Table 1. Oct-PEG Component (mol %) for Each Liposome Formulation

formulation	Oct-PEG_{3400}-DSPE	PEG_{2000}-DSPE
CL	0	0
0.25Oct-CL	0.25	0
0.8Oct-CL	0.8	0
1.0Oct-CL	1.0	0
1.2Oct-CL	1.2	0
1.4Oct-CL	1.4	0
1.6Oct-CL	1.6	0
SL	0	1.6

1:1,000 in 1% Triton. About 84%, 85%, and 70% of Oct ligand was inserted into 0.25Oct-CL, 0.8Oct-CL, and 1.6Oct-CL, respectively.

2.3. Cytotoxicity Assay. TT cells were seeded onto 96-well plates at a density 10^4 cells/well for 72 h before addition of the drug. Culture medium was replaced with fresh medium containing various concentrations of liposomal CPT-11, free CPT-11 or empty liposomes. After 96 h incubation at 37 °C, the cells were washed with PBS three times and cultured with fresh medium for 48 h. Then, cell viability was determined using a WST-8 test (Dojindo Laboratories, Kumamoto, Japan). All measurement was carried out in quadruplicate. The 50% growth-inhibitory concentration (IC50) was calculated using the bootstrap method.[27]

2.4. Analysis of Cellular Uptake of Liposomes by Flow Cytometry. Cells were seeded onto 6-well plates at a density 10^4 cells/well for 72 h before addition of the drug. Cells were incubated with medium (2 mL/well) containing 0.25Oct-CL, 0.8Oct-CL, 1.0Oct-CL, 1.2Oct-CL, 1.4Oct-CL, 1.6Oct-CL, or SL at a concentration of 50 μg of DXR/ml for 1 or 2 h. In flow cytometry and confocal laser scanning microscopy studies, as described below, DXR was used instead of CPT-11 because CPT-11 is not excited at 488 nm. Subsequently, cells were washed three times with PBS (pH 7.4) to remove unbound liposomes, and the cellular uptake of liposomes was analyzed using a FACS Calibur flow cytometer (Becton Dickinson, CA, USA) equipped with a 488 nm argon ion laser and using CELL Quest software (Becton-Dickinson Immunocytometry System, CA, USA). A total of 10,000 events per sample were analyzed. The autofluorescence of cells was taken as a control. The cells were incubated without liposomes.

In competitive inhibition experiments, a 20-fold molar excess of free Oct (84 nmol/mL medium) was added to 1.6Oct-CL loaded with DXR (Figure 3). The medium in each well (2 mL) contained 50 μg of DXR/ml, 4.2 nmol of Oct originating from 1.6Oct-CL/ml, and 112 μg of phospholipid/ml. A two volume excess of empty 1.6Oct-CL or SL (258 μL) was added to 1.6Oct-CL loaded with DXR. Because all liposomes have a lipid concentration 194 μg of phospholipid/mL, the final concentration of phospholipid in medium was 582 μg/mL (Figure 4Bi,ii). This was nearly the maximum concentration because greater than 0.6 mg of phospholipid/mL induces cytotoxicity. The cells were incubated at 37 °C for 2 h.

2.5. Confocal Laser Scanning Microscopy. Cells were seeded onto 6-well plates at a density 10^4 cells/well for 72 h before addition of the drug. Cells were washed three times with PBS and then incubated with 1.6Oct-CL loaded with DXR in the presence or absence of a two volume excess of empty 1.6Oct-CL or SL for 2 h at 37 °C, as described above. After incubation, the cells were washed three times with PBS and fixed with 10% formaldehyde in PBS at room temperature for 15 min. Then, the cells were washed two times with PBS and coated with Aqua Poly/Mount (Polyscience, Warrington, PA, USA) to prevent fading and covered with coverslips. The fixed cells were observed using a Radiance 2100 confocal laser scanning microscope (Bio-Rad, Hercules, CA, USA) with an excitation wavelength at 488 nm and an emission wavelength at 560 nm utilizing a LP560 filter.

2.6. Cellular Distribution of Liposomal CPT-11 Observed by Fluorescence Microscopy. Cells were seeded onto 35 mm glass dishes at 10^4 cells/dish for 72 h before addition of the drug. Cells were treated with medium containing 1.6Oct-CL loaded with CPT-11 for 2 h at 37 °C. After incubation, the cells were fixed and coated as described above. Cells were examined using an inverted microscope, ECRIPS TS100 (Nikon, Tokyo, Japan) with an Epi-Fluorescence Attachment (Nikon) a utilizing a UV1A filter.

2.7. *In vitro* Drug Release. The release of the drug from liposomes into phosphate-buffered saline (PBS, pH 7.4) was monitored by a dialysis method. Dialysis was carried out at 37 °C under sink conditions using seamless cellulose tube membranes Spectra/Por CE (MWCO 2000, Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA). The initial concentration of CPT-11 was 500 μg/mL. The sample volume in the dialysis bag was 1 mL, and the sink solution was 200 mL. After various time intervals, aliquots were withdrawn and the CPT-11 concentrations were analyzed as described above.

2.8. Therapeutic Study. To generate TT tumor xenografts, 1 $\times$ 10^7 TT cells suspended in 100 μL of PBS containing 50% Matrigel (Collaborative Research, Bedford, MA, USA) were inoculated subcutaneously into the flank region of four female ICR nu/nu mice (6 weeks of age, purchased from Oriental Yeast Co., Ltd. Tokyo, Japan). The tumor volume was calculated using this formula: tumor volume = $0.5ab^2$, where a and b are the larger and smaller diameters, respectively. When the average volume of xenograft tumors reached about 100 mm^3, therapy was started (day 1). Antitumor activity was assessed by measuring tumor volume change after intravenous injection of CPT-11. Liposomal CPT-11 (10 mg/kg Oct-CL and 10 mg/kg CL) was administered on days 1 and 4, and 30 mg/kg free CPT-11 and saline were administered on days 1, 4, and 7. The data of free CPT-11-treated or saline-treated mice were referring to our previous data.[24] Tumor volume and body weights were measured for individual animals. The mean increase in life span (% ILS) was calculated using the formula 100 $\times$ {(median day of death in treated tumor bearing mice) − (median day of death in control tumor-bearing mice)}/(median day of death in control tumor-bearing mice). Animal experiments were performed with approval from the Industrial Animal Care and Use Committee at Hoshi University.

2.9. Statistical Analysis. Data are expressed as mean ± SD. The statistical significance of data was evaluated using Student's *t* test. $P < 0.05$ was considered as significant.

3. RESULTS

3.1. Characterization of Oct-Targeted Liposomes. Liposome size and zeta-potential of CL, Oct-CL and SL are listed in Table 2. The average diameter of prepared liposomes was approximately 134−154 nm with a narrow, monodisperse distribution (less than 0.2 polydispersity indexes). As the Oct concentration increased from 0 to 1.6 mol %, the zeta-potential of liposomes decreased. The Oct amount of each Oct-CL was

Table 2. Size and Zeta-Potential of Oct-Targeted Liposomes[a]

formulation	size (nm)	zeta-potential (mV)
CL	151.9 ± 5.5	−5.1 ± 0.1
0.25Oct-CL	141.3 ± 6.1	−11.2 ± 3.5
0.8Oct-CL	141.6 ± 9.4	−17.8 ± 6.9
1.0Oct-CL	153.9 ± 6.2	−18.1 ± 4.3
1.2Oct-CL	134.2 ± 5.4	−15.3 ± 1.9
1.4Oct-CL	147.6 ± 2.4	−17.3 ± 4.7
1.6Oct-CL	136.6 ± 3.2	−19.5 ± 1.3
SL	144.7 ± 1.6	−20.1 ± 1.6

[a] Mean ± SD (n = 3).

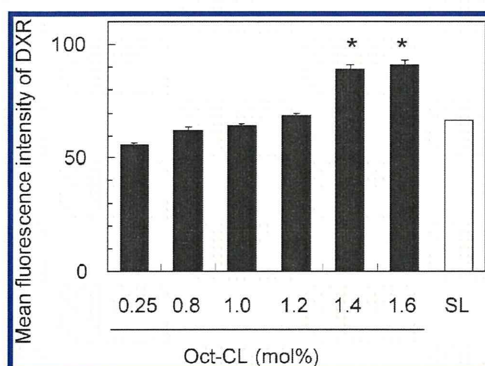

Figure 2. Effect of Oct surface density on cellular association of Oct-CL. DXR-loaded liposomes modified with various Oct-PEG-DSPE concentrations (mol %) were incubated with TT cells at 50 μg/mL DXR for 2 h at 37 °C. Each value represents the mean ± SD of three experiments. (*) Differences are statistically significant from SL at $P < 0.05$.

more than 70% of the theoretical values. The loading efficiencies of CPT-11 were approximately >82% in all liposomes except CL (data not shown). The average diameter and amount of CPT-11 loaded in all types of liposomes did not change for at least 1 month at 4 °C in the dark (data not shown).

3.2. Effects of Oct Surface Density of Oct-CL on Cellular Uptake. We examined the selectivity of Oct-CL for delivery into TT cells, which highly overexpress SSTR2, by flow cytometry. As shown in Figure 2, the mean fluorescence intensities of 0.8Oct-CL, 1.0Oct-CL, 1.2Oct-CL, 1.4Oct-CL and 1.6Oct-CL were approximately 1.1-fold, 1.2-fold, 1.2-fold, 1.7-fold and 1.7-fold greater than for SL, respectively, after a 2 h incubation. The cellular uptake of free DXR was ~3 times higher than 0.25Oct-CL (data not shown). When paying attention to the effects of the Oct surface density of liposomes on cellular association, a higher Oct surface density, more than 1.4 mol % of liposomes was more effectively associated with TT cells. The fluorescence intensities of 1.6Oct-CL after a 2 h incubation increased 2-fold more than after 1 h, whereas that of SL did not increase (data not shown). This finding indicated that the cellular association of Oct-CL increased in an incubation-time-dependent manner, but that of SL was not. From this result, 1.6Oct-CL was used in the following experiments as Oct-CL, and SL was used as a control for the 2 h incubation.

3.3. Competitive Inhibition Study. First, to investigate the cellular association of Oct-CL *via* SSTR, a competitive inhibition study was performed using free Oct as a competitive inhibitor. In the presence of a 20-fold excess of free Oct (84 nmol/mL

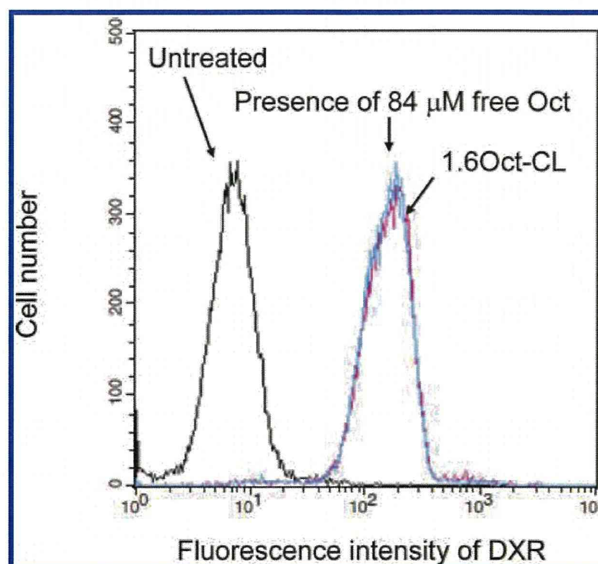

Figure 3. Association of Oct-CL loaded with DXR with TT cells in the presence or absence of free Oct by flow cytometry. 1.6Oct-CL loaded with DXR was incubated in the presence of a 20-fold excess of free Oct (84 μM) at 50 μg/mL DXR for 2 h at 37 °C. Untreated indicates autofluorescence of untreated cells.

medium) with a 2 h incubation, a competitive effect, a decrease of cellular uptake of Oct-CL, was not observed (Figure 3). Next, we tried to use Oct-CL without drug loading (empty Oct-CL) as a competitive inhibitor, because addition of drug-loaded liposomes has the possibility of increasing the cytotoxicity due to DXR. Figure 4A illustrates the scheme of the competitive cellular association of Oct-CL loaded with DXR with empty Oct-CL. The cellular uptake of Oct-CL loaded with DXR in the presence of two excess volumes of empty Oct-CL (i) was compared with that of empty SL (ii) by flow cytometry (Figure 4B) and confocal microscopy (Figure 5). As shown in Figure 4B, the mean fluorescence intensities of DXR loaded with Oct-CL in the presence of empty Oct-CL decreased by approximately half compared with in the presence of empty SL. This finding indicated that the cellular uptake of Oct-CL loaded with DXR was blocked significantly by empty Oct-CL compared with empty SL.

To confirm differences in the cellular uptake of Oct-CL loaded with DXR in the presence of empty Oct-CL or SL, intracellular localizations were observed by confocal microscopy (Figure 5). The presence of empty Oct-CL decreased the localization of DXR-loaded Oct-CL in the nucleus (red fluorescence of DXR) compared with that of empty SL, corresponding to the results of Figure 4Bi and Figure 4Bii, respectively.

3.4. Drug Release from Liposomal CPT-11. Before the investigation of cellular uptake of 1.6Oct-CL and SL, the release of drug from each liposome was examined. The profiles of CPT-11 release versus time are presented in Figure 6. Both 1.6Oct-CL and SL showed slow drug release, about 17% drug release for 24 h in PBS at 37 °C. There were no significant differences between 1.6Oct-CL and SL in drug release at each time point. This result suggested that 1.6 mol % Oct-modification did not affect drug release from liposomes.

3.5. Cellular Uptake Oct-CL Loaded with CPT-11. Next, we tried to observe the cellular distribution of liposomes loaded with CPT-11 by fluorescence microscopy (Figure 7). TT cells were

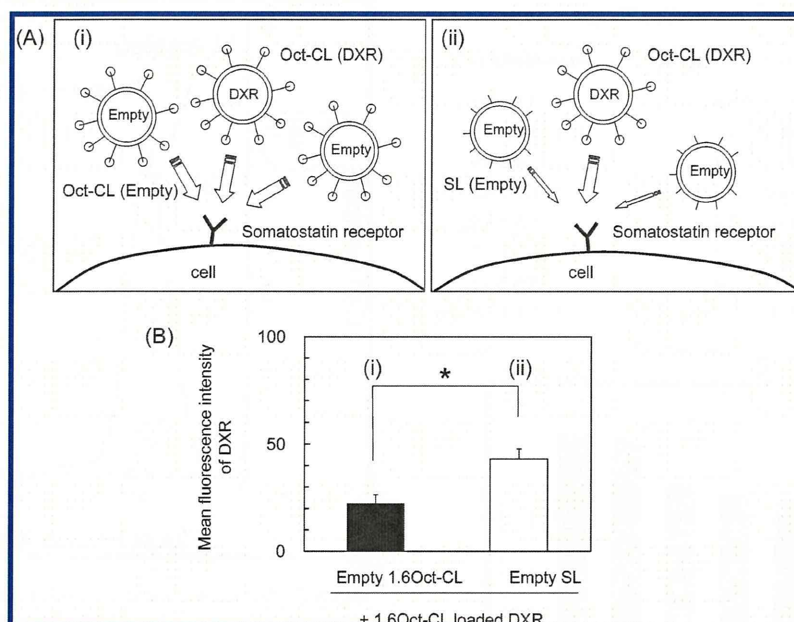

Figure 4. Scheme (A) and DXR fluorescence (B) of cellular association of 1.6Oct-CL loaded with DXR with TT cells in the presence of two excess volumes of empty 1.6Oct-CL (i) or empty SL (ii) for 2 h at 37 °C by flow cytometry. Each value is the mean ± SD for three experiments. (*) Differences are statistically significant at $P < 0.05$.

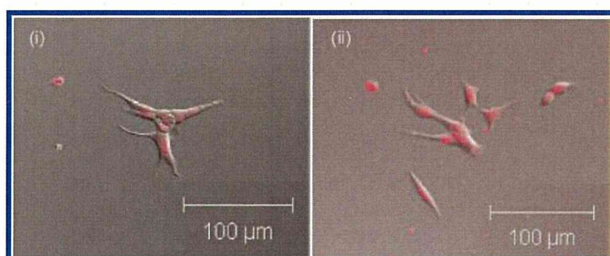

Figure 5. Effects of empty 1.6Oct-CL (i) or SL (ii) on the cellular association of 1.6Oct-CL loaded with DXR with TT cells determined by confocal microscopy. The experimental conditions were the same as for Figure 4B. Scale bar denotes 100 μm.

incubated with Oct-CL loaded with CPT-11 for 2 h at 37 °C. Blue fluorescence due to CPT-11 (Figure 7A) was observed weakly in Oct-CL loaded with CPT-11 at the same location as TT cells (Figure 7B). This finding indicated that Oct-CL loaded with CPT-11 was taken up into the cells as well as Oct-CL loaded with DXR.

3.6. Effect on Cytotoxicity of Oct-Targeted Liposomes. To evaluate the cellular uptake of Oct-CL loaded with CPT-11, the cytotoxicity with TT cells was measured using a WST-8 assay. The doubling time of TT cells is 83 h; therefore, a 96 h incubation was set in this experiment. As shown in Table 3 and Figure S1 in the Supporting Information, free CPT-11, Oct-CL, and SL increased cytotoxicity in an incubation-time-dependent manner. Free CPT-11 showed higher cytotoxicity than liposomal CPT-11 with 48 h and 72 h incubations. After a 96 h incubation, the IC50 value of Oct-CL was the highest (1.05 ± 0.47 μM), whereas free CPT-11 (3.76 ± 0.61 μM) and SL (3.05 ± 0.28 μM) gave similar cytotoxicity results. Therefore cytotoxicity of Oct-CL loaded with CPT-11 may be due to cellular uptake of liposomal CPT-11.

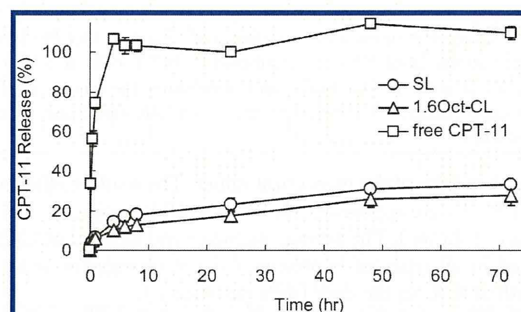

Figure 6. Percentage of CPT-11 released from 1.6Oct-CL and SL as a function time at 37 °C. CPT-11 concentrations were measured as described in Materials and Methods using PBS as a sink solution at pH 7.4. Each value represents the mean ± SD of three experiments.

In addition, 8.4 μM Oct originating from empty 1.6Oct-CL significantly decreased TT cell viability to 60% after a 96 h incubation compared with empty SL (corresponds to the amount of PEG-DSPE of Oct-CL) (Figure 8). Cytotoxicity of liposomes modified with more than about 12 μM Oct was observed due to the lipids. On the other hand, cytotoxicity of free Oct was not observed independent of the Oct concentration (data not shown).

3.7. Antitumor Effect of Oct Modified Liposomes on TT Tumor Xenografts. To examine the effect of Oct modification of liposomes on cytotoxicity *in vivo*, the antitumor activity of Oct-targeted liposome and nontargeted CL loaded with CPT-11 was evaluated following two intravenous injections into TT tumor bearing mice (Figure 9). 1.6Oct-CL reduced the tumor size in mice after the final injections, and the reduced tumor size was maintained until day 23, whereas CL maintained tumor growth suppression only until day 10. 1.6Oct-CL suppressed tumor growth significantly compared with CL, free CPT-11, or saline.

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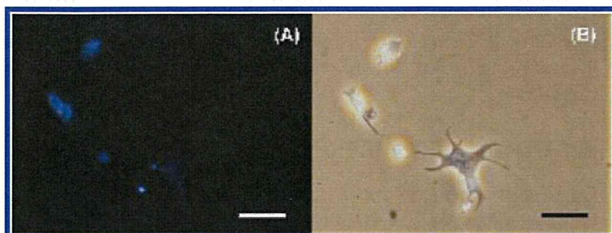

Figure 7. Cellular uptake of liposomal CPT-11 observed by fluorescence microscopy. TT cells were incubated with 1.6Oct-CL loaded with CPT-11 for 2 h at 37 °C. Blue fluorescence; location of CPT-11. Dark field (A), and bright field (B). Scale bar, 50 μm.

Table 3. IC50 of Free CPT-11 and Liposomes Loaded with CPT-11 on TT Cells after Various Incubation Times[a]

	IC50 (μM)		
formulation	48 h	72 h	96 h
free CPT-11	7.43 ± 6.73	5.10 ± 1.85	3.76 ± 0.61
1.6Oct-CL	29.05 ± 19.40	8.72 ± 1.14	1.05 ± 0.47*
SL	22.50 ± 19.50	8.65 ± 2.26	3.05 ± 0.28

[a] Mean ± SD (n = 4). (*) Differences are statistically significant from SL at $P < 0.05$.

Body weight loss was not observed in any of the groups (data not shown). Median survival for mice treated with saline was 68 days, compared with 88 days for free CPT-11-treated, 103 days for CL-treated, and 217 days for 1.6Oct-CL-treated mice. Treatment with liposomal CPT-11 significantly increased survival time. Therefore, %ILS of 1.6Oct-CL treated group was significantly improved compared with that of the CL-treated and free CPT-11-treated groups. This finding indicated that Oct modification of liposomal CPT-11 enhanced antitumor effect *in vivo*.

4. DISCUSSION

In this study, we prepared Oct-CL loaded with CPT-11 and demonstrated that high Oct-surface-density significantly increased the cellular association of Oct-CL *via* SSTR and approximately 2-fold higher cytotoxicity when compared with free CPT-11 and PEGylated liposomes with TT cells using a 96 h exposure period. Recently, it was reported that 0.5 mol % Oct-modified liposomes loaded with anticancer drug were selectively taken by the cells and were effective for the treatment of SSTR-positive breast cancer and gastric cancer.[22,23] In the case of transferrin-targeted liposomes modified with transferrin-PEG-DSPE, the cellular uptake of liposomes was dependent on the concentration of transferrin-PEG-DSPE.[28] Therefore, we increased the concentration of Oct-PEG-DSPE in the liposomes and found that the cellular uptake of the Oct-CL increased more effectively with high Oct-surface-density, more than 1.4 mol % (Figure 2).

Two striking observations in this study were as follows. First, the cellular uptake of Oct-CL was significantly inhibited by empty Oct-CL. The competitive inhibition study of cellular uptake of ligand-modified liposomes was generally done using excess free ligands, not using ligand-liposomes such as Oct-CL. For example, it was reported that the cellular uptake of transferrin-targeted liposomes was inhibited by 20-fold excess transferrin.[28] In this study, 20-fold excess free Oct did not inhibit the cellular uptake of Oct-CL (Figure 3). In the competitive inhibition study *via*

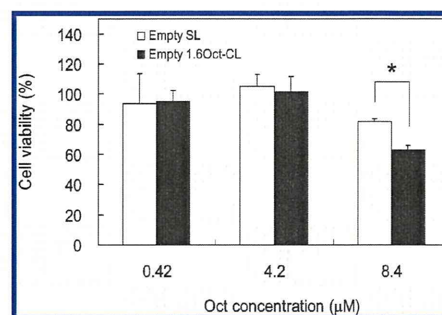

Figure 8. Effect of Oct concentrations on cell viability of Oct-CL compared with SL. TT cells were incubated with empty 1.6Oct-CL or empty SL for 96 h at 37 °C. (*) Differences are statistically significant at $P < 0.05$.

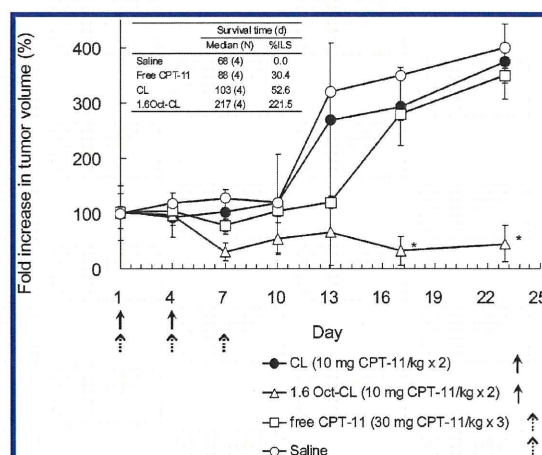

Figure 9. Effect of Oct-modification of liposomal CPT-11 on antitumor activity in mice bearing TT cells. Antitumor activity of Oct-CL loaded with CPT-11 was compared to that of CL or free drug. Liposomal CPT-11 (△, 10 mg/kg Oct-CL; ●, 10 mg/kg CL) was administrated on days 1 and 4, and free CPT-11 (□, 30 mg/kg) and saline (○) were administrated on days 1, 4, and 7. Arrows indicate the day of drug injections. Each value represents the mean ± SD (n = 4). (*) Differences are statistically significant from free CPT-11 at $P < 0.05$.

SSTR, 100-, 1,000- or 1,000,000-fold excess of Oct was used.[29−31] These experiments had a very high cost, and Oct has been reported to be used as an anticancer drug.[32,33] This information suggested that the use of such an excess Oct was not suitable for competition of the cellular uptake of Oct-CL because of increases in cytotoxicity. Gabizon et al.[34] reported that liposome binding is multivalent, in other words, several ligands contribute to cellular uptake, and the overall affinity for the target cell is the product of the individual affinities of the ligands participating in binding. It could be predicted that the affinity of free Oct and Oct-CL to SSTR are not the same. In this regard, we used empty Oct-CL and empty SL as a competitive inhibitor and as a control, respectively (Figure 4B). The cellular uptake of Oct-CL loaded with drug (Oct concentration 7.2 μM) was inhibited significantly by empty Oct-CL with 14.4 μM Oct, compared with empty SL. This finding indicated that Oct-CL associated *via* SSTR, and the affinity of Oct-CL to SSTR was substantially higher than that of free Oct.

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Second, the cytotoxicity of Oct-CL loaded with CPT-11 incubated for 96 h was higher than that of free CPT-11 and SL loaded with CPT-11. The cytotoxicity of free CPT-11 increased in an incubation-time-dependent manner from 24 to 96 h in TT cells, as reported previously.[4] The long incubation times may lead to metabolization of CPT-11 to SN-38, the active form of CPT-11 in TT cells. As a result, the cytotoxicity of free CPT-11 after the 96 h incubation increased 6-fold compared with at 48 h, and that of Oct-CL increased ~25 times, resulting in the highest cytotoxicity among free CPT-11 and SL. These findings suggested that a long incubation time caused CPT-11 release from the inner liposomes, which was converted to the active form SN-38, and consequently increased the cytotoxicity. The receptor-mediated endocytosis mechanism of Oct-CL significantly facilitated cellular uptake and the cytotoxic potential of CPT-11 compared with SL.

The question remains why 1.6Oct-CL showed higher cytotoxicity than free CPT-11, which freely diffuses into cells. Empty 1.6Oct-CL as a control of 1.6Oct-CL (correspond to 4.2 μM of Oct-PEG-DSPE) at 96 h incubation in the cytotoxicity experiments was shown to have no effects (Figure 8). However, empty 1.6Oct-CL showed higher cytotoxicity than empty SL liposomes modified with PEG-lipid corresponded to Oct-PEG-DSPE (8.4 μM) of 1.6Oct-CL at 96 h incubation, at the concentration where free Oct did not show cytotoxicity, suggesting that Oct as a ligand showed cytotoxicity.

With regard to Oct activity, Oct was reported to produce an antiproliferative action in insulinoma cells and pituitary tumor cells.[12,35] These findings suggested that Oct may show an antiproliferative effect in TT cells. From this, Oct may lead empty Oct-CL to showing a stronger cytotoxicity than empty SL because Oct-targeted liposomes were taken up effectively *via* SSTR. However, further experiments are needed to clarify these points *in vitro*.

To examine the effect of Oct-modification of liposomal CPT-11 on cytotoxicity *in vivo*, the antitumor activity of 1.6Oct-CL loaded with CPT-11 was compared with that of free and liposomal CPT-11 in mice bearing TT cells (Figure 9). In an *in vitro* study, Oct increased the cellular association of liposomal CPT-11 (Figure 2), and empty 1.6Oct-CL decreased the viability of TT cells (Figure 8). Therefore, it was estimated that 1.6Oct-CL selectively associated with TT tumor xenografts, led to significantly higher antitumor activity, prolonged the survival time and improved % ILS, compared with CL and free CPT-11 at a one-third dose and lower administration times with free CPT-11. This finding suggested that Oct-modification of liposomal CPT-11 improved therapeutic efficacy for MTC.

5. CONCLUSION

In conclusion, the present study showed that higher concentrations of modified Oct-CL associated effectively with TT cells *via* the somatostatin receptor and had higher cytotoxicity than free CPT-11 or PEGylated liposome SL. These findings indicated that Oct-targeted liposomes loaded with CPT-11 may offer considerable potential for MTC chemotherapy because cytotoxicity of both CPT-11 and Oct was enhanced by effective cellular uptake *via* the somatostatin receptor.

■ ASSOCIATED CONTENT

Supporting Information. Figure S1 depicting cytotoxicity of free CPT-11, SL loaded with CPT-11, and 1.6Oct-CL loaded with CPT-11 for TT cells incubated for 48, 72, or 96 h. This material is available free of charge via the Internet at http://pubs.acs.org.

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PEGylated lipidic systems with prolonged circulation longevity for drug delivery in cancer therapeutics

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Cancer therapy is hampered by severe dose-limiting side-effects that reduce the efficacy of cancer treatments. In improving cancer therapy, lipidic nanoparticle systems for drug delivery, such as liposomes and emulsions, have received much attention because they are capable of delivering their drug payload selectively to cancer cells and of circulating for a long period in the bloodstream. The physicochemical properties of particles are crucial for prolonging their longevity in the circulation. PEG modification, PEGylation of particles is one of the methods able to provide this functionality. This review provides an overview of current strategies for more effective cancer therapy using PEGylated lipidic particle systems for drug and gene delivery and for their improvement in the future. Here, we focus on cancer treatment via systemic injection of drugs or genes capable of prolonged maintenance in the circulation in PEGylated lipidic particles with passive targeting. In addition, we discuss the requirements for longevity in the circulation even for drug systems with active targeting using particles with a tumor-specific moiety.

Key words: PEG lipid – Prolonged circulation – Targeting – Drug delivery – Gene delivery – Cancer – Liposome – Emulsion – Diagnosis – Lipidic particle.

Cancer is a class of disease in which a group of cells displays uncontrolled growth that is different to that of normal cells. Most cancer drugs in current use target the high proliferation rate of cancer cells by acting on cellular targets associated with cell division. Therefore, severe side-effects can occur due to the non-selective nature of anticancer drugs on normal and cancer cells, and dose reduction may be necessary, which reduces the efficacy of cancer treatment. To overcome this problem, new targeted-therapy drugs, such as molecular target drugs, have been developed. Another approach is to develop cancer cell specific drug delivery systems using particle carriers in conjunction with unmodified, conventional drugs.

Anticancer drugs, in general, have a low molecular weight, which following i.v. injection, distribute throughout the body immediately because they can leak easily out from blood vessels. On the other hand, particulate drugs cannot extravasate through the walls of blood vessels because their size is larger than the gaps of the tight junctions of epithelial cells. In addition, particulate drugs cannot be eliminated through kidney filtration. Therefore, particles can circulate for a prolonged period. Furthermore, particles have the potential to act as a delivery system to carry drugs to the desired site by simple modifications to the particle surface with functional molecules. Among such carriers, liposomes are the most widely studied. Recently, these systems, especially liposomes, have been applied extensively for the delivery of genes and diagnostic agents as well as anticancer drugs. The attraction of lipidic particles lies in their composition, which makes them both biocompatible and biodegradable.

When injected intravenously, particles are rapidly cleared by mononuclear phagocytotic cells (MPS), mainly represented by Kupper cells in the liver and spleen macrophages. The recognition of particles by macrophages usually occurs through specific recognition by cellular receptors specific for plasma proteins that have been adsorbed on the particle surface. Polyethylene glycol (PEG)-coated particles, called PEGylated particles or sterically stabilized particles, evade uptake by MPS and show enhanced accumulation in solid tumors as a result of increased microvascular permeability and defective lymphatic drainage, a process also referred to as the enhanced permeability and retention (EPR) effect [1]. This is a passive and nonspecific process of extravasation that is improved by the prolonged residence time of nanoparticles in the circulation, which is also referred to as passive targeting.

In this review, we discuss of PEGylated lipidic systems, such as liposomes and emulsions, for use in drug and gene delivery with an emphasis on liposomes.

I. PASSIVE DRUG TARGETING

1. PEGylated liposomes

Liposomes were discovered by Bangham *et al.* [2]. Liposomes are self-assembling colloid structures composed of lipid bilayers surrounding an aqueous compartment, which can entrap hydrophilic and hydrophobic drugs (*Figure 1*). Conventional (neutrally and negatively charged) liposomes after i.v. administration are rapidly cleared from the blood circulation by MPS as described in the introduction. Small size and specific physicochemical properties of particles, such as rigidity, surface charge, and surface modification, are required for prolonged maintenance in the circulation for use in liposomal drug delivery systems. Small, rigid liposomes with high entrapment efficiency for drugs are required. Particles with a size of less than ~200 nm

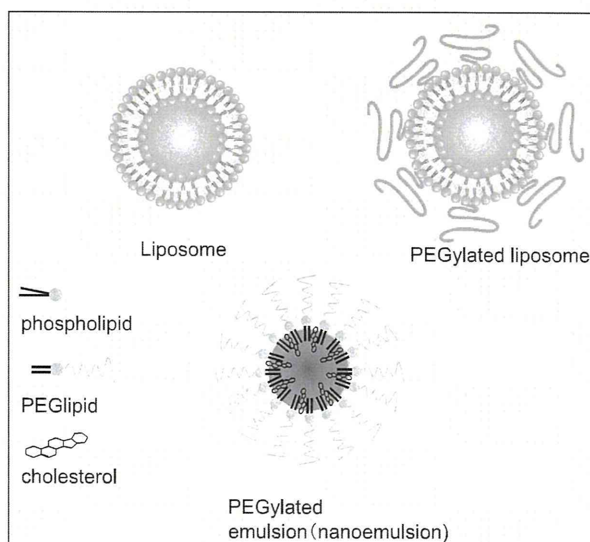

Figure 1 - Structure of nanocarriers.

are preferable for prolonged longevity in the circulation. Liposomes composed of rigid lipids with a higher phase transition temperature reduce serum protein interactions, which destabilize the liposomal structure, resulting in reduced release of the entrapped drug while in the blood circulation.

The development of liposomes led to the finding that the incorporation of hydrophilic polymers, such as polyethylene glycol (PEG) lipids, on the surface of liposomes led to their evasion of uptake of MPS and prolonged the duration of liposomes in the systemic circulation significantly [3, 4]. The structure of PEGylated liposomes and emulsions is shown in *Figure 1*. Liposomes coated with PEG are referred to as sterically stabilized liposomes, PEG-coated liposomes, PEGylated liposomes, or stealth liposomes. PEGylation has been performed using PEG-polymers and PEG-lipids. The former are adsorbed to the particle. The latter are often used for two different approaches; one is a pre-coating method with the preparation of liposomes using lipids and lipid derivatives of PEG; the second is a post-coating method. For ligand-linked liposomes, PEG-lipids are introduced by incubation with pre-formed liposomes at temperatures above the phase transition temperature because these PEG-lipids form micelles (critical micelle concentration, CMC; ~3 and 6 µM of PEG2000-DSPE and PEG5000-DSPE, respectively, by a fluorescence probe method using pyrene) and then transferred to the liposomes. Drugs are usually loaded after PEGylation. The presence of amphipathic PEG does not interfere with the loading of drug. For immunoliposomes, the former pre-coating method does not allow for coupling of the antibody due to steric hindrance with PEG, which has already been introduced on to the liposome surface. In the post-coating method, immunoliposomes are generated via a maleimido linker conjugated as described in section 5.1.

PEGylated liposomal doxorubicin (Doxil in USA, Caelyx in Europe) was the first and is still the only stealth liposome formulation for the treatment of Kaposi's sarcoma and recurrent ovarian cancer. Myocet was also approved as liposomal doxorubicin (DXR) without PEGylation. A listing of approved liposome formulations is given in *Table I*. Liposomal anthracyclines have been formulated to attenuate drug-related toxicity, such as cardiomyopathy and bone marrow depression, rather than to enhance antitumor efficacy.

The hydration layer of PEG stabilizes liposomes [5] and increases the circulation longevity of drug carriers by reducing protein binding. Serum proteins of the immune system, acting as opsonins, attach to particles and increase their subsequent uptake by macrophages. Among them, the C3 protein of the complement system plays a major role in the immune system's recognition of foreign particles. PEG increases circulation longevity by decreasing protein binding; a finding supported by both *in vitro* studies that have demonstrated a masking effect of PEG against protein adsorption to liposome surfaces [6] and *in vivo* studies, in which low protein binding in the bloodstream correlated with longer circulation times [7]. However, there is sufficient conflicting data to warrant a reassessment of the mechanism of PEGylation. Other studies have shown that the presence of bound serum proteins did not result in increased macrophage uptake and that pre-incubating the liposomes with serum lowered macrophage uptake [8]. To our current knowledge, the absorbed amount of serum albumin on PEGylated liposomes was not greatly different to that of non-PEGylated liposomes following incubation with albumin *in vitro*, and pre-incubating the PEGylated liposomes with serum albumin circulated for much longer than PEGylated liposomes alone [9]. Regardless of the mechanism, however, it is clear that PEG significantly increases the circulation longevity of liposomes, and substantial tumor accumulation by passive targeting occurs.

1.1. Characterization of PEGylated liposomes *in vitro*

Small-sized liposomes (small unilamellar vesicles; SUV) have a small inner water volume, in which hydrophilic drugs are entrapped (*Figure 1*). The size and lamellarity, i.e., inner water volume of liposomes can be varied by the preparation method of liposomes. In entrapping drug into liposomes, hydrophilic drugs are entrapped in the large inner water capacity of liposomes and hydrophobic drugs in the lipid bilayers of liposomes, multilamellar vesicles (MLV). Remote loading methods are used extensively for amphiphilic drugs because the sizes of liposomes are already adjusted by sonication or extrusion, and then drug can be loaded via, for example, pH gradient. In this case, the amount of unentrapped drug is minimal.

PEGylated liposomes containing low cholesterol (Chol) levels decreased the encapsulation efficacy of DXR, but PEGylated liposomes containing high Chol levels did not decrease it. Incorporation of PEG-lipid aids in the formation of uniform, small, mono-disperse particles. The zeta potential decreased substantially after PEG-coating; however, the PEGylated liposomes were stable due to steric repulsion and exhibited less aggregation. Release is important in liposomal drugs with prolonged maintenance in the blood, depending on the encapsulation method. Johnston *et al.* [10] reported that because the release of drug from liposomes is critical, prolonged circulation did not reflect the high antitumor effect of liposomal drugs. The half-life of vincristine entrapped in liposomes prepared with different drug to lipid ratios varied from 6.1 h to 117 h. The antitumor effect in a xenograft model was most pronounced with an intermediate release half-life 15.6 h. Highly entrapped liposomal vincristine may barely be released. Vincristine is a cell cycle specific drug and, therefore, the release rate may influence antitumor efficacy markedly.

1.2. Pharmacokinetics

PEGylation decreased the volume of distribution, slowed the elimination of liposomal drug, and thereby increased the AUC, but this effect seemed to plateau between 3 and 6 % and at a molecular weight for PEG of above 1900. Further increases in PEG percentage may not alter the pharmacokinetic properties of the DSPC/Chol liposome system significantly [11]. PEGylated, sterically stabilized liposomes show dose-independent, first order pharmacokinetics [12].

Surprisingly, it was reported that prolonged maintenance in the circulation did not always increase tumor accumulation. Accumula-

Table I - Clinically approved liposome-based therapeutics.

Active agent	Trade name	Composition	Indication
Liposomal amphotericin B	AmBisome	HSPC/DSPG/Chol	Fungal and protozoal infections
Liposomal cytarabine	DepoCyt	DOPC/DPPG/Chol/triolein	Malignant lymphomatous meningitis
Liposomal daunorubicin	DaounoXome	DSPC/Chol	HIV-related Kaposi's sarcoma
Liposomal doxorubicin	Myocet	EPC/Chol	Combination therapy with cyclophosphsmide in metastatic breast cancer
Liposome-PEG doxorubicin	Doxil/Caelyx	HSPC/Chol/PEG-DSPE	HIV-related Kaposi's sarcoma, metastatic breast cancer, metastatic ovarian cancer

All agents: i.v. administration. HSPC: hydrogenated soy phosphatidylcholine. DSPG: distearoylphosphatidylglycerol. Chol: cholesterol. DOPC: dioleylphosphatidylcholine. DPPG: dipalmitoylphosphatidylglycerol. EPC: egg phosphatidylcholine.

tion of liposomal lipid and encapsulated DXR in murine Lewis lung carcinoma showed a lack of beneficial effects with PEGylated liposomes [13]. Extended time-course studies evaluating lipid and drug levels in plasma and tumors after i.v. administration indicated that the tumor-accumulation efficiency (AUC(Tumor)/AUC(Plasma)) was greater for non-PEGylated liposomes. The AUC(Plasma) and antitumor effects after administration of DXR encapsulated in liposomes and in PEGylated liposomes were similar [13]. Hong *et al.* [14] reported that to enhance the therapeutic effect of liposomal drugs, simply increasing plasma AUC by PEG-coating may not be satisfactory. PEGylated liposomal DXR showed two-fold higher AUC than liposomal DXR. The tumor-accumulation efficiency (AUC(Tumor)/AUC(Plasma)) of non-PEGylated liposomal DXR was more than two-fold higher than that of the PEGylated liposomal DXR. The survival of both liposomal drug groups was not significantly different. Recently, Cui *et al.* reported that enhanced drug accumulation of PEGylated liposomal DXR with different sizes did not correlate with increased antineoplastic effect [15].

1.3. Subcutaneous (s.c.) and intraperitoneal (i.p.) administration

Administration route is an important for drug distribution. PEGylated liposome levels were significantly higher in the draining lymph nodes after s.c. administration in mice. The liposomes subsequently appeared in blood and had similar biodistribution, pharmacokinetics, and half-lives to liposomes given by the i.v. route [16, 17]. This method was applied for the lymphatic delivery of methotrexate [18].

In the case of PEGylated liposomal ganciclovir by i.v. and i.p. injection, i.v. injection exhibited a 1.2-fold higher AUC value than the i.p. injection. I.p. injection was also suitable for longevity in the circulation as well as i.v. injection [19].

2. PEGylated emulsion (nanoemulsion)

Emulsions usually refer to oil droplets dispersed in a continuous liquid phase. Emulsion is stable for sterilization, and the safety of fat emulsion has been confirmed from its use as total parenteral nutrition since 1968. Microemulsion has a bicontinuous structure of water and oil in a colloidal form [20]. In pharmaceutical terms, nanoemulsion refers to an oil droplet with a diameter in the nano-size range, which is also called injectable emulsions. Microemulsions and nanoemulsions allow the delivery of larger doses of poorly water-soluble drugs compared with liposomal or miceller formulations. The use of these emulsions for i.v. drug delivery is limited due to the requirement for high levels of biocompatible surfactants in their formulations and associated risks of toxicity. PEGylated nanoemulsions are of interest in passive drug targeting delivery.

Liu *et al.* [21] published the first report on emulsions with prolonged circulation times (oil-in-water) as carriers for lipophilic drugs. Inclusion of PEG derivatives, such as Tween-80 or dioleoyl N-(monomethoxy-polyethyleneglycol succinyl)phosphotidylethanolamine (PEG-PE), into emulsions composed of castor oil and phosphatidylcholine decreases MPS uptake and increases the blood residence time of the emulsion. The activity of PEG derivatives in prolonging the circulation time of emulsions depends on the PEG chain length (PEG2000 ≥ PEG5000 > PEG1000, Tween-80) [21]. Lipospheres with prolonged circulation time containing 6-mercaptopurine were prepared by solidification of a microemulsion at low temperature and incorporating palmitoyl PEG. By increasing the circulation time of lipospheres, tissue accumulation of the drug correlated with the pharmacokinetic behavior of the lipospheres [22].

Recently, we reported injectable PEGylated emulsions composed of vitamin E, Chol, and PEG2000-DSPE and acracinomysine or vincristine. These emulsions showed prolonged circulation times, high accumulation of drug in tumor tissue, and effective anticancer effect *in vivo* compared with free drug. Furthermore, we reported an optimized design for folate-linked emulsion for active targeting [23, 24]. PEGylated parenteral emulsion encapsulated etoposide composed of soybean oil, egg lecithin, Chol, and PEG2000-DSPE [25], and PEGylated nanoemulsion incorporated chlorambucil [26] produced improved pharmacokinetic profiles with longer circulation times than that of non-PEGylated controls.

A new approach has been reported using reconstituted chylomicron emulsion with gadolinium incorporated acetylacetonate (GdAcAc) composed of commercially available natural and biocompatible lipids as an artificial chylomicron remnant for gadolinium (Gd) neutron capture therapy (NCT) for tumor therapy. A significant proportion of the PEGylated, GdAcAc-incorporated emulsion remained circulating in the blood 5 h after injection into mice, while the PEG-free emulsion mainly accumulated in the liver [27].

II. PASSIVE GENE TARGETING

Nucleic acid-based drugs acquire other benefits from liposomal encapsulation. Research has illustrated that systemic administration rarely achieves meaningful cell transfection. This is most likely due to the high nuclease levels present in serum. Among various synthetic carriers currently in use in gene therapy, cationic liposomes are the most suitable transfection vectors. Cationic liposome vectors can stabilize the DNA, protect it from serum nucleases, and ideally interact with biological systems when liposomes are modified with ligands by helping to target the DNA to particular cells, leading to endocytosis. However, a major limitation of these systems is their inability to generate tumor accumulation following i.v. injection. While cationic complexes interact with anionic components of cell membranes and thus trigger cellular uptake by absorptive mediated endocytosis, they also interact with blood components and are subject to clearance by MPS. These problems can be overcome using PEGylation of cationic liposomal vesicles by reducing nonspecific charge interactions and thus prolonging circulation time, e.g. DNA entrapped in PEGylated liposomes, cationic liposome/DNA complex (lipoplex) coated with PEG, and polymer/DNA complex (polyplex) entrapped in PEGylated liposome. Furthermore, gene transfer activity after i.v. injection of a lipoplex is most prevalent in the lung. Finally, there is the challenge of tumor-specific targeting, and active targeting using gene-delivery systems are used to decrease this nonspecific gene transfer in the lung while simultaneously maintaining or increasing the level of gene transfer to the tumor tissues. This will be discussed in section 5 as an active targeting.

With regard to the systemic delivery of carrier and nucleic acid into tumors, as assessed using fluorescently labeled markers, it was reported that cationic polymer PEI polyplex of ODN entrapped into biotinylated PEGylated liposomes showed longer circulation times than complexes between ODN and PEGylated PEI derivatives [28]. PEGylated cationic liposomes (DOTAP/DOPE) loaded with cyclooxygenase-2 (COX-2)-specific siRNA delivered this nucleic acid in tumors [29]. PEG coated lipid nanocapsules containing complexes of DOTAP/DOPE with DNA (lipoplex) with less positive surface charge showed increased circulation time with the density of PEG 2000-DSPE chains, resulting in five-fold greater circulation longevity than for non-coated DNA lipid nanocapsules in mice [30]. Another approach using exchangeable PEG stabilized lipid particle is described in section 4.3.

There are few reports of systemic injection for the delivery of DNA and siRNA into tumors for gene expression and siRNA function. PEGylated liposomes improved the plasma retention, uptake by s.c. tumors, and antitumor activity of encapsulated siRNA. Li and Huang [31] reported a tumor-targeted LPD formulation (liposome-polycation-DNA complex) for siRNA. Downregulation of survivin in human lung cancer cells by targeted LPD induced 90 % apoptosis. The 20-25 mol % PEGylated LPD formulation also significantly improved the tumor localization of siRNA in a human lung cancer xenograft model.

In a mouse model of human prostate cancer, three-fold higher accumulation of siRNA in the tumors was achieved when i.v. ad-

ministration of PEGylated liposome incorporating a PEGylated lipid with longer acyl chains rather than non-PEGylated liposomes, and sequence-specific antitumor activity was observed [32]. Preclinical data was reported for Atu027, a siRNA-lipoplex directed against protein kinase N3, currently under development for the treatment of advanced solid cancer, which is in phase I in 2009. Using systemic administration of Atu027, Aleku *et al.* [33] showed the efficacy of Atu027 in orthotopic mouse models for prostate and pancreatic cancers with significant inhibition of tumor growth and lymph node metastasis.

III. PROBLEMS OF PEGYLATED PARTICLE DELIVERY

1. Clinical side-effects

It was observed with Doxil [34] and other PEGylated liposomal formulations [35] that their infusion into a substantial percentage of human subjects triggers hand-foot syndrome and immediate non-IgE-mediated hypersensitivity reactions; shortness of breath, facial swelling, headache, chills, hypo- and hypertension, chest pain, and back pain. Recently, it has been shown that methylation of the phosphate oxygen of phospholipid-PEG conjugate, and hence the removal of the negative charge, totally prevented complement activation by PEG-lipids [36].

2. ABC phenomena

PEG is non-toxic and non-immunogenic, resulting in particles with prolonged circulation longevity. However, PEGylated empty liposomes are known to lose their prolonged circulation properties with multiple dosing. Recently, it has been reported that the first dose of PEGylated liposomes injected i.v. caused a loss of the prolonged circulation properties and extensive accumulation in the liver following a second dose injected several days later in patients [37], a phenomenon known as accelerated blood clearance (ABC). Besides PEGylated liposomes, other nanocarriers, such as nanoparticles containing PEG, also produced this phenomenon [38]. Recent data obtained in our group showed that ABC was not induced by repeated injections of gadolinium-containing polymeric micelles [39]. It is believed that macrophages in the MPS play an important role in ABC, whereas the mechanism of the immune response on repeated injections of liposomes has not been fully elucidated yet. The components and structures of PEGylated nanocarriers have an essential effect on the induction and activation phase of the ABC phenomenon resulting in recognition by a PEG antibody.

The ABC phenomenon was also reported in gene delivery using PEGylated liposome vectors for systemic administration of ODN, DNA, or siRNA, which can raise an antibody-response against PEG, resulting in rapid elimination following repeated injections [40, 41]. Nucleic acid/PEGylated liposome induced an immunoresponse against PEG because nucleic acid can act as a potent adjuvant.

3. Release from particles

For successful cancer therapy via i.v. administration, it is essential to optimize the stability of particle carriers in the systemic circulation and cellular association after the accumulation of particles in tumor tissue. PEG is useful for conferring stability in the systemic circulation but is undesirable for the cellular uptake and release of the encapsulated drug from the endosomes of the target cells into the cytoplasm because the presence of PEG on the particle surface hinders the entry of liposomes into tumor tissue.

Attempts have been made to enhance the therapeutic efficacy of sterically stabilized particles by shedding, i.e., loss of the coating, after arrival at the target site. This cleavage and exchangeable PEG may facilitate drug release and interaction with target cells. Other methods to increase the release of drug or gene in endosomes have used the incorporation of pH-sensitive phosphatidylethanolamine (PE) (pH-sensitive liposomes).

Chemical stimuli, such as the presence of low pH or reducing agents, and proteases can induce cleavage of the linker between the PEG chains and the anchor lipid. This shedding approach was reviewed by Romberg *et al.* [42]. Liposomes stabilized with either a non-cleavable PEG (PEG-DSPE) or PEG-lipid derivative, in which PEG was attached to a lipid anchor via a disulfide linkage (PEG-S-S-DSPE), retained an encapsulated dye at pH 5.5. However, treatment at pH 5.5 of liposomes stabilized with PEG-S-S-DSPE with dithiothreitol or cytoplasmic and lysosomal enzymes caused release of contents due to cleavage of the PEG chains and concomitant destabilization of DOPE in the liposomes. Exchangeable PEG-lipids rely on slow diffusion from the particle surface at a rate determined by the size of their lipid anchors [43].

pH-sensitive liposomes are stable at physiological pH (pH7.4) but undergo destabilization and acquire fusogenic properties under acidic conditions, thus leading to release of their aqueous contents because these undergo a phase transition under acidic conditions [44]. However, the incorporation of PEG-DSPE into pH-sensitive liposomes decreased the pH-dependent release of content from the liposomes. Hong *et al.* [45] showed that liposomes composed of DOPE, DPSG, and PEG-DSPE (up to 5 %) were pH sensitive, plasma stable, and had a prolonged circulation times in the blood. Furthermore, they released the entrapped markers rapidly in tumor tissue homogenates where the pH is lower than normal healthy tissues. The therapeutic studies performed in a murine model of B-cell lymphoma demonstrated that the anti-CD19-targeted pH-sensitive formulation was superior to stable, long-circulating non-pH-sensitive liposomes despite the more rapid drug release and clearance of the pH-sensitive formulation [46].

Application of pH-sensitive liposomes to gene therapy has not been widely reported. To remove PEG from the carriers via cleavage by a matrix metalloproteinase (MMP), which is especially expressed in tumor tissues, a novel PEG-peptide-DOPE was used. PEG-peptide-DOPE modified liposomal DNA (MEND) stimulated DNA expression in tumor tissues, as compared with conventional PEGylated liposome [47]. Recently, siRNA/cationic liposome complexes were postcoupled to PEG with an oxime linkage, which is pH sensitive, to facilitate acidic pH-triggered release of nucleic acids from endosomes. Hepatitis B virus (HBV) transgenic mice suppressed makers of HBV replication markers by up to three-fold relative to controls over a 28 day period via repeated systemic administration of triggered PEGylated siRNA-nanoparticles [48].

5. PEGylated lipidic system for active targeting

Tumor cell active targeting is a promising strategy for enhancing the therapeutic potential of chemotherapy agents. A variety of targeting moieties capable of recognizing cells, including antibodies, peptides, glycoproteins, and receptor ligands, such as folate, transferring, and RGD, etc., targeting moieties are important for the mechanism of cellular uptake. Longevity in the circulation will allow for effective delivery of liposomes to the tumor site via EPR effect, and the targeting moiety can increase the endocytosis of liposomes. Amphiphilic anticancer drug released into the circulation can be delivered and penetrate into cancer cells. In most cases, cell internalization of the liposomes is important for delivery of other anticancer drugs and for gene delivery.

Ligands are usually attached to the distal end of the PEG chain to extend outside of the PEG surface layer, as shown in *Figure 2A*. The importance of longer PEG chain length of the ligand was demonstrated by Lee RJ and Low PS [49]. However, when targeting moieties are employed, circulation times are often decreased *in vivo* due to recognition by the MPS [50, 51].

5.1. Antibodies and antibody fragments

Among the ligands used for active targeting, antibodies and antibody fragments have superior specificity towards tumor tissues but disadvantages include instability during storage and higher produc-

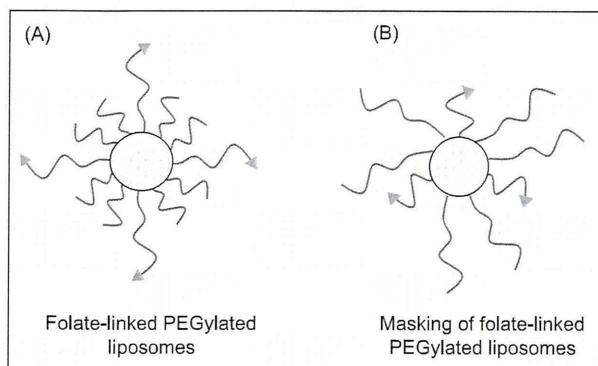

Figure 2 - Structure of folate-linked PEGylated liposomes. Folate ligand with sufficiently long PEG spacers (PEG5000) of PEGylated liposome (PEG2000) (A) increased the folate receptor association *in vitro*. Masking of folate-linked PEGylated liposomes (B) showed a higher tumor killing effect than folate-linked non-PEGylated liposome *in vivo* [65].

tion costs. Liposomes coated with monoclonal antibodies (mAbs) (immunoliposomes) can provide target-specific binding to cells.

Prolonged circulation immunoliposomes (Fab'-PEG immunoliposomes) prepared from a dipalmitoylphosphatidylethanolamine derivative of PEG with a terminal maleimidyl group and a conjugated Fab' fragment of antibody allowed liposomes to evade MPS uptake and remain in the circulation for a long time, resulting in enhanced accumulation of liposomes in the solid tumor [52]. Recently, several *in vivo* studies of gene delivery demonstrated incorporation of PEG into targeted immunolipoplex, resulting in a more efficient delivery of the complex to tumor cells. Antibody-targeted liposomes increased the anticancer efficacy, as compared with passively targeted liposomes [53]. Similarly, sterically stabilized immunoliposomes with a Fab fragment conjugated to the liposome with PEG-DSPE [54], and DNA packaged into neutral PEG-stabilized immunoliposome targeted to the mouse transferrin receptor exhibited prolonged circulation time and tumor targeted gene delivery [55]. Elbayoumil and Torchilin [56] reported that tumor-targeted prolonged-circulation-time PEG-liposomes with Doxil attached to a nucleosome-specific 2C5 antibody demonstrated prolonged circulation and enhanced antitumor efficacy against a broad range of different tumors.

However, in a study, it was shown that antibody targeting of prolonged circulation time liposomes to HER2 does not increase tumor localization but does increase internalization in an animal model. The enhanced antitumor effect was related to increased cancer cell drug uptake rather than increased tumor accumulation [57]. Similarly Hatakeyama *et al.* [58] reported that the enhanced efficacy by immunoliposome Doxil was related to the increase in cellular uptake not tumor accumulation of immunoliposomes.

5.2. Folate

Folate receptor has been found to be overexpressed in a wide range of tumors, such as ovarian, lung, and head and neck cancers, and is only minimally distributed in normal tissues [59]. Therefore, it presents an attractive target for tumor-selective delivery. The vitamin, folic acid, is necessary for the synthesis of purines and pyrimidines. Folate receptor-targeting materials can continuously accumulate into cells due to receptor recycling. On the other hand, antibodies, hormones, and other related ligands are normally internalized to clear the ligand from the receptor in order to discontinue the activated signaling and are shuttled to the lysosome for destruction [60]. Three isoforms of folate receptor have been identified, and two, folate receptor-α and -β, are attached to the cell by a 38 kDa glycosylphosphatidylinositol (GPI)-anchor, while folate receptor-γ is secreted.

Folic acid is an inexpensive, nontoxic, nonimmunogenic, and high-affinity ligand for folate receptor, which retains its receptor-binding and endocytosis properties even if it is covalently linked to a wide variety of molecules. Thus, liposomes conjugated to the folate ligand via a PEG spacer have been used to deliver chemotherapeutic agents, oligonucleotides, and markers to folate receptor-bearing tumor cells [61]. The targeting efficiency of folate-linked vesicles was affected by the amount of folate-PEG-lipid because it was reported that a higher molar fraction of folate-PEG-lipid in folate-linked liposomes reduced liposome uptake into cells [62,63]. Furthermore, as described above, steric hindrance by PEGylation reduced the association of the liposome-bound ligand with its receptor [64]. Therefore, the density and PEG-spacer length of the targeting ligand and PEGylation of liposomes are known to be critical characteristics for ligand-receptor interaction [65].

In a non-solid tumor, the high therapeutic efficacy of folate-linked liposomal DXR was reported in a mouse ascites leukemia model, in which the treatment route was i.p. injection [66]. In addition, the therapeutic efficacy of i.v. treatment with folate-linked liposomal DXR was improved in mice inoculated i.p. with lymphoma cells [67]. In solid tumors, we evaluated folate-mediated association of liposomal DXR with cells overexpressing folate receptor, in terms of the effect of PEG-spacer length and the density of the folate ligand of liposomes with or without PEG-coating, and of the degree of masking of folate ligand on liposomes by adjacent PEG [65] (*Figure 2*). Folate-linked non-PEGylated liposomes showed the highest folate receptor association and cytotoxicity *in vitro*. On the contrary, folate-linked PEGylated liposomes and masking of folate-linked PEGylated liposomes showed a higher tumor killing effect than folate-linked non-PEGylated liposomes *in vivo*. As a result, the advantage of increased targeting from folate-linked PEGylated liposome is obscured by accelerated clearance of ligand-linked formulations.

5.3. Transferrin

The transferrin receptor is a carrier protein for transferrin and is located at the cell surface where it mediates uptake of iron bound to transferrin glycoproteins via receptor-mediated endocytosis. This receptor is necessary for the import of iron into the cell and is regulated in response to intracellular iron concentration. Transferrin receptors are overexpressed in rapidly dividing cells, such as many cancer cells, which is likely to be related to the fact that iron is required for the functioning of an enzyme involved in DNA synthesis [68]. The overexpression of transferrin receptor on several cancer cells enables active tumor targeting using transferrin or transferrin receptor antibodies as liposome-targeting ligands [69]. Therefore, targeting liposomes to transferrin receptors facilitates cellular uptake of liposomes by receptor mediated endocytosis, similarly to folate receptor targeting.

Ishida *et al.* [70] demonstrated that transferrin (Tf)-PEG-liposomes (Tf-PEG-liposomes) showed a prolonged residence time in the circulation and enhanced extravasation of the liposomes into solid tumor tissues in Colon 26 tumor-bearing mice. On application of this system for boron neutron capture therapy, decahydrodecaborate-(10) B-encapsulating Tf-PEG liposomes injected into tumor-bearing mice, showed prolonged retention in the circulation, and enhanced accumulation of (10)B in solid tumors measured by gamma-ray spectrometry [71]. Tf-PEG liposomes encapsulating cisplatin in an i.p. administered group maintained high liposome and cisplatin levels in ascites and showed prolonged residence time in the peripheral circulation. Mice receiving Tf-PEG liposomes by i.p. injection showed significantly higher survival rates compared with those receiving PEG liposomes without Tf, bare liposomes, or free cisplatin solution [72]. Tf-PEG liposomes encapsulated oxaliplatin also showed increased antitumor efficacy via i.v. injection against Colon26 mouse colon carcinoma [73].

5.4. RGD for angiogenic blood vessels

When tumors reach a size of a few millimeters, they need to create new tumor blood vessels through angiogenesis by recruiting new blood vessels from the preexisting vasculature. Angiogenic blood vessels in tumors express several proteins that are attractive targets for ligand-targeted liposomal cancer therapy. Compared with targets that are located on the cancer cell surface, such as folate and transferrin receptors, the targeting of tumor vasculature has certain advantages: antigens on the tumor vasculature are more accessible than antigens on the cancer cell surface for nm-sized liposomes. Ligands for targeting angiogenic blood vessels include peptides containing the RGD (Arg-Gly-Asp) or NGR (Asn-Gly-Arg) sequences. Peptides with the RGD amino acid sequence show affinity for $\alpha(v)$ $\beta(3)$-integrin, which is overexpressed on the angiogenic endothelium as compared with resting endothelial cells of blood vessels in non-diseased tissue.

RGD-peptides coupled to the distal end of PEGylated prolonged circulation time liposomes containing DXR showed superior efficacy over non-targeted PEGylated liposomes in inhibiting C26 DXR-insensitive tumor outgrowth [74]. Gene delivery with integrin $\alpha(v)$ $\beta(3)$-targeted lipidic nanoparticles in tumor-bearing mice by systemic injection resulted in apoptosis of the tumor-associated endothelium, ultimately leading to tumor cell apoptosis and sustained regression of the tumors [75]. Furthermore, it was reported that the neutralized lipoplexes in mice showed decreased accumulation of the particles in the lung as compared with PEGylated cationic lipoplexes. Tumor targeting for DNA delivery via i.v. injection was achieved by the addition of RGD-PEG-Chol as a lipid-ligand in postgrafted lipoplex formation by a chemical, postgrafting reaction [76].

6. Diagnostic agent delivery

The application of particles comprises an emerging field of cellular MR imaging. Small gadolinium (Gd)-based particles are widely used as contrast agents for clinical MRI to detect tumors. In terms of relaxation properties for MRI, an optimal formulation would be liposomes of small size with a permeable bilayer. These liposomes usually are less stable in the serum than liposomes with a more rigid bilayer. Contrast agents delivered into tumor may be not enough to achieve enough contrast. Targeted drug delivery systems that combine imaging and therapeutic modalities in a single particle may offer advantages in the development and application of nanomedicines.

Magnetic-fluid-loaded liposomes sterically stabilized by PEG exhibited prolonged circulation longevity, whereas iron oxide loading tended to favor uptake by the MPS [77]. Novel, bimodal fluorescent and paramagnetic liposomes using a novel Gd lipid designed for liposomal cell labeling with Gd (Gd-liposome) have been described for the labeling of xenograft tumors [78]. Gd-liposomes formulated with low charge and a PEG layer for long systemic circulation, accumulated in tumor tissue according to MRI analysis [78].

Quantum dots are semiconductors, the smaller size of a crystal, have been used in investigations as agents for medical imaging because of their excellent fluorescent properties [79]. The use of these dots has been limited *in vivo* due to the difficulty in obtaining biocompatible forms. *In vivo* imaging of a quantum dot encapsulated in a phospholipid micelle was reported recently [80]. *In vivo* fluorescence imaging shows the luminescent quantum dot (QDs)-conjugated to immunoliposome-based nanoparticles (QD-ILs) of anti-HER2 scFv exhibited efficient receptor-mediated endocytosis in HER2-overexpressing cells. In athymic mice, QD-ILs significantly prolonged the circulation of QDs. In xenograft models, localization of QD-ILs at tumor sites was confirmed by *in vivo* fluorescence imaging [81]. Most recently, a strategy to target hybrid vesicles in solid tumor tissues of tumor-bearing mice has been explored. "Rigid" PEGylated functionalized quantum dot-liposome (f-QD-L) (DSPC:Chol:PEG2000 -DSPE) hybrid vesicles led to rapid tumor accumulation of peak values (approximately 5 % of the injected dose per gram tissue) of QD. More interestingly, this hybrid vesicle tumor retention persisted for at least 24 h [82].

*

Systemic stability in blood flow and local release of the contents from particles in cancer cells are contradictory properties for particles. PEG-modification provides this property in particles. Without external force, PEG can regulate body-controlled particle behavior. More precisely, the distribution of PEGylated particle carriers in tumor cells will be related to the antitumor effect. PEGylated particle carriers of drugs that do not permeate through cell membrane, such as genes, will elucidate the mechanism of the EPR effect and open the way to the next generation of PEGylated particles.

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Research Article

Effects of Polyethylene Glycol Spacer Length and Ligand Density on Folate Receptor Targeting of Liposomal Doxorubicin In Vitro

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The folate receptor is an attractive target for selective tumor delivery of liposomal doxorubicin (DXR) because it is abundantly expressed in a large percentage of tumors. This study examined the effect of polyethylene glycol (PEG) spacer length and folate ligand density on the targeting ability of folate-modified liposomes. Liposomes were modified with folate-derivatized PEG-distearoylphosphatidylethanolamine with PEG molecular weights of 2000, 3400, or 5000. The association of DXR-loaded liposomes with KB cells, which overexpress the folate receptor, was evaluated by flow cytometry at various ratios of folate modification. A low ratio of folate modification with a sufficiently long PEG chain showed the highest folate receptor-mediated association with the cells, but did not show the highest in vitro cytotoxicity. DXR release from folate-modified liposomes in endosomes might be different. These findings will be useful for designing folate receptor-targeting carriers.

1. Introduction

Antitumor drug delivery systems with nanoscopic dimensions have received much attention due to their unique accumulation behavior at the tumor site. Various nanoparticulate carriers such as liposomes, polymer conjugates, polymeric micelles, and nanoparticles are utilized for selective delivery of various anticancer drugs to tumors in a passive targeting manner [1]. However, a more effective and active targeting system is needed to enhance the uptake of drugs using nanocarriers into cancerous cells at the tumor site.

Receptor-mediated endocytosis pathways have been exploited for tumor-specific targeting of nanocarriers and intracellular delivery of their contents. Modification of carriers with a ligand directed to an overexpressed receptor in cancer cells can improve selectivity and facilitate the movement of carriers into the intracellular compartment. One such candidate ligand is folic acid because the folate receptor-α is overexpressed in a number of human tumors, including ovarian, lung, brain, head and neck, and breast tumors [2–4]. Folic acid has been widely employed as a targeting moiety for various anticancer drugs through covalent conjugation to anticancer drugs and nanocarriers [5–8]. Liposomes modified with folic acid showed selective targeting toward human carcinomas along with enhancement of doxorubicin (DXR) cytotoxicity in vitro [9].

Ligand density per drug carrier and spacer length are important in designing suitable carriers for targeting. However, the optimal ligand density on liposomes is controversial. Different densities of folate in liposomes (ligand/total lipid molar ratio) ranging between 0.01% and 1.0% have been reported in the literature as sufficient to promote liposome binding to the folate receptor on cells [10–12]. These differences may be related to the accessibility of the folate ligand [13] or to the differences in the polyethylene glycol (PEG)-folate chemical linkage [14]. Because PEGylated liposomes, called sterically stabilized liposomes, reduce the association of liposome-modified ligands with their receptors by steric hindrance of the PEG polymer [13], we used non-PEGylated liposomes to examine the optimum number and spacer length of the targeting ligand.

In this study, folate-mediated association of DXR-loaded liposomes with human oral carcinoma KB cells, which overexpress the folate receptor, was evaluated in terms of PEG spacer length and the ratio of modification with the folate ligand. Enhanced association of DXR in KB cells was

shown with an extremely low ratio of folate modification and a sufficiently long PEG spacer length, but high cytotoxicity of DXR was observed with a high ratio of folate modification.

2. Materials and Methods

2.1. Materials. Hydrogenated soybean phosphatidylcholine (HSPC), aminopoly(ethyleneglycol)-distearoylphosphatidylethanolamine (amino-PEG-DSPE, PEG mean molecular weight of 2000, 3400, and 5000), and methoxy-PEG_{5000}-DSPE (mPEG_{5000}-DSPE) were obtained from NOF Corporation (Tokyo, Japan). Cholesterol (Ch), doxorubicin (DXR) hydrochloride, folic acid, and HPLC-grade acetonitrile were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Folate-derivatized PEG-DSPE (F-PEG_{2000}-, F-PEG_{3400}-, and F-PEG_{5000}-DSPE), which are conjugates of folic acid and amino-PEG-DSPE, were synthesized as reported previously [13, 15]. Ionophore A23187 and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) were purchased from Sigma (St. Louis, MO, USA) and Lambda Probes and Diagnostics (Graz, Austria), respectively. Folate-deficient RPMI 1640 medium and fetal bovine serum (FBS) were obtained from Invitrogen Corp., (Carlsbad, CA, USA). Other reagents used in this study were reagent grade.

2.2. Preparation of Folate-Modified Liposomes. Liposomes were prepared from HSPC/Ch (55/45 mol/mol). All lipids were dissolved in chloroform, which was removed by evaporation. Lipophilic fluorescent marker DiI-labeled liposomes were prepared by the same procedure, but with the addition of DiI (0.4 mol% of total lipid) to the lipid mixture and without DXR loading. The film was hydrated with $MgSO_4$ aqueous solution (300 mM, adjusted to pH 3.5 with HCl) and sonication. The resulting mean diameter of liposomes was about 130 nm, as determined by the dynamic light scattering method (ELS-800; Otsuka Electronics Co., Ltd., Osaka, Japan) at 25°C after diluting the liposome suspension with water.

DXR was encapsulated into liposomes using the ionophore-mediated loading method [16, 17]. The $MgSO_4$ gradient was formed by exchange of the external solution with sucrose buffer (300 mM sucrose, 20 mM HEPES, and 15 mM EDTA; pH 7.4) by gel filtration chromatography. Subsequent addition of ionophore A23187 to the liposome dispersion results in the outward movement of 1 metal cation in exchange for 2 protons, thus establishing a transmembrane pH gradient. A23187 was used at a concentration of 0.1 μg/μmol lipid and liposomes were incubated with the ionophore at 60°C for 5 min prior to the addition of drug. DXR was then added to the liposomes at a final drug-to-lipid ratio of 0.2 : 1 (wt/wt) and incubated at 60°C for 20 min.

For comparison of loading procedures, DXR was encapsulated in liposomes by the pH gradient method [18]. Briefly, the lipid film was hydrated with citrate buffer (300 mM; pH 4.0) and sonicated. After the external pH was adjusted to 7.4, liposomes were incubated with DXR (drug : lipid = 0.2 : 1, wt/wt) at 60°C for 20 min.

The folate ligand was inserted into preformed liposomes by the postinsertion technique [19]. Briefly, liposomes (DXR-loaded or DiI-labeled) were incubated with an aqueous dispersion of F-PEG-DSPE (from 0.01 to 1 mol% of total lipid) at 60°C for 1 h. In the case of unmodified liposomes (NF-L), water was added instead of F-PEG-DSPE solution. Liposomes modified with F-PEG_{2000}-, F-PEG_{3400}-, F-PEG_{5000}-, or mPEG_{5000}-DSPE will henceforth be designated as F2-L, F3-L, F5-L, and M5-L, respectively. After heating, the liposomes were cooled to room temperature. The suspension was then passed through a Sephadex G-50 column to remove any leaked DXR and unincorporated folate ligand. DXR loading efficiency was determined and significant DXR leakage was not observed with incubation of F-PEG-DSPE at these concentrations. DXR concentration was determined by measuring absorbance at 480 nm (UV-1700 Phamaspec, Shimadzu Corp., Kyoto, Japan).

2.3. In Vitro Assay for Drug Retention. The release of drug from the liposomes in phosphate-buffered saline (PBS, pH 7.4 or 5.0) was monitored by a dialysis method. The dialysis was done at 37°C using seamless cellulose tube membranes (Spectrum, Houston, TX, USA) with a molecular weight cutoff of 300,000 Da and PBS as the sink solution. The initial concentration of DXR-loaded liposomes was 0.2 mg/mL. The sample volume in the dialysis bag was 1 mL, and the sink volume was 100 mL. The concentration of drug was analyzed at various times points during dialysis.

2.4. Cellular Association of Liposomes Determined by Flow Cytometry. KB cells were obtained from the Cell Resource Center for Biomedical Research, Tohoku University (Miyagi, Japan). The cells were cultured in folate-deficient RPMI 1640 medium with 10% heat-inactivated FBS and kanamycin sulfate (50 μg/mL) in a humidified atmosphere containing 5% CO_2 at 37°C.

The cells were prepared by plating 3×10^5 cells/well in a 12-well culture plate 1 day before the assay. The cells were incubated with DXR-loaded liposomes or DiI-labeled liposomes containing 20 μg/mL DXR or 100 μg/mL lipid diluted in 1 mL of serum-free medium for 2 h or the indicated time at 37°C. For free ligand competition studies, 1 mM folic acid was added to the medium. After incubation, the cells were washed with cold PBS (pH 7.4), detached with 0.02% EDTA-PBS, and then suspended in PBS containing 0.1% bovine serum albumin and 1 mM EDTA. The suspended cells were directly introduced into a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) equipped with a 488 nm argon ion laser. Data for 10,000 fluorescent events were obtained by recording forward scatter, side scatter, and 585/42 nm fluorescence. The autofluorescence of cells incubated with serum-free medium without drug for 2 h was used as the control.

2.5. Cytotoxicity of Liposomes in KB Cells. KB cells were incubated with DXR-loaded liposomes (20 μg/mL) for 2 h. After incubation, the cells were washed with cold PBS and cultured in fresh medium for 48 h. Cytotoxicity was determined using the WST-8 assay (Dojindo Laboratories,

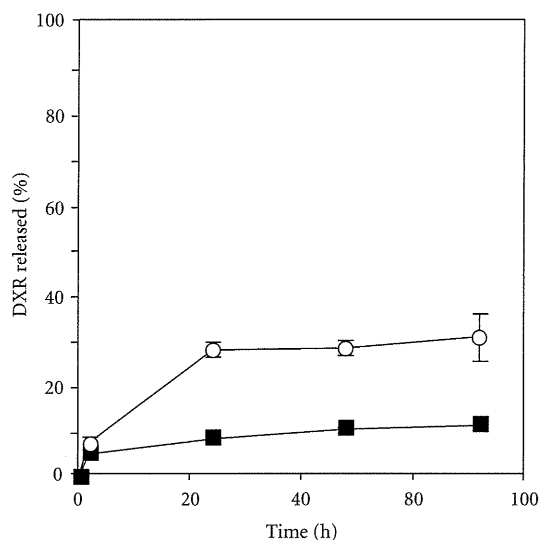


Figure 1: DXR release profile of liposomes loaded by the $MgSO_4$/ionophore method (■) and pH gradient method using citrate buffer (○) in PBS (pH 7.4) at 37°C. Each value represents the mean ± SD ($n = 3$).

Kumamoto, Japan) based on enzymatic reduction of a tetrazolium salt, WST-8, to water-soluble formazan. The number of viable cells was then determined by absorbance at 450 nm.

3. Results and Discussion

3.1. Characterization of DXR-Loaded and Folate-Modified Liposomes. For efficient drug delivery to the target site, drugs should be stably entrapped in liposomes. In this study, an ionophore-mediated pH gradient method utilizing $MgSO_4$ was applied to load DXR into liposomes because this method can effectively encapsulate drugs [17]. More than 95% of DXR was incorporated in liposomes using this system at a drug-to-total lipid ratio of 1 : 5 (wt : wt). The drug retention in the liposomes was examined by incubation in PBS (pH 7.4) at 37°C. For comparison, DXR-liposomes loaded by the remote loading method using citrate buffer were used. As shown in Figure 1, DXR-liposomes loaded using $MgSO_4$ showed significantly lower DXR leakage during the 72-h incubation compared with those loaded using citrate buffer, indicating that the DXR-liposomes produced by the ionophore/$MgSO_4$ loading method were more stable than those produced by the pH gradient method. Therefore, we applied the ionophore/$MgSO_4$ method to load DXR into liposomes for evaluation of folate receptor-targeted liposomes.

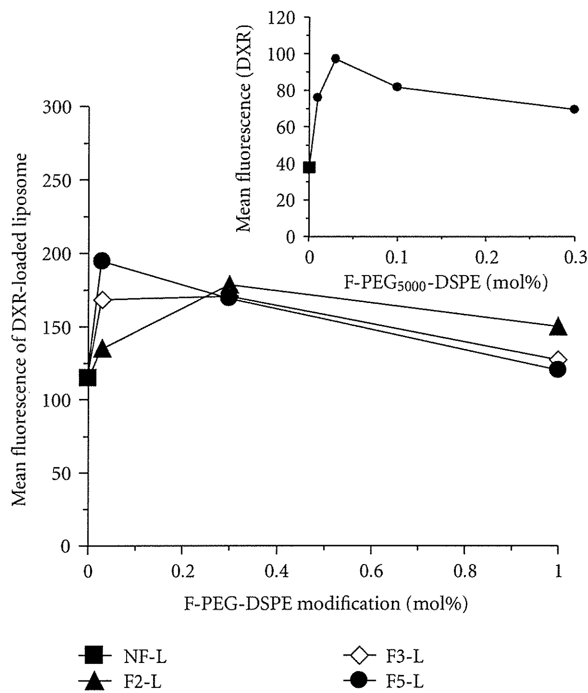
The average particle size of liposomes used in this study was approximately 130 nm, and the folate modification did not change the sizes of liposomes. More than 80% of folate ligand was inserted in liposomes at each ratio, which was confirmed after the separation of folate-modified liposomes by ultracentrifugation (100,000 × g, 1 h, 4°C).

3.2. Effects of Spacer Length and Modification Ratio of F-PEG-DSPE on Liposome Association with KB Cells. In this study, the cellular association of folate-modified liposomes was evaluated in KB cells with respect to PEG spacer length and modification ratio by flow cytometry based on DXR fluorescence (Figure 2(a)) and DiI-labeled liposomes (Figure 2(b)). Folate modification with F-PEG_{2000}-, F-PEG_{3400}-, or F-PEG_{5000}-DSPE (F2-, F3-, and F5-L) at 0.03 to 1.0 mol% enhanced the cellular association compared to that of unmodified liposomes (NF-L), indicating that differences in the density and PEG spacer length of folate ligands resulted in different liposome associations with KB cells. The highest association of liposomes was observed with 0.03 mol% folate modification with the PEG_{5000} spacer, which was 1.7-fold and 160-fold higher than that of unmodified liposomes by measurement of DXR and DiI, respectively (Figure 2(a) inset and Figure 2(b)). The large discrepancy in the value might be due to the difference of distribution of DXR and DiI in liposomes, that is, DXR was entrapped in the water phase of liposomes, but DiI was incorporated in the liposomal membrane. Both the drug incorporated into liposomes and the lipid membrane of liposomes revealed a similar enhancement in association, suggesting that the DXR was associated in the liposomal form, not as drug released from liposomes.

Next, we confirmed whether folate might mediate cellular association with KB cells by mPEG-DSPE modification and free ligand competition (Figure 3). F5-L with 0.03 mol% folate modification showed higher cellular association than NF-L and M5-L (0.03 mol% mPEG-DSPE modification) did. Furthermore, the cellular association of F5-L could be blocked by 1 mM free folic acid and reduced to the level of NF-L. These results indicated that enhancement was due to folate-mediated cellular association.

The effect of incubation time on the cellular association of liposomes was then examined (Figure 4). As the incubation time increased, cellular associations increased and higher association was observed with F5-L modified at 0.03 mol% than at 0.3 mol%. Cellular association of liposomes modified at 0.3 mol% seemed to be saturated after a 2-h incubation. It has been reported that the folate receptor recycling system is downregulated as a result of satisfaction of the cellular folate requirement [11]. Therefore, liposomes modified with more folate ligands would lead to a larger intracellular folate content than those with fewer targeting ligands. Our data showed that liposomes containing fewer folate ligands per liposome had higher association efficiencies compared to liposomes containing large numbers of folate ligands per liposome. As a result, liposomes with minimal folate ligands may be efficient in enhancing drug accumulation in cells.

3.3. Effect of Folate Modification Ratio on Cytotoxicity. The effect of the folate modification ratio on cytotoxicity in KB cells was evaluated using the WST-8 assay (Figure 5). Cell viability was compared with untreated control. All folate-modified liposomes showed higher cytotoxicity than NF-L. The cytotoxicity of F5-L was sharply enhanced from 0 mol% to 0.03 mol% folate modification, which correlated with



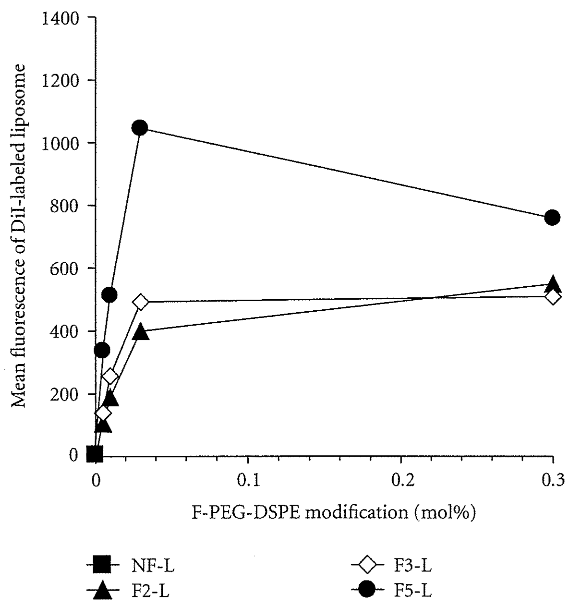


Figure 2: Association of folate-modified liposomes with KB cells with 2-h incubation was determined by fluorescence of DXR-loaded liposomes (a) and DiI-labeled liposomes (b) using flow cytometry. (a) Folate modification from 0.03 to 1.0 mol% of F2-, F3-, and F5-PEG-DSPE. Inset: F5-PEG-DSPE modification from 0.01 to 0.3 mol%. (b) Folate modification from 0.01 to 0.3 mol% of F2-, F3-, and F5-PEG-DSPE.

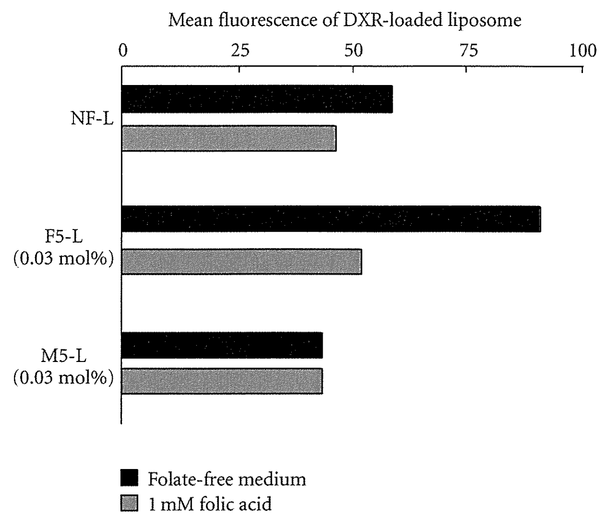


Figure 3: Association of DXR-loaded liposomes with KB cells with 1-h incubation was determined by flow cytometry. Cells were incubated with each liposome in folate-free medium or medium containing 1 mM folic acid.

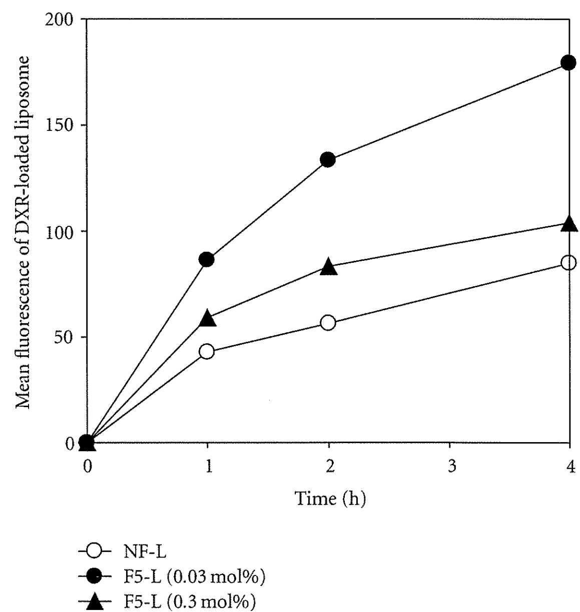


Figure 4: Cellular association of DXR-loaded liposomes with time was determined by flow cytometry. KB cells were incubated with F5-L modified at 0.03 or 0.3 mol% or without folate modification (NF-L).

the result of their cellular associations (Figure 2). However, modification with concentrations greater than 0.03 mol% gently enhanced the cytotoxicity, which did not correlate with cellular associations. As in the case of F5-L, 0.3 mol% folate-modified F2-L and F3-L showed higher cytotoxicity than with 0.03 mol% modification.

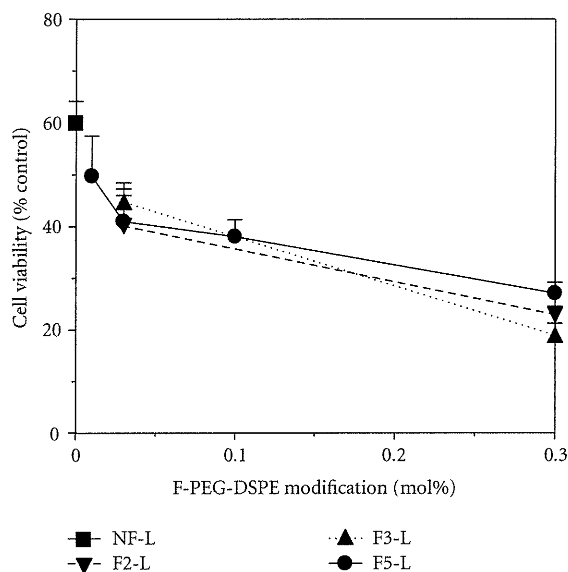


FIGURE 5: Cytotoxicity of DXR-loaded liposomes against KB cells. Cells were incubated with each liposome at a DXR concentration of 20 μg/mL for 2 h, then in fresh medium for 48 h. Each value represents the mean ± SD ($n = 3$).

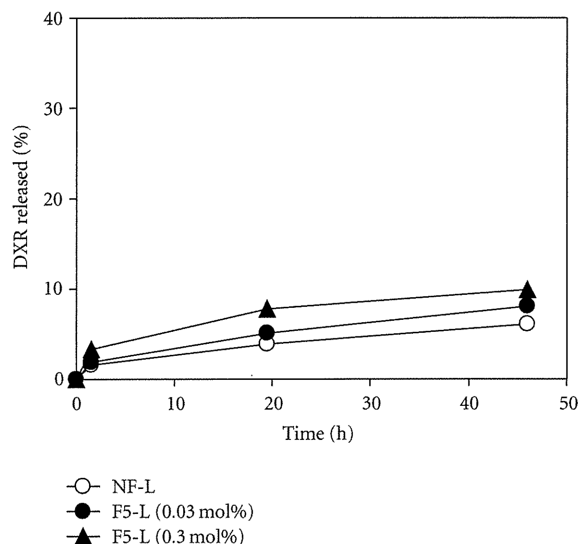


FIGURE 6: DXR release profile of folate-modified liposomes (F5-L) in PBS (pH 5.0) at 37°C. Each point represents the mean of 2 experiments.

The release of free drug from liposomes is involved in cytotoxicity or antitumor activity [20, 21]. Thus, we measured DXR release from liposomes with different modification ratios at pH 5.0, which resembled endosome content (Figure 6). Liposomes with high modification showed slightly higher drug release than those with low or no modification, although the release from all formulations was very low. Because DXR was stably loaded in the liposome using ionophore/$MgSO_4$, it may be difficult to evaluate drug release differences under these conditions. Taken together, the enhanced cytotoxicity might reflect changes in drug release from liposomes by folate modification. Further evaluation of folate-modified drug carriers will be needed to optimize cellular association, cytotoxicity, and/or antitumor effects.

4. Conclusions

In this study, the effects of PEG spacer length and ligand density on folate receptor-targeted liposomes were evaluated. A low ratio of folate modification with a sufficiently long PEG chain (F-PEG_{5000}-DSPE) increased folate receptor-mediated association, but a high ratio of folate modification enhanced in vitro cytotoxicity. This information will be useful for designing folate receptor-targeting carriers.

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