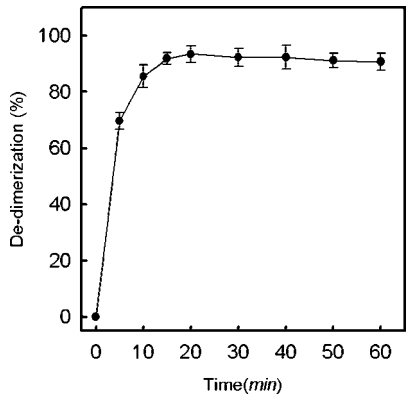

Figure 3. De-dimerization degree of ACD.

rapidly increased up to 85.5% in 10 min and further increased to 91.9% for the next 10 min in a saturation manner. Coumarin and its derivatives are known to be dimerized through the formation of cyclobutane bridges under the irradiation of a light ($\lambda > 310$ nm), and the de-dimerization reversibly takes place under the irradiation of a light ($\lambda < 260$ nm).[28] On the other hand, the de-dimerization degree slightly decreased during the rest of irradiation period. This is possibly because not only de-dimerization but also dimerization can occur by the irradiation of $\lambda = 254$ nm; thus, the photo reactions reach a photostationary state under prolonged irradiation.[29]

Photoresponsive Release from Liposomes. Figure 4A shows the release degree of EPC liposome, EPC/ACD liposome, EPC/ACD/DSPE-PEG_{2000}-amine liposome, and EPC/ACD/DSPE-PEG_{2000}-folate liposome for 60 min under the irradiation of UV light (254 nm, 6 W). EPC liposome released 3.7% of its content for 60 min. EPC/ACD liposome released 10.9% for the same period. The higher release degree can be ascribed to the photocleavage of ACD. Upon the irradiation of UV light, ACD will be cleaved to AC monomers (Figure 3). Since the monomer is totally different from the dimer in terms of size and polarity, the orientation of monomer will be quite different from that of dimer. Thus, under the irradiation of UV light, the liposomal membrane would be disturbed by the reorientation of AC residue, so the release from the liposome would be promoted. The releases from the EPC/ACD/DSPE-PEG_{2000}-amine liposome and EPC/ACD/DSPE-PEG_{2000}-folate liposome were much more promoted by UV irradiation than the release from EPC/ACD liposome. For example, the release degree of EPC/ACD/DSPE-PEG_{2000}-amine liposome and that of EPC/ACD/DSPE-PEG_{2000}-folate

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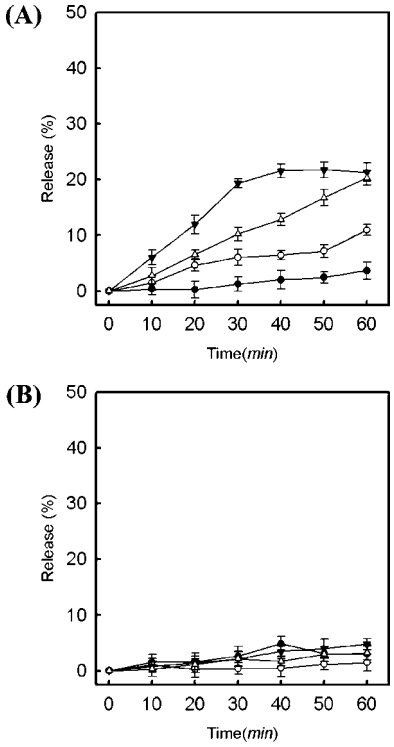

Figure 4. Release degree of EPC liposome (●), EPC/ACD liposome (○), EPC/ACD/PEG_{2000}-amine liposome (▼), and EPC/ACD/PEG_{2000}-folate liposome (△) for 60 min with (A) and without (B) irradiation of UV light (254 nm, 6 W).

liposome in 60 min were 21.4% and 20.3%, respectively, which were much higher than the release degree of EPC/ACD liposome, 10.9%. DSPE-PEG_{2000}-amine and DSPE-PEG_{2000}-folate are a kind of surfactant and they can fluidize the liposomal membrane. Accordingly, the photoinduced reorientation of AC residue in the liposomal membrane bearing either DSPE-PEG_{2000}-amine or DSPE-PEG_{2000}-folate will be more favorable than in the liposomal membrane without them.

The initial CF concentration in the liposomal aqueous core could be different from each other. So the concentration gradient across the liposomal membrane, one of major factors affecting the CF release rate, would hardly be the same. Even though the quenching degree and the initial CF concentration could differ from each other, they would not significantly affect the release degree for 60 min because EPC-based liposomal membranes are known to be stable at room temperature (23 °C) during the short period in terms of release.[30] Figure 4B shows the release degree of EPC/ACD liposome, EPC/ACD/DSPE-PEG_{2000}-amine liposome, and EPC/ACD/DSPE-PEG_{2000}-folate liposome for 60 min without the irradiation of UV light (254 nm, 6 W). The release degrees of the liposome in 60 min were less than 4%, and they were not significantly different from each other, indicating that the quenching degree and the initial CF concentration had little effect on the release degree for 60 min, and they were much less than the values obtained under UV irradiation. Therefore, the promoted releases from EPC/ACD liposome, EPC/ACD/DSPE-PEG_{2000}-amine liposome, and EPC/ACD/DSPE-PEG_{2000}-folate liposome shown in Figure 4A are possibly due to the phototriggered de-dimerization of ACD.

***In Vitro* Targeting of Liposomes.** Figure 5 shows the confocal laser scanning microscopic images of KB cells treated with EPC/ACD/DSPE-PEG_{2000}-amine liposome and EPC/ACD/DSPE-PEG_{2000}-folate liposome. The KB cells treated with EPC/ACD/DSPE-PEG_{2000}-amine liposome exhibited no CF fluorescence, indicating that there was no interaction between the folate-free liposomes and the cancer cells. On the other hand, the KB cells treated with EPC/ACD/DSPE-PEG_{2000}-folate liposome exhibited CF fluorescence of liposome at the position where DAPI fluorescence of the cell nucleus was

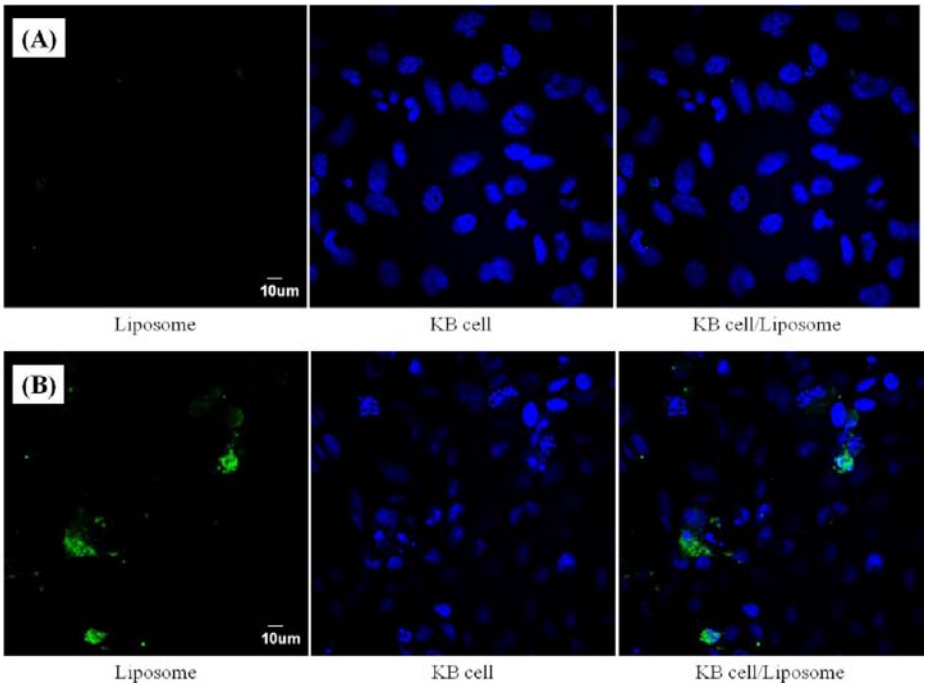

Figure 5. Confocal laser scanning micrographs of KB cells treated with EPC/ACD/DSPE-PEG_{2000}-amine liposome (A) and EPC/ACD/DSPE-PEG_{2000}-folate liposome (B).

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shown. It means that the folate-decorated liposomes were bound to the cancer cells. The binding of the liposomes with the cells can be ascribed to the specific interaction of folate on the liposomes and the receptor on the cells.[14,25] Figure 6 shows

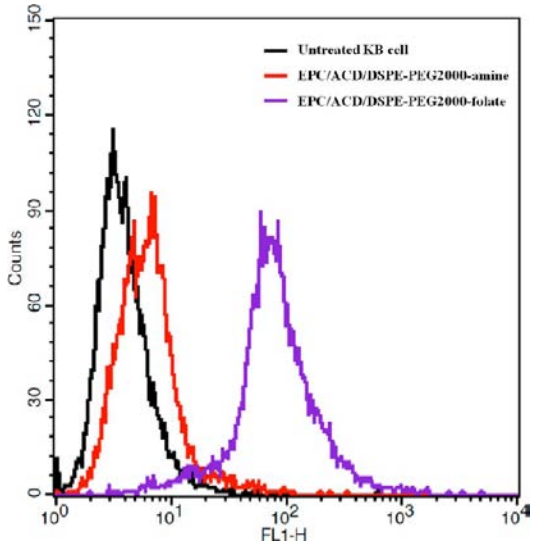

Figure 6. Flow cytometry of untreated KB cells and those treated with EPC/ACD/PEG_{2000}-amine liposome and EPC/ACD/PEG_{2000}-folate liposome.

the flow cytometric analysis of untreated KB cells and those treated with EPC/ACD/DSPE-PEG_{2000}-amine liposome and EPC/ACD/DSPE-PEG_{2000}-folate liposome. The cells treated with EPC/ACD/DSPE-PEG_{2000}-amine liposome showed a slightly higher fluorescence than the untreated cells, possibly because the folate-free liposomes can nonspecifically interact with the cancer cells or they can be taken up by the cells via endocytosis. The cells treated with EPC/ACD/DSPE-PEG_{2000}-folate liposome exhibited a much higher fluorescence than the untreated cells or the cells treated with EPC/ACD/DSPE-PEG_{2000}-amine liposome. The folate-decorated liposomes can be tightly bound to the cancer cell, possibly because of the specific interaction of folate on the liposomes and the receptor on the cells, and this may account for the reason the cells treated with the folate-decorated liposomes exhibited a higher fluorescence.

***In Vitro* Anti-Cancer Efficacy and Toxicity of Liposomes.** Figure 7A shows the viability of KB cell treated with free DOX and DOX-loaded liposomes (EPC/DSPE-PEG_{2000}-amine liposome, EPC/ACD/DSPE-PEG_{2000}-amine, EPC/DSPE-PEG_{2000}-folate liposome, and EPC/ACD/DSPE-PEG_{2000}-folate liposome) with the irradiation of UV light. The viability of KB cell decreased with increasing concentration of DOX. When the concentration of DOX is relatively low (e.g., 0.5 μg/mL), there was no significant difference in the viability among the cells treated with the above-mentioned DOX preparations. However, the difference became marked as the concentration increased. At the concentration of 0.5, 1.0, 2.0, 4.0, and 8.0 μg/mL, the viability was lower in the order of KB cells treated with EPC/ACD/DSPE-PEG_{2000}-folate liposome > EPC/DSPE-PEG_{2000}-folate liposome > free DOX > EPC/ACD/DSPE-PEG_{2000}-amine liposome > EPC/DSPE-PEG_{2000}-amine liposome. Folate-decorated liposomes (e.g., EPC/ACD/DSPE-PEG_{2000}-folate liposome, EPC/DSPE-PEG_{2000}-folate liposome) were more efficacious in inhibiting

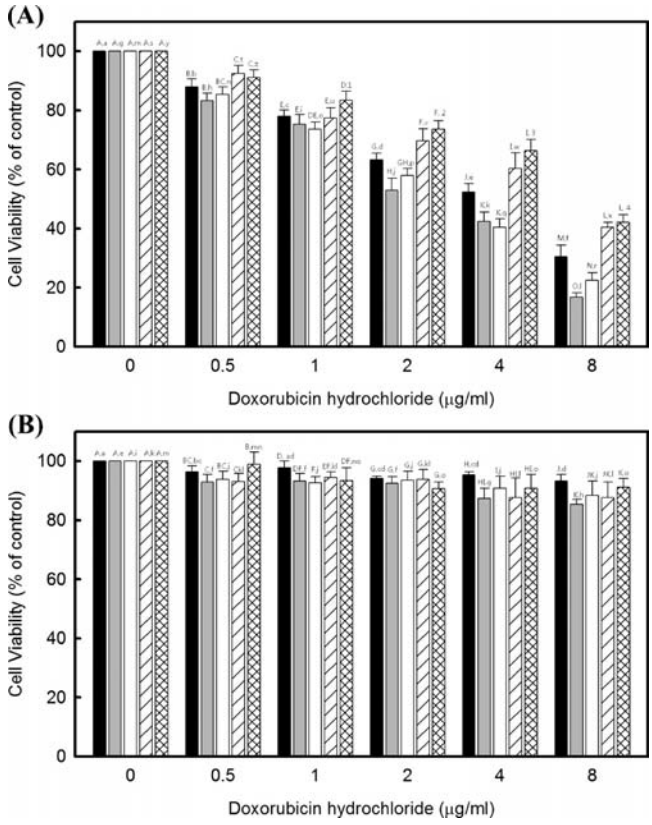

Figure 7. Viability of KB cell (A) and Raw264.7 cell (B) treated with free DOX (solid black bar) and DOX-loaded liposomes (EPC/ACD/DSPE-PEG_{2000}-folate liposome (solid gray bar), EPC/DSPE-PEG_{2000}-folate liposome (white bar), EPC/ACD/DSPE-PEG_{2000}-amine (hashed bar), and EPC/DSPE-PEG_{2000}-amine liposome (cross-hatched bar)) with the irradiation of UV light. Capitals denote statistical significant difference in cell viability among samples whose DOX concentrations were the same. Small letters and number denote statistical significant difference in cell viability among the same kind of samples whose DOX concentrations were different from one another. The same characters indicate no significant difference and the different ones indicate a significant difference.

the cancer cell growth than free DOX and their corresponding folate-free liposomes (e.g., EPC/ACD/DSPE-PEG_{2000}-amine liposome, EPC/DSPE-PEG_{2000}-amine liposome), possibly because the folate-decorated liposomes can target the cancer cell (Figure 5 and Figure 6). In addition, ACD-containing liposomes (e.g., EPC/ACD/DSPE-PEG_{2000}-folate liposome, EPC/ACD/DSPE-PEG_{2000}-amine liposome) were more efficacious in inhibiting the cancer cell growth than their corresponding ACD-free liposomes (e.g., EPC/DSPE-PEG_{2000}-folate liposome, EPC/DSPE-PEG_{2000}-amine liposome), possibly because the former liposomes could actively release their payload in response to UV irradiation (Figure 4A). On the other hand, since EPC/DSPE-PEG_{2000}-folate liposome was more efficacious than EPC/ACD/DSPE-PEG_{2000}-amine liposome, it is believed that targeting is more critical than phototriggered release in inhibiting the cancer cell growth. Figure 7B shows the viability of Raw264.7 cell treated with free DOX and DOX-loaded liposomes (EPC/DSPE-PEG_{2000}-amine liposome, EPC/ACD/DSPE-PEG_{2000}-amine, EPC/DSPE-PEG_{2000}-folate liposome, and EPC/ACD/DSPE-PEG_{2000}-folate liposome) with the irradiation of UV light. The viability of Raw264.7 cells treated with the above-mentioned DOX

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preparations decreased with increasing concentration of DOX; however, it did not decrease as much as the viability of KB cells did. This is possibly because the normal cell is less susceptible to the anti-cancer drug than KB cell,[26] and there was no significant difference in the viability among cells treated with the DOX preparations at all the concentration tested. Since there is no folate receptor on Raw264.7 cell, folate-decorated liposomse would hardly target the normal cell; thus, the folate-decorated liposomes would have almost the same growth inhibitory effect on the normal cell as the folate-free liposomes. In addition, although ACD-containing liposomes released their payload more than ACD-free liposomes under the UV irradiation (Figure 4A), the former liposomes showed almost the same growth inhibitory effect on Raw264.7 cell as the latter ones. This is possibly because the susceptibility of the normal cell to the anti-cancer drug is relatively low.[26] In summary, among the DOX-loaded liposomes tested, EPC/ACD/DSPE-PEG_{2000}-folate liposomes most promoted the *in vitro* anti-cancer efficacy of DOX under the irradiation of UV light without causing an acute *in vitro* toxicity. The targeting ability and the phototriggered active release property of the liposome could be reasons they were so efficacious against the anti-cancer cell.

CONCLUSION

ACD-incorporated and folate-decorated liposomes were prepared from the dry mixed thin film of EPC, ACD, and folate conjugate (DSPE-PEG_{2000}-folate) by a film hydration method, where the molar ratio of EPC/ACD/DSPE-PEG_{2000}-folate were 10/0/0, 9/1/0, 9/1/0.05, and 9/0/0.05. The membranes of EPC liposomes were multilamellar and the size was hundreds of nanometers, whether ACD and folate conjugate were included in the liposomal preparations. The quenching degree of CF in EPC liposomes was fairly high, 89.3%, and the inclusion of ACD in EPC liposome had little effect on the fluorescence quenching degree. However, the inclusion of DSPE-PEG_{2000}-folate in the liposome resulted in a significant reduction in the fluorescence quenching degree, possibly because it is a kind of surfactant and can disintegrate some liposomal membranes. The zeta potential of EPC/ACD/DSPE-PEG_{2000}-folate liposomes, −10.2 mV, was much higher than that of EPC/ACD liposomes, −36.2 mV, possibly due to the amino groups of folate. The mean diameter of EPC liposome was 198.1 nm, and the inclusion of DSPE-PEG_{2000}-folate resulted in a significant increase in the size. The hydrophilic surface modifier (PEG_{2000}-folate) and the agglomeration caused by the lower absolute value of zeta potential would contribute to the size increase. Without the irradiation of UV light (254 nm, 6 W), the release degrees of CF from EPC/ACD/DSPE-PEG_{2000}-folate liposome in 60 min were less than 4%. Under the irradiation of UV light, it was more than 20%, possibly due to the phototriggered de-dimerization of ACD. On the image of confocal laser scanning microscopy, KB cells treated with EPC/ACD/DSPE-PEG_{2000}-folate liposome showed CF fluorescence of liposome at the position where DAPI fluorescence of the cell nucleus appeared, indicating that EPC/ACD/DSPE-PEG_{2000}-folate liposome targeted the cancer cell. In flow cytometric analysis, the cell treated with EPC/ACD/DSPE-PEG_{2000}-folate liposome exhibited much higher fluorescence than the untreated cells did, also indicating that the liposome specifically interacted with the cancer cell. With the irradiation of UV light, EPC/ACD/DSPE-PEG_{2000}-folate liposomes promoted markedly the *in vitro* anti-cancer efficacy of DOX against KB cell without causing an acute *in vitro* toxicity toward Raw264.7 cell.

MATERIALS AND METHODS

Materials. 7-Hydroxycoumarin, 5(6)-carboxyfluorescein, anhydrous dimethylsulfoxide, 4′6-diamidino-2-phenylindole (DAPI), 3-4,5 dimethylthiazol-2,5 diphenyl tetrazolium bromide (MTT), and dicyclohexyl carbodiimide were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). 1,2-Disteraroyl-*sn*-glycero-3-phosphoethanolamine-*N*-[amino(polyethylene glycol)$_{2000}$] (ammonium salt) (DSPE-PEG_{2000}-amine) was purchased from Avanti Polar Lipids (Alabaster, Alabama, USA). *N*-[2-Hydroxyethyl] piperazine-*n*′-[2-ethanesulfonic acid] (HEPES) was obtained from USB corporation (Cleveland, OH, USA). KB cell and Raw264.7 were purchased from Korean cell line bank (Seoul, Korea). All other reagents were in analytical grade.

Preparation of 7-Acetoxycoumarin Dimer. 7-Acetoxycoumarin (AC) was prepared following a method described elsewhere.[11] 12.5 g of 7-hydroxycoumarin and 9.5 g of sodium acetate were dissolved in 45 mL of acetic anhydride, and then 2−3 drops of pyridine was added to the solution. The reaction mixture was heated slowly to 144 °C, and it was stirred at the same temperature for 7 h with reflux. After being cooled to a room temperature, the reaction mixture was filtered through a filter paper (Whatman No. 2). The cake was washed with 1 L of distilled water and 1 L of ethanol. The washed product (AC) was recrystallized in ethanol for the purification. 0.5 g of AC was spread out on a glass dish (Ø100 × 10 mm), covered with a cover glass, and it was subjected to a UV irradiation (400 W, 365 nm) for 12 h while it was turned over every 1 h. The UV-treated AC was washed with 1 L of diethyl ether and the residue (7-acetoxycoumarin dimer (ACD)) was recrystallized in acetic acid.

Preparation of DSPE-PEG_{2000}-Folate. 12.5 mg of folate was dissolved in 1 mL of anhydrous DMSO. In parallel, 50 mg of DSPE-PEG_{2000}-amine and 16.3 mg of dicyclohexyl carbodiimide were dissolved in 0.25 mL of pyridine.[14] The latter solution was dropped to the former solution and the reaction mixture was gently stirred at room temperature for 4 h. The reaction was ascertained by ninhydrin-positive DSPE-PEG_{2000}-amine spot on a silica gel TLC plate. After pyridine was removed on a rotary evaporator, 6.25 mL of distilled water was added to the reaction mixture to precipitate out impurities. Then the mixture was filtered by a syringe filter (0.22 μm PTFE membrane). The filtered solution was dialyzed for 12 h against 2 L of sodium chloride (50 mM) with two times exchange of the dialysate, and then dialyzed for 18 h against 2 L of distilled water with three times exchange of the dialysate. The dialyzed solution was freeze−dried to obtain dry DSPE-PEG_{2000}-folate.

^{1}H NMR Spectroscopy. ^{1}H NMR spectra of AC and ACD in $CDCl_3$ and DSPE-PEG_{2000}-Folate in DMSO were taken on a Bruker Avance 400 spectrometer (Karlsruhe, Germany, in the Central Laboratory Center of Kangwon National University).

Preparation of Liposomes. Liposomes were prepared by a film hydration.[8] EPC solution in chloroform (100 mg/mL), ACD solution (1 mg/mL), DSPE-PEG_{2000}-amine solution (10 mg/mL), and DSPE-PEG_{2000}-folate solution (1 mg/mL) were put in a 30 mL-round-bottom flask. The molar ratio of EPC/ACD/DSPE-PEG_{2000}-amine/DSPE-PEG_{2000}-folate are 10/0/0/0, 9/1/0/0, 9/1/0/0.05, 9/1/0.05/0, 9/0/0/0.05, and 9/0/0.05/0. The solvent of mixture solution was evaporated in a rotary evaporator operating at 90 rpm under reduced pressure

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at 40 °C to obtain thin lipid film on the wall of the flask. Two mL of 5(6)-carboxyfluorescein (CF) solution (100 mM) in HEPES buffer (pH 8.0, 30 mM) was put in the flask and it was swirled by hand until lipid film was taken off the wall. When DOX-loaded liposomes were prepared, doxorubicin (DOX) solution (2 mL, 0.1% (w/v)) in the HEPES buffer (pH 7.4, 30 mM) was used instead of the CF solution. Then, the suspension was subjected to homogenization for 20 min in a bath-type sonicator (Sonics & Materials, USA) operated at room temperature in a cyclic pulse on and off mode with 30 s duration. The liposomal membrane was annealed by keeping the suspensions at 4 °C overnight. Unentrapped CF and DOX were removed by gel permeation chromatography through a Sephadex G-100 column (1.8 cm × 40 cm).

Transmission Electron Microscopy. The shapes and the structures of EPC liposomes, EPC/ACD liposomes, EPC/ACD/DSPE-PEG_{2000}-folate liposomes, EPC/ACD/DSPE-PEG_{2000}-aimne liposomes, EPC/DSPE-PEG_{2000}-folate liposomes, and EPC/DSPE-PEG_{2000}-amine liposomes were investigated by taking the TEM photos of the negatively stained liposomes. The liposomal suspension was mixed with phosphotungstic acid solution (2%, pH 6.8) in an equivolumetric ratio and the mixture was allowed to stand at room temperature for 3 h.[15] Then, an aliquot of the stained liposomal suspension was deposited on a Formvar/copper-coated grid (200 mesh, Electron Microscopy Sciences) and it was air-dried under dust-free condition. The TEM photos were taken on a transmission electron microscope (JEOL JEM-2010 Luminography (Fuji FDL-5000) in the Central Laboratory Center of Kangwon National University).

Specific Loading of DOX in Liposomes. The calibration curve of DOX in HEPES buffer (pH 7.4, 30 mM) was obtained by measuring the fluorescence intensity of DOX solutions (0−0.002 mg/mL) at 580 nm using the excitation wavelength of 475 nm on a fluorescence spectrophotometer (F-2500, HITACHI, Tokyo, Japan). The amount of DOX loaded in EPC liposomes, EPC/ACD liposomes, EPC/ACD/DSPE-PEG_{2000}-folate liposomes, EPC/ACD/DSPE-PEG_{2000}-amine liposomes, EPC/DSPE-PEG_{2000}-folate liposomes, and EPC/DSPE-PEG_{2000}-amine liposomes were determined by measuring the fluorescence intensity after the liposomes were solubilized by Triton X-100. The specific loading was defined as the amount of DOX loaded per unit mass of lipid.

Fluorescence Quenching of CF. The phospholipid concentration of the liposomal suspension was adjusted to 0.014% for the determination of the fluorescence quenching degree of CF entrapped in liposomes. It was determined by following equation.[8−10,16]

$$\%\text{Quenching} = (1 - F_i/F_f) \times 100$$

where F_i is the initial fluorescence of liposomal suspension after free CF was removed, and F_f is the total fluorescence after liposomes were disintegrated by Triton X-100. The fluorescence intensities were measured at 517 nm with excitation at 492 nm on a fluorescence spectrophotometer (F-2500, HITACHI, Tokyo, Japan).

Zeta Potentials and Sizes. The zeta potentials and the mean diameters of liposomes were observed on a particle size analyzer (ZetaPlus 90, Brookhaven Instrument Co., USA). The liposomal suspensions were diluted with buffer solutions so that the light scattering intensities were 50−140 Kcps.

De-Dimerizations of ACD. ACD solution in DMSO (1 mg/mL) was diluted 100 times with distilled water and the solution was subjected to the irradiation of UV light (λ = 254 nm, 6 W) for 60 min. The absorbance of the UV irradiation-treated solution was measured at 310 nm to determine the amount of monomer (7-acetoxycoumarin) formed by the photocleavage. The degree of de-dimerization was determined as follows.

$$\text{de-dimerization}(\%) = (A_t/A_o) \times 100$$

where A_o is the absorbance at 310 nm of monomer (7-acetoxycoumarin) solution of which molar concentration is two times that of ACD solution, and A_t is the absorbance at 310 nm of ACD solution after being subjected to the UV irradiation for a certain period.

Photoresponsive Release from Liposomes. EPC liposome, EPC/ACD liposome, EPC/ACD/DSPE-PEG_{2000}-amine liposome, and EPC/ACD/PEG_{2000}-folate liposome were used for the experiment of photoresponsive release. The concentration of EPC in the suspension of the liposomes was adjusted to 0.01 mg/mL by diluting the suspension with HEPES buffer (pH 8.0). UV light (254 nm, 6 W) was irradiated to the liposomal suspension for 60 min and the % release was determined as follows[8−10,16,17]

$$\%\text{release} = [(F_t - F_i)/(F_f - F_i)] \times 100$$

where F_t is the intensity of fluorescence after a UV light (254 nm, 6W) is irradiated to liposomal suspension for a certain period and F_i is the initial intensity before the UV light irradiation. F_f is the total fluorescence after liposome is completely solubilized by Triton X-100. Fluorescence intensity was determined at 517 nm with excitation at 492 nm using a fluorescence spectrophotometer (F-2500, HITACHI, Japan). As controls, the % release from UV light-untreated liposomes was observed.

***In Vitro* Targeting of Liposomes.** KB cells (Human epidermal carcinoma) were seeded in 24-well plates so that the density was 3 × 10^5 cells/well, and then they were incubated in a CO_2 incubator at 37 °C for 24 h. After the cells were carefully washed twice with PBS (pH 7.4), 100 μL of liposomal suspension was added to the cells contained in the wells and the culture mixtures were incubated at 37 °C for 3 h in the CO_2 incubator. Then, the KB cells were washed twice with PBS (pH 7.4) in order to remove unbound liposomes, and the cells were detached by a trypsin/EDTA treatment.

Flow cytometric analysis (FACS analysis) for liposomal fluorescence was performed using a FACSCalibur (Becton Dickinson, USA, in the Central Laboratory Center of Kangwon National University) with an argon laser of which the exciting wavelength was 488 nm. 10 000 events were collected for each sample by list-mode, and the center of fluorescence emission was 530 nm (FL1) and 585 nm (FL2), respectively. The fluorescence was obtained on a logarithmic scale with a 1024 channel resolution. Cell Quest Pro software (Becton Dickinson, USA) was utilized for the analyses. The fluorescence intensity of liposomes bound to cells was determined at 517 nm with excitation wavelength of 492 nm using flow cytometry.

The fluorescence images of cells were taken on a confocal laser scanning microscope (FV1000 SPD, Olympus Co.). KB cells were incubated with liposomes under the same conditions as those for flow cytometry. The cells were washed twice with PBS (pH 7.4) and then fixed in 4% formaldehyde in PBS (pH 7.4) for 30 min at room temperature. After fixation, KB cells were washed with PBS (pH 7.4) and then stained with 4′,6-diamidino-2-phenylindole (DAPI) for 20 min at room

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temperature. The cells were washed twice with the buffer solution and mounted on glass slides with CC/MountTM. Slides were investigated using a confocal laser scanning microscope installed with a 488 nm Ar, 543 nm HeNe(G), and 633 nm HeNe laser (FV1000 SPD, Olympus Co.). Images were analyzed by using FV1000 v 1 application software (FV10-ASW).

***In Vitro* Anti-Cancer Efficacy and Toxicity of Liposomes.** KB cells was chosen as a cancer cell for the evaluation of the *in vitro* anti-cancer efficacy of DOX-loaded liposomes, and Raw264.7 was chosen as a normal cell for the evaluation of the *in vitro* toxicity of the liposomes. The cells in RPMI1640 were put in 24-well plates so that the density was 2×10^5 cells/well, and then they were incubated in a CO_2 incubator at 37 °C for 24 h. The supernatants were discarded and the cells were washed twice with PBS (pH 7.4). Either free DOX or DOX-loaded liposome (e.g., EPC/DSPE-PEG_{2000}-amine liposome, EPC/ACD/DSPE-PEG_{2000}-amine liposome, EPC/DSPE-PEG_{2000}-folate liposome, and EPC/ACD/DSPE-PEG_{2000}-folate liposome) was put in the culture medium so that the concentration of DOX was 0.5, 1.0, 2.0, 4.0, and 8.0 μg/mL.

500 μL of the culture medium containing either free DOX or DOX-loaded liposomes was put in the wells containing cells so the concentration of DOX in each well was 0.5, 1.0, 2.0, 4.0, and 8.0 μg/mL. The culture media containing no DOX were put in the wells for the preparation of control groups. The cells were incubated in a CO_2 incubator at 37 °C for 1 h under UV irradiation (254 nm, 6 W) and incubated under the same condition for 23 h without UV irradiation. After the cells were washed twice with PBS (pH 7.4), 100 μL of MTT working solution (5 mg/mL) was put in each well and the cells were incubated in a CO_2 incubator at 37 °C for 4 h. After the cells were washed with PBS (pH 7.4), 500 μL of DMSO was put in each well and the plate was whirled by hand and shaken for 30 min, the absorbance was measured at 540 nm. The *in vitro* efficacy and *in vitro* toxicity of free DOX and DOX-loaded liposomes were expressed as cell viability, defined as the percent of the cell counts when the cells were treated with free DOX and DOX-loaded liposomes versus the cell counts of control groups.

Statistical Analysis. Multiple comparisons among all the groups were performed using two-way ANOVA (Statistical Analysis System (SAS) software). The data were analyzed using general linear model (GLM) and least significant difference (LSD) procedures in SAS software. GLM and LSD were used to compare the mean properties at 5% of significance level.

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Notes

The authors declare no competing financial interest.

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