at the end of the incubation, the dishes were washed 3 times with 1 mL of PBS and fixed with 10% formaldehyde PBS at room temperature for 20 min, and washed three times with PBS. Then, the cells were coated with Aqua Poly/Mount (Poly science, Warrington, PA) to prevent fading and covered with coverslips. The fixed cells were observed with an ECLIPSE TS100/100-F for Epi-fluorescence Observations. The contrast level and brightness of the images were adjusted.

2.8. Data Analysis

Significant differences in the mean values were evaluated using Student's unpaired *t*-test. A *p*-value of less than 0.05 was considered significant.

3. RESULTS AND DISCUSSION

3.1. Characterization and Transfection Efficiency of Arg10-PEG-BDB

In order to determine the CMC of Arg10-PEG-BDB in water, we monitored fluorescence intensity as we added different concentrations of Arg10-PEG-BDB into an aqueous dispersion of pyrene. The CMC value of Arg10-PEG-BDB was 20.9 μM or 83.6 μg/mL at room temperature (Fig. 2).

Particle size and the zeta-potential of Arg10-PEG-BDB (25 μM)/DNA (N/P = 42.5/1) complex were about 1500 nm and 42.7 mV, respectively (Table I). The zeta-potential of the complex (5 μM, N/P = 8.5/1, 27.1 mV) decreased by about 10 mV compared with that of Arg10-PEG-BDB micelles (38.0 mV) due to the negative charge of DNA.

We examined the influence of the concentration of Arg10-PEG-BDB on transfection efficiency by luciferase activity. The highest transfection efficiency was observed at the concentration of 5 μM of Arg10-PEG-BDB (N/P = 8.5/1), which is significantly (1.5-fold) higher than that of

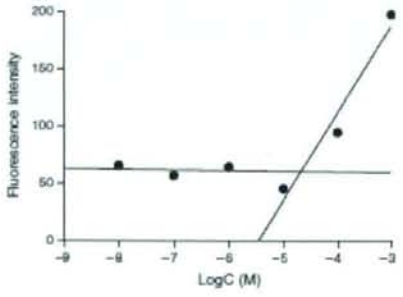

Fig. 2. CMC measurements of Arg10-PEG-BDB by fluorescence probe method using pyrene.

Table I. Particle size and zeta-potential of Arg10-PEG-BDB complexed with or without DNA.

Lipid concentration and complex[a]	N/P	Size (nm)	Zeta-potential (mV)
Arg10-PEG-BDB (25 μM)		N.D.[b]	+38.0
Arg10-PEG-BDB (5 μM)/DNA	8.5/1	N.D.[b]	+27.1
Arg10-PEG-BDB (25 μM)/DNA	42.5/1	~1500	+42.7

[a]Complex with 2 μg of DNA.
[b]N.D., not detected by dynamic light scattering method.

25 μM (Fig. 3(A)), superficially suggesting that micelle formation is not necessary for high transfection efficiency. To examine the distribution of transfection in cells, we observed the transfection efficiency of Arg10-PEG-BDB with the plasmid pEGFP-C1 using flow cytometry and fluorescence microscopy. 5 μM of Arg10-PEG-BDB showed about 7-fold higher transfection efficiencies than 25 μM (Fig. 3(B)). A significantly higher level of GFP protein was observed in the cells treated with 5 μM of Arg10-PEG-BDB than 25 μM, corresponding to the results of luciferase expression (Fig. 3(C)).

The higher concentration of Arg10-PEG-BDB/DNA could not be used because cytotoxicity was increased with an increase of lipid concentration.[12] The cytotoxicity of 5 μM of Arg10-PEG-BDB/DNA, therefore, was lower than that of 25 μM. To clarify the underlying mechanisms that dictated the remarkable differences between 5 and 25 μM in lipid-mediated transfection efficiency, the properties of lipid complexes with DNA were investigated.

3.2. Investigation of the Interaction of Arg10-PEG-BDB with DNA by FIDA

To examine the interaction of Arg10-PEG-BDB with DNA, Arg10-PEG-BDB and 2 μg rhodamine-DNA was mixed and characterized using FIDA with a MF20 microplate reader (Olympus Corp. Tokyo, Japan).[9] A theoretical probability distribution of photon count numbers is fitted against the obtained histogram, yielding specific brightness values Q and concentrations C for all different species in a sample. Decreased C value and increased Q value were observed at the concentration of 1~5 μM of Arg10-PEG-BDB, suggesting that more DNA bound to Arg10-PEG-BDB in this concentration range (Figs. 4(A, B)). The data showed that the interaction of 5 μM of Arg10-PEG-BDB with DNA (N/P = 8.5/1) was stronger than that of 25 μM (N/P = 42.5/1). DNA might help the self-aggregation of Arg10-PEG-BDB even at low concentrations of lipid. This effect may be reflected in the highest transfection efficiency at the 5 μM concentration of Arg10-PEG-BDB to 2 μg DNA. To examine whether this difference of interaction of lipid with DNA in complexes by the concentration of lipid correlated with the differences in structural feature, the complexes were further characterized by electron microscopy.

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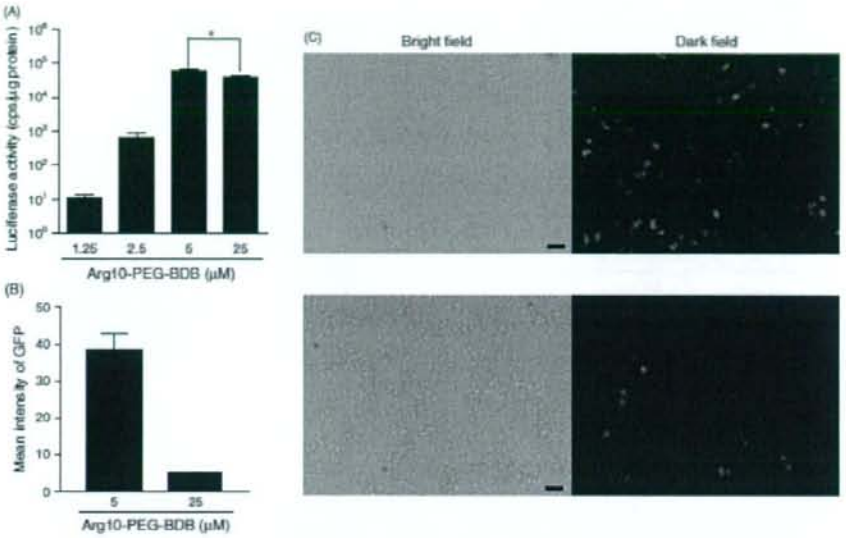

Fig. 3. Influence of the concentration of Arg10-PEG-BDB complexed with DNA on transfection efficiency. Arg10-PEG-BDB/DNA complexes (prepared by mixing 2 μg of pCMV-luc or pEGFP-C1 with various concentrations of Arg10-PEG-BDB) were diluted with serum-free DMEM to a final volume of 1 mL. After incubation for 3 h at 37 °C in serum-free DMEM, DMEM (1 mL) containing 10% FBS was added, and the cells were further incubated for 21 h. (A) Luciferase activity of various concentrations of Arg10-PEG-BDB. (B) GFP expression of 5 or 25 μM of Arg10-PEG-BDB. Each value is the mean ± S.D. of three separate determinations. (C) Analysis of GFP expression by fluorescence microscopy (magnification ×100). 5 μM of Arg10-PEG-BDB (top) and 25 μM of Arg10-PEG-BDB (bottom) are shown. Scale bar = 50 μm.

3.3. The Morphology of Arg10-PEG-BDB/DNA Complexes Determined by Electron Microscopy and Microscopy

To reveal the nanostructure of Arg10-PEG-BDB/DNA complexes under various concentrations of lipid, we observed the Arg10-PEG-BDB/DNA complex using phase contrast cryo-TEM. Free DNA papered was observed as open circular one (data not shown). To observe the lipid structure, two higher concentrations of Arg10-PEG-BDB were examined at the same (N/P) ratio as the transfection experiments. Micellar structures with several nm sizes were observed at 1.25 mM of Arg10-PEG-BDB above CMC (Fig. 5(A)). In Arg10-PEG-BDB/DNA complex (N/P = 8.5/1), a net-like structure was observed in which DNA was involved (Fig. 5(B)). These net-like structures may contribute to high transfection efficiency, but the particle size of Arg10-PEG-BDB (5 μM, 0.25 mM)/DNA (N/P = 8.5/1) in water was not detected by dynamic light scattering method. Surprisingly, in the Arg10-PEG-BDB/DNA complex (N/P = 42.5/1), heterogeneous nanostructures were observed. Other than net-like structures, large fibrous nanostructures were visible (Fig. 5(C)). Their particle size of Arg10-PEG-BDB (1.25 mM)/DNA (N/P = 42.5/1) in water was about 1.5 μm by dynamic light scattering method. In both cases we can clearly see DNA molecules around the edge of the net-like structures.

At a lower magnification, we observed the structure at the same condition as the transfection by the microscopy. Cells were exposed for 1 h to the 5 or 25 μM of Arg10-PEG-BDB/DNA complex in the absence of serum. Next, the unfixed cells were visualized by microscopy. It was hardly observed in 5 μM of Arg10-PEG-BDB/DNA, but a large aggregation was observed in 25 μM (Figs. 6(A, B)). The large aggregation of 25 μM of Arg10-PEG-BDB/DNA, therefore, might inhibit the cell internalization or the release of DNA from endosomes into cytoplasm.

Increase of lipid concentration appeared to tend to convert from net-like structures into a large fibrous one. Previously, we reported that the structure of PEG-BDB became fiber with the increase of lipid concentration.[20] At higher concentration of Arg10-PEG-BDB, DNA might induce the fibrous nanostructure by partial neutralization of Arg10-PEG-BDB, suggesting that DNA may modulate the net-like structure and fibrous nanostructure of Arg10-PEG-BDB. The former reflected a stronger interaction between DNA and lipid than the latter. To further investigate effect of the difference of nanostructures of complexes

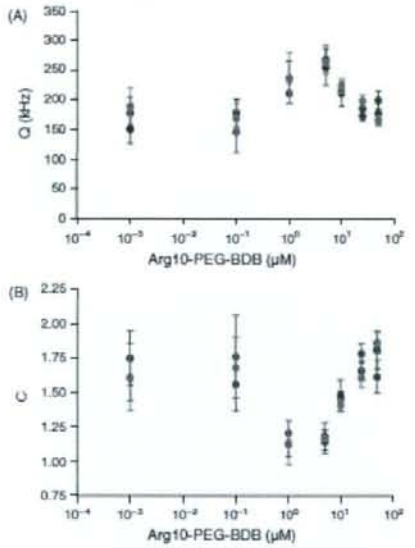

Fig. 4. Fluorescence intensity distribution analysis (FIDA) of Arg10-PEG-BDB and 2 μg rhodamine-DNA complex. (A) Brightness per complex; Q value. (B) Fluorescent DNA number; C value. Each value is the mean ± S.D. of three separate determinations.

on transfection efficiency, cellular uptake mechanism was subsequently examined.

3.4. Cellular Uptake Mechanism

The difference of nanostructures of complexes may cause difference in the ability to deliver DNA into HeLa cells. At first, to examine the association of Arg10-PEG-BDB/DNA complex with cells, we assayed the cell internalization of 5 or 25 μM of the Arg10-PEG-BDB/DNA 3 h after transfection with serum by flow cytometry (Fig. 7(A)). To remove bind Arg10-PEG-BDB/DNA on the surface of plasma membrane, we washed the cells with PBS and treated them with trypsin.[21] Associated amount of 5 or 25 μM of Arg10-PEG-BDB/DNA with the cells was almost same, indicated that both concentrations of Arg10-PEG-BDB were able to carry similar amount of rhodamine-DNA into cells.

The cellular uptake pathway is reported to be different depending on the density of octaarginine (Arg8) in liposome containing Arg8.[22] Hence, there is a possibility that the cellular uptake mechanism might change depending on the concentration of Arg10-PEG-BDB. The translocation of Tat and Arg8 peptide are suggested to occur through macropinocytosis which is dependent on lipidic

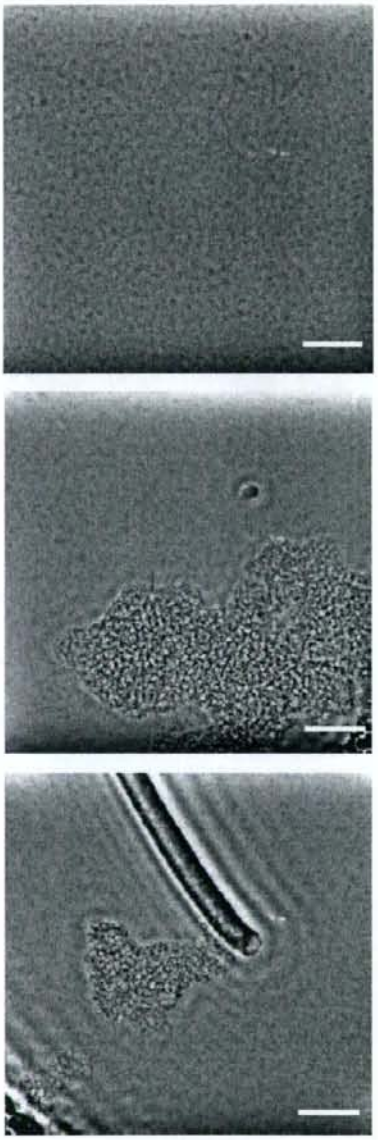

Fig. 5. Phase contrast cryo-TEM analysis of the complex structure of Arg10-PEG-BDB and DNA. (A) 1.25 mM of Arg10-PEG-BDB. (B) 0.25 mM of Arg10-PEG-BDB/DNA (N/P = 8.5/1). (C) 1.25 mM of Arg10-PEG-BDB/DNA (N/P = 42.5/1). Scale bar = 100 nm.

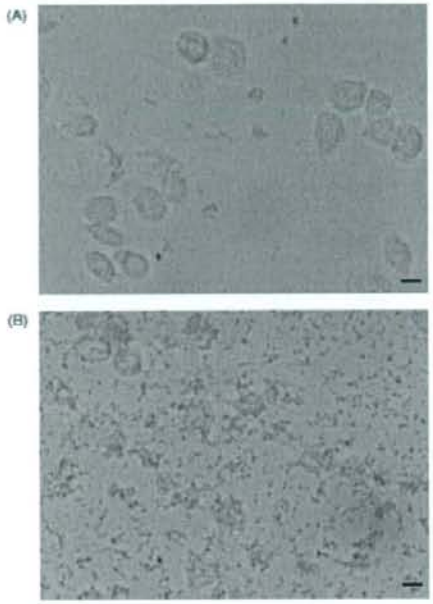

Fig. 6. Microscopic analysis of the Arg10-PEG-BDB/DNA complex incubated with the cells for 1 h at 37 °C in serum-free DMEM. The unfixed cells were observed with a microscope. (A) 5 μM of Arg10-PEG-BDB/DNA (N/P = 8.5/1). (B) 25 μM of Arg10-PEG-BDB/DNA (N/P = 42.5/1). Scale bar = 20 μm.

microdomains.[23,24] Macropinocytosis is a kind of endocytosis as a cellular uptake pathway.[25] Macropinosomes are formed by actin-driven ruffling of the plasma membrane, followed by folding and pinching off of irregular-sized vesicles.[26] Macropinocytosis is inhibited by 5-(*N*-ethyl-*N*-isopropyl) amiloride (EIPA), which inhibits Na^+/H^+ exchange protein.[23] To examine the internalization mechanism of 5 and 25 μM Arg10-PEG-BDB/DNA (N/P = 8.5/1 and 42.5/1), we investigated the effect of EIPA on the cellular uptake of complexes, using Arg10-PEG-BDB-NBD (Fig. 7(B)). Arg10-PEG-BDB-NBD (5 and 25 μM)/DNA showed about 70% lower internalization efficiency at 50 μM of EIPA than in its absence. This finding suggests that 5 and 25 μM of Arg10-PEG-BDB/DNA were taken up via a macropinocytosis pathway although their structures were different (Figs. 5(B, C)). DNA may be released easily in the cytoplasm because it is reported that macropinosomes are leaky.[27] The large fibrous nanostructure, therefore, might inhibit the release of DNA from macropinosomes into cytoplasm. At the present research technique, it is difficult to examine it further since the research of CPP should be observed at unfixed cells.

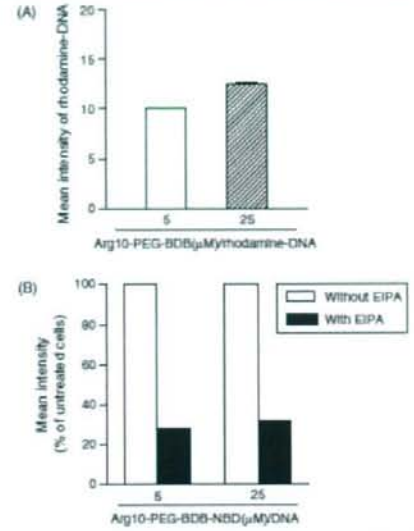

Fig. 7. (A) Cellular uptake of 5 or 25 μM of Arg10-PEG-BDB/rhodamine-DNA incubated for 3 h at 37 °C. The cells were treated with trypsin before flow cytometry. (B) Effect of EIPA on their cellular uptake. Cells were pretreated with EIPA (50 μM) at 37 °C for 30 min. Medium was replaced with fresh medium containing 5 or 25 μM Arg10-PEG-BDB-NBD/2 μg DNA. Cells were incubated for 1 h at 37 °C in serum DMEM containing EIPA (50 μM). Each value is the mean ± S.D. of three separate determinations.

4. CONCLUSIONS

Arg10-PEG-BDB at the concentration below CMC showed higher transfection efficiency in HeLa cells than that above CMC. In Arg10-PEG-BDB/DNA complex below CMC, a net-like structure was observed. On the other hand, in the Arg10-PEG-BDB/DNA complex above CMC, heterogenous structures composed of net-like and large fibrous structures were observed. It is very important that plasmid DNA is able to help Arg10-PEG-BDB to form supramolecular structures. DNA-assisted Arg10-PEG-BDB nanostructure formation may result in concentration-dependent transfection efficiency.

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Effect of sugars on storage stability of lyophilized liposome/DNA complexes with high transfection efficiency

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Abstract

Cationic lipid-based gene delivery systems have shown promise in transfecting cells in vitro and in vivo. However, liposome/DNA complexes tend to form aggregates after preparation. Lyophilization of these systems, therefore, has become of increasing interest. In this study, we investigated the feasibility of preserving complexes as a dried preparation using a modified dehydration rehydration vesicle (DRV) method as a convenient and reliable procedure. We also studied storage stability of a lyophilized novel cationic gene delivery system incorporating sucrose, isomaltose and isomaltotriose. Liposomes were composed of 3β-[*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol) and L-dioleoylphosphatidylethanolamine (DOPE), plus sucrose, isomaltose or isomaltotriose. Lyophilized liposome/DNA complexes were stored at −20, 25, 40 and 50 °C and their stability was followed for 50 days. Liposome/DNA complexes with sucrose could be stored even at 50 °C without large loss of transfection efficiency. The transfection efficiency of formulations stored at various temperatures indicated that the stabilizing effect of sugars on plasmid DNA was higher in the following order: isomaltotriose < isomaltose < sucrose, which was inverse to the order of their glass transition temperature (T_g) values. It was concluded that we could prepare novel lyophilized liposome/DNA complexes with high transfection efficiency and stability, which might be concerned that sucrose stabilized plasmid DNA in liposomes by directly interacting with plasmid DNA rather than by vitrifying to a high T_g solid.

Keywords: Cationic liposome; Sucrose; Dehydration rehydration vesicle; Transfection; Storage stability; Lyophilization

1. Introduction

Cationic liposome-mediated transfer of DNA is a promising approach, because of low immunogenicity and toxicity, ease of preparation, and potential applications for active targeting. The disadvantages include poor efficiency of transfection in vivo. Therefore, many cationic lipid-based transfection reagents have been developed for the efficient delivery of DNA into cells (Gao and Huang, 1991; Vigneron et al., 1996). Commercially available cationic liposomes or particles are mixed with plasmid DNA, and tend to form large liposome/DNA aggregates in solution, especially at high DNA concentrations. They form as a result of electrostatic binding between cationic liposomes and negatively charged DNA, and are inherently difficult to manipulate, resulting in a decrease of transfection (Sternberg et al., 1994; Lai and van Zanten, 2002). Because of this problem, cationic liposome/DNA complexes have to be freshly prepared when they are used. This would make it demanding to prepare them, and make quality control very difficult due to the fact that preparation of cationic liposome/DNA complexes is a process that is poorly defined and difficult to control.

To produce stable gene delivery systems that avoid these problems, lyophilization is suitable for long-term storage. There are many studies about lyophilization of liposome vectors using sugars (Anchordoquy et al., 1997; Li et al., 2000; Molina et al., 2004). Disaccharides were used in most studies. Especially, sucrose, which has a high glass transition temperature (T_g), is known to be effective to maintain the stability of liposomes, presumably by forming glasses under the typical freezing conditions used for lyophilization (Molina et al., 2001). To develop lyophilized liposome complexes with plasmid DNA vector, we used a modified dehydration rehydration vesicle (DRV) method as a convenient and reliable procedure (Perrie and Gregoriadis, 2000). The technique of the DRV method, employing sucrose at

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the lyophilization stage, has been evaluated for a range of solutes (Zadi and Gregoriadis, 2000; Kawano et al., 2003) and plasmid DNA (Perrie et al., 2004). The effects of sugars on the stability of lyophilized liposomes, sizes of liposomes and entrapment efficiency of solutes using DRV methods have been reported (Zadi and Gregoriadis, 2000; Kawano et al., 2003), but there have been a few reports about the effect of sugars on the stability of plasmid DNA in liposomes during storage (Li et al., 2000).

In this study, we examined stability of lyophilized liposome/DNA complexes with sucrose, isomaltose or isomaltotriose at different temperatures over 50 days, and determined which sugars could inhibit aggregation and maintain the transfection activity of plasmid DNA during preservation at temperatures above T_g. We found that DRV/DNA complexes with sucrose could be stored even at 50 °C without a large loss of transfection activity. Isomaltose and isomaltotriose were selected as excipients because their T_g values were higher than that of sucrose and therefore, they were expected to exhibit a greater stabilizing effect.

2. Materials and methods

2.1. Materials

3β-[*N*-(*N*′,*N*′-Dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol) was purchased from Sigma Chemical Co. (St. Louis, MO, USA), and L-dioleoylphosphatidylethanolamine (DOPE) was obtained from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Lipofectamine 2000 was purchased from Invitrogen Corp. (Carlsbad, CA, USA). The Pica gene luciferase assay kit was purchased from Toyo Ink Mfg. Co. Ltd. (Tokyo, Japan). BCA protein assay reagent was purchased from Pierce (Rockford, IL, USA). All other chemicals used were of reagent grade. The plasmid DNA encoding the luciferase marker gene (pAAV-CMV-Luc) was supplied by Dr. S. Tanaka in Mt. Sinai School of Medicine (NY, USA). All reagents were of analytical grade. RPMI1640 medium and fetal bovine serum (FBS) were purchased from Life Technologies, Inc. (Grand Island, NY, USA).

2.2. Preparation of DRV

The preparation method has been reported previously (Perrie and Gregoriadis, 2000). Briefly, lipids (e.g., DC-Chol:DOPE = 3:2 and 1:2 mol/mol) were dissolved in chloroform and a dried film was formed by rotary-evaporation. The preparation was hydrated with filtered water and vortexed at room temperature. The resulting multilamellar vesicle (MLV) suspension was extruded through a series of polycarbonate membranes with pore sizes of 0.6 and 0.2 μm (Millipore, Billerica, MA) to yield about 200-nm-sized vesicles. A sugar/total lipid (w/w) of 5, and 12.5–100 μg of plasmid DNA at a charge ratio of (+/−) of 2 and 16 were carefully added to the vesicle suspension, and the mixture was transferred to polypropylene tubes (10 mm in diameter and 40 mm in length), frozen by immersing in liquid nitrogen for 10 min, and lyophilized (DRVs) using a Freezevac C-1 lyophilizer (Tozai Tsusho Co., Tokyo, Japan) at a vacuum level below 5 Pa. Shelf temperature was controlled at −40 °C for 12 h, at −20 °C for 12 h, at 0 °C for 8 h, at 20 °C for 4 h, and at 30 °C for 4 h. After lyophilization, dry nitrogen was introduced in the drying chamber, and vials of DRVs were sealed with screw caps in a nitrogen atmosphere. Water contents of formulations obtained were less than 0.5%, as determined by the Karl Fischer method.

Prior to the measurement of transfection efficiency, the dry cake of DRVs was rehydrated with milli-Q water (1 ml of water per vial) and ultracentrifuged at 45,000 rpm for 45 min to partition sugars from the liposome suspension. The supernatant was collected and then milli-Q water was added to the DRV pellets to achieve 100 μg DNA/ml (DRV pellet suspension).

2.3. Measurement of size

The mean particle size of the DRVs suspended in water was determined using a light scattering instrument (DLS-7000, Otsuka Electronics Co. Ltd., Osaka, Japan) by a dynamic laser light scattering method at 25 ± 1 °C. The reported particle size was the average value of two measurements.

2.4. Stability test

Vials of DRVs were transferred to vessels containing P_2O_5 and were stored at −20, 25, 40 and 50 °C for 50 days.

2.5. Measurement of T_g

A T_g of DRV formulation was measured by using a model 2920 differential scanning calorimeter (DSC) with a refrigerator cooling system (TA Instruments, Newcastle, DE, USA). Approximately 3 mg of DRV cake was put in an aluminum sample pan, dried in vacuum at 25 °C for 16 h and sealed hermetically in a nitrogen atmosphere in order to prevent water sorption during sample preparation. DSC traces were measured at a heating rate of 20 °C/min. An empty pan was used as a reference sample. Temperature calibration of the instrument was carried out using indium. T_g values reported were obtained for first heating scan. The T_g values and changes in the heat capacity at T_g of stored samples were similar to those before storage, indicating that crystallization of amorphous excipient in the formulations did not occur during stability studies.

2.6. Entrapment efficiency of plasmid DNA in DRV

The plasmid DNA in the supernatant after ultracentrifugation of the rehydrated DRV suspension at 45,000 rpm for 45 min was measured as free plasmid DNA using a PicoGreen dsDNA Quantitation Kit *200-2000 assays* (Molecular Probes, Inc., OR, USA).

2.7. Cell culture

Human cervical carcinoma HeLa cells were kindly provided by Toyobo Co. Ltd. (Osaka, Japan) and grown in DMEM supplemented with 10% FBS at 37 °C in a humidified 5% CO_2

atmosphere. Cell cultures were prepared by plating cells in 35-mm culture dishes 24 h prior to each experiment.

2.8. Transfection of cells

The DRV pellet suspension prepared as described was diluted with medium without FBS to a final concentration of 2 μg plasmid DNA per well. After transfection in the medium without FBS for 3 h, 1 ml of the growth medium containing 10% FBS was added to the wells and culturing was continued for an additional 21 h.

2.9. Expression assays

Luciferase expression was measured using the luciferase assay system. Incubation was terminated by washing the plates three times with cold phosphate-buffered saline (pH 7.4, PBS). Cell lysis solution (Pica gene) was added to the cell monolayers, which were then subjected to freezing (−80 °C) and thawing at 37 °C, followed by centrifugation at 13,000 rpm for 10 min. The supernatants were frozen and stored at −80 °C until the assays. Aliquots of 10 μl of the supernatants were mixed with 100 μl of luciferin solution (Pica gene) and counts per second (cps) were measured with a chemoluminometer (Wallac ARVO SX 1420 Multilabel Counter, Perkin Elmer Life Science, Japan, Co. Ltd., Kanagawa, Japan). The protein concentration of the supernatants was determined with BCA reagent, using bovine serum albumin as a standard, and cps/μg protein was calculated.

2.10. Statistical analysis

Statistical significance of the data was evaluated by Student's *t*-test. A p value of 0.05 or less was considered significant. All experiments were repeated at least two times. Duplicate determinations of luciferase expression values typically differed by less than 10%.

3. Results and discussion

Notably, liposomes composed of DC-Chol together with DOPE (DC-Chol/DOPE liposomes) have been classified as one of the most efficient vectors for the transfection of plasmid DNA into cells (Zhou and Huang, 1994; Farhood et al., 1994, 1995) and in clinical trials (Nabel et al., 1993, 1994). It has been demonstrated that a 3:2 or 1:1 molar ratio of DC-Chol/DOPE in liposomes results in high transfection efficiency (Farhood et al., 1995). Recently, we reported that DC-Chol/DOPE liposomes with molar ratio 1:2 showed more efficient transfection than those with molar ratio 3:2 or 1:1 in medium with FBS, having transfection efficiency comparable to that of Lipofectamine 2000, a commercial transfection reagent. Also, these lipoplexes showed a maximum at (+/−) 2:1 of transfection efficiency (Maitani et al., 2007). Therefore, we selected two kinds of DRV formulations: the conventional one (DC-Chol/DOPE = 3:2, A1–A3) and the novel one (DC-Chol/DOPE = 1:2, B1–B6), and prepared DRV/DNA complexes at sugar/total lipid (w/w) of 5, and charge ratio (+/−) of cationic lipid (DC-Chol) to DNA of 2 or 16 (Table 1).

3.1. DRVs without sugars

In preliminary experiments of the preparation of DRVs without sugars, the size was increased to over 1 μm. For the process of freeze-drying, fast freezing and addition of sugars in the freezing state resulted in less aggregation. This finding agreed with that reported by Molina et al. (2001). Moreover, in this study, since some preparations of dry cakes of DRVs after lyophilization were needed to perform the subsequent procedures, sugars were added to the suspension of liposomes and plasmid DNA at a weight ratio of 5:1 (sugar:total lipids) and mixed before freezing.

3.2. T_g measurement of DRVs

Fig. 1 shows representative DSC traces of DRV formulations containing plasmid DNA (formulations B1–B3) and lyophilized sugar. The formulations were considered to be amorphous because they exhibited base line shifts due to the glass transition. The T_g values of the lyophilized sugar (T_g^*) were higher in the order: sucrose < isomaltose < isomaltotriose. The T_g values of DRV formulations increased in the order: B1 < B2 < B3, indicating that the molecular mobility of the formulation containing sucrose was higher than that of the formulations containing isomaltose or isomaltotriose. The T_g values of DRV formulations and lyophilized sugars with and without liposomes are summarized in Table 2. The T_g values of lyophilized sugars with cationic liposomes (DRV formulation without DNA) were slightly lower than those of the corresponding lyophilized sugars. In contrast, a

Table 1
Composition of DRV formulation

Formulation	DC-Chol:DOPE (mol:mol)	Charge ratio, lipid:DNA (+/−)	Sugar
A1	3:2	16:1	Sucrose
A2	3:2	16:1	Isomaltose
A3	3:2	16:1	Isomaltotriose
B1	1:2	2:1	Sucrose
B2	1:2	2:1	Isomaltose
B3	1:2	2:1	Isomaltotriose
B4	1:2	16:1	Sucrose
B5	1:2	16:1	Isomaltose
B6	1:2	16:1	Isomaltotriose

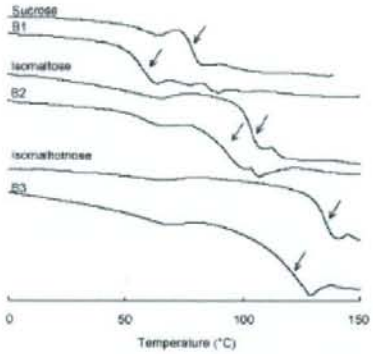

Fig. 1. Representative DSC traces of DRV formulations and sugars for first heating scan. Arrows in the figure represent T_g.

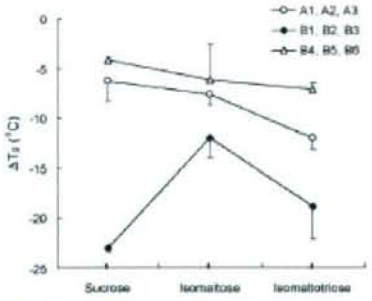

Fig. 2. Difference in $T_g^{midpoint}$ between DRV formulations and corresponding lyophilized sugars in Table 2. Error bars represent standard deviation ($n = 3$).

large decrease in T_g of the DRV/DNA complexes was observed, as indicated by the T_g values of formulations B1–B3, as shown in Fig. 2. The T_g values of the formulations can be expressed by the Gordon–Taylor equation (Eq. (1)) assuming that miscibility of the sugars and the liposome/plasmid DNA complex is complete:

$$T_g = \frac{kW_C T_g^C + W_s T_g^s}{W_s + kW_C} \quad (1)$$

where W_s, W_C, T_g^s, and T_g^C are the weight fraction and T_g of sugar and complex, respectively, and k is a constant. Differences in the T_g between the formulations and the corresponding sugars (ΔT_g) are expressed by the following equation:

$$\Delta T_g = T_g - T_g^s = -\frac{kW_C(T_g^s - T_g^C)}{W_s + kW_C} \quad (2)$$

T_g^C (not determined) is expected to be lower than T_g^s, since T_g values of the formulations studied were lower than those of corresponding lyophilized sugar. For formulations B1–B3, the T_g^C value is considered to be the same, since the formulations contain the same amount of plasmid DNA in liposomes. A smaller $T_g^s - T_g^C$ value is consequently expected for the formulation containing the sugar with lower T_g. Assuming that k is not largely different between sugars, the smallest difference in T_g between a formulation and the corresponding lyophilized sugar should

Table 2
T_g of DRV formulations and lyophilized sugars

	$T_g^{midpoint}$ (°C)	S.D.	T_g^{onset} (°C)	S.D.
DRV formulation[a]				
A1 (sucrose (DC/DOPE = 3/2))	72.0	2.1	68.8	2.6
A2 (isomaltose (DC/DOPE = 3/2))	94.4	1.1	87.3	4.9
A3 (isomaltotriose (DC/DOPE = 3/2))	121.1	1.2	108.2	1.6
B1 (sucrose (DC/DOPE = 1/2))	55.2	0.5	50.4	0.5
B2 (isomaltose (DC/DOPE = 1/2))	90.7	0.5	83.1	1.6
B3 (isomaltotriose (DC/DOPE = 1/2))	114.2	3.3	104.6	2.7
B4 (sucrose (DC/DOPE = 1/2))	74.0	0.4	71.1	0.5
B5 (isomaltose (DC/DOPE = 1/2))	96.6	3.6	86.9	1.4
B6 (isomaltotriose (DC/DOPE = 1/2))	125.9	0.6	118.3	0.9
Lyophilized sugar with cationic liposomes				
Sucrose + (DC/DOPE = 3/2)	75.1	0.1	73.7	0.8
Isomaltose + (DC/DOPE = 3/2)	98.1	0.8	97.5	1.0
Isomaltotriose + (DC/DOPE = 3/2)	129.5	2.0	123.3	2.7
Sucrose + (DC/DOPE = 1/2)	77.8	0.8	72.5	0.5
Isomaltose + (DC/DOPE = 1/2)	101.8	0.1	94.2	3.1
Isomaltotriose + (DC/DOPE = 1/2)	129.1	0.1	123.9	0.5
Lyophilized sugar				
Sucrose	78.2	0.2	74.5	0.2
Isomaltose	102.7	0.6	98.6	0.2
Isomaltotriose	133.0	0.8	128.8	0.2

T_g values reported were obtained for first heating scan ($n = 3$).
[a] Sugar, liposome and plasmid DNA used in Table 1.

Table 3
Particle size of liposomes after rehydration of DRVs stored at −20 °C for 50 days

Formulation	Diameter (nm)	Dispersion (%)	Diameter (nm)	Dispersion (%)
A1	179	62	1274	38
A2	178	69	1269	31
A3	191	70	1558	30
B1	448	10	3495	90
B2	364	8	3826	92
B3	487	11	3161	88
B4	238	30	1451	70
B5	256	32	1591	68
B6	205	32	1130	68

be observed for sucrose-based formulations. Formulation B1, however, exhibited a larger difference in T_g than formulation B2 or B3 (Fig. 2), suggesting that the Gordon–Taylor equation was not applicable to formulation B1. This deviation from the Gordon–Taylor equation suggests that plasmid DNA may interact with sucrose more strongly than with isomaltose or isomaltotriose.

3.3. Size distribution of DRV suspension after rehydration and entrapment efficiency

DRVs stored at −20 °C for 50 days were rehydrated. The size distribution of DRV/DNA complex suspensions was heterogeneous, depending on the charge ratio (+/−), but not depending on sugars (Table 3). The DC-Chol/DOPE = 3/2 complex (formulations A1–A3) showed the smallest size. In the DC-Chol/DOPE = 1/2 complex, formulations B1–B3 with low cationic charge (+/−) of 2 showed larger size than formulations B4–B6 with that of 16. It seemed that the charge of DRVs was critical in determining sizes because the electric repulsion of the complex opposed aggregation. Also sugar amount might affect size of DRV. Molina et al. (2001) reported that formulations with high sucrose/DNA ratios are capable of maintaining particle size during the freezing step, and suggested that the separation of individual particles within sugar matrices is responsible for the protection of cationic lipid DOTAP/DOPE liposome vectors during the freezing step of a typical lyophilization protocol.

DRV methods in which lyophilization is performed after the addition of sucrose to the liposome suspension increase the entrapment efficiency of DNA in DRVs. Crowe and Crowe (1993) reported that trehalose outside and inside liposomes prevented the aggregation of liposomes and stabilized liposomes to entrap solutes. Zadi and Gregoriadis (2000) reported that a small amount of sugar outside liposomes disturbs the liposomal membrane and makes solute outside liposomes enter them. In our case, free plasmid DNA was not detected in the supernatant of any DRV suspensions using a PicoGreen Kit. The cationic charge of DRVs was so high that whether plasmid DNA was entrapped and/or adsorbed on DRVs was not clear.

3.4. Effect of sugars on transfection efficiency (TE)

In preliminary experiments, when lyophilized formulations B1–B3 of DRV/DNA complexes were preserved at room temperature for 24 h and then rehydrated, they showed similar TE values with nonlyophilized ones. To investigate the effect of sugars on lyophilization of the DRV/DNA complexes, the stability of plasmid DNA was evaluated by measuring TE after preservation at various temperatures.

At −20 and 25 °C, formulations B1–B3 showed the highest TE, and then formulations B4–B6 showed intermediate TE higher than those of formulations A1–A3 (Fig. 3). This finding indicated that TE was affected by the cationic lipid ratio in liposomes, and by the charge ratio (+/−) of cationic lipid to plasmid DNA, more than by the sugar. Molina et al. (2004) reported that progressive degradation of DOTAP lipoplex in terms of TE was observed during storage in the dried state at −20 °C, and the presence of DOPE enhanced degradation under these conditions. To the contrary, formulations B1–B3 with rich DOPE and the low charge ratio (+/−), exhibited high TE. This difference may be due to difference of cationic lipids.

The TE of formulations at various temperatures indicated that the stabilizing effect of sugars on DNA was higher in the following order: isomaltotriose < isomaltose < sucrose, except for formulation B1. This order of the stabilizing effects of sugars was inverse to the order of their T_g values. About the effects of the glassy state on liposome in the freeze-drying state, it was reported that the solute retention in dry liposomes may be prolonged by increasing the T_g of the dry liposome preparation (Sun et al., 1996; Crowe et al., 1997; van Winden and Crommelin, 1999). On the other hand, in cationic lipid/DNA complexes during lyophilization, sample vitrification did not correlate with maintenance of transfection efficiency (Allison and Anchordoquy, 2000). It was likely that sucrose might stabilize plasmid DNA by directly interacting with plasmid DNA rather than by vitrifying to a high T_g solid. Supporting our inference, it was reported that the efficacy of transfection of lipoplexes was enhanced by mixing medium and disaccharides (Tseng et al., 2007).

Formulation B1 maintained a high TE value even during storage at 50 °C for 50 days, having about one-fourth of the TE of a commercially available transfection reagent, Lipofectamine 2000. We examined the cytotoxicity of the DRV/DNA complex, as indicated by the protein concentration after transfection (data not shown). All formulations showed low cytotoxicity compared with Lipofectamine 2000.

Entrapment of plasmid DNA inside DRVs, rather than a greater association at the surface of liposomes, may offer a more

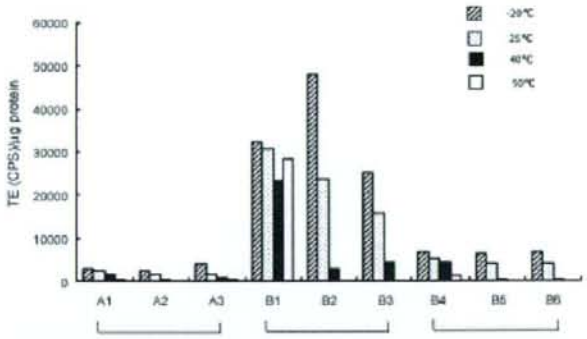

Fig. 3. Transfection efficiency in HeLa cells transfected with DRV/DNA complex with sugars after storage at various temperatures for 50 days. Each DRV/DNA complex was diluted with medium without FBS to a final concentration of 2 μg of plasmid DNA in 1 ml of medium per well. Each column represents the mean ($n = 2$). TE of B5 and B6 at 50 °C was not detected.

controlled approach to vesicle formation. This cryo-protective effect may be desirable when preparing DRV/DNA complexes without aggregation and with entrapment of DNA.

4. Conclusion

We developed lyophilized formulations of liposome (DRV)/plasmid DNA complex vectors prepared with the DRV method. DRV/DNA complexes lyophilized with sucrose could be stored even at 50 °C for 50 days without a large loss of transfection efficiency. This finding suggests that sucrose might stabilize plasmid DNA by directly interacting with plasmid DNA rather than by vitrifying to a high T_g solid. Further long-term stability studies will be required to determine the shelf life of lyophilized liposome/DNA complexes at room temperature. These findings provide new information about the effects of physicochemical changes of nonviral vectors during lyophilization.

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Artificial Lipids Stabilized Camptothecin Incorporated in Liposomes

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Camptothecin (CPT) has anticancer activity. While only the lactone form of CPT is biologically active, this form exhibits poor aqueous solubility. Pharmaceutical formulation of CPT incorporated in liposomes is of significant importance to develop the therapeutic utilization of CPT. The aim of this study was to increase incorporation efficiency and stability of CPT in liposomes composed of hydrogenated soybean phosphatidylcholine, cholesterol, and oleic acid (7 : 3 : 1, molar ratio), by incorporating three kinds of artificial lipids (DBs) (DB-liposome); 4-*n*-(M12B), 3,5-bis(B12B) and 3,4,5-tris(dodecyloxy)benzoic acid (T12B). The interaction of CPT with DB in the state of liposomes, was examined. In DB-liposomes presenting mean diameters of 150 nm, incorporation efficiency of CPT up to 55% and final drug to lipid molar ratio up to 0.07 were obtained when the liposomes were prepared at a feeding ratio of 1/30 (w/w) CPT/total lipid. However, in the optimal formulations, incorporated DB mol% was different; T12B and D12B were incorporated about one third and half mol% of M12B, respectively. Moreover, we demonstrated that T12B stabilized CPT in liposomes significantly compared with other DBs as measured by CPT release, and by steady state fluorescence polarization degree of CPT using intrinsic fluorescence of CPT. These findings suggested that in addition of contribution of phenyl group of DB, dodecyloxy group may interact strongly with lactone ring of CPT. The capacity to contain CPT interacted with DBs may be limited in liposomes. T12B may be incorporated in the interior of the bilayers, resulting in increase of incorporation stability of CPT. This finding demonstrates a potential application of the novel liposome formulation of CPT in drug delivery.

Key words camptothecin; liposome, artificial lipid; release test; incorporation efficiency; incorporation stability

Camptothecin (CPT) is a naturally occurring cytotoxic alkaloid isolated from the Chinese plant *Camptotheca accuminate*.[1] CPT and some of its analogs have shown a broad spectrum of antitumor activity against many solid tumors in xenografts including colorectal cancer.[2,3] CPT inhibits the enzyme DNA topoisomerase I, initially by noncovalent binding and subsequently by stabilization of the complex through a nucleophilic attack by the enzyme at the acyl position of the CPT lactone ring.[4]

Of significant importance for pharmaceutical formulation is that, while only the lactone form of CPT is biologically active, this form exhibits poor aqueous solubility. The lactone of CPT is converted to carboxylate in a pH-dependent equilibrium.[5] To overcome the aforementioned solubility problems and hydrolytic processes of CPT, several approaches have been investigated. Numerous attempts have been made to prepare water-soluble CPT analogs. The majority of these analogs were less potent in assays both *in vitro* and *in vivo* than the parent drug. In addition to the synthesis of new derivatives and pro-drug products,[6—10] the development of adequate drug carriers is gaining increasing attention. There are many reports about effective formulation and utilization of CPT in cancer therapy by using drug delivery technologies such as liposomes,[11,12] microemulsions,[12] microspheres,[13,14] and inclusion complexes with cyclodextrins.[15] Previously we have reported that the stability of CPT loaded polymeric micelles *in vivo* was increased by benzyl esterification of hydrophobic segment of block copolymer.[16—18] However, long circulation of CPT loaded polymeric micelles *in vivo* was not achieved yet. Other carriers such as liposomes were examined because release of drugs encapsulated in carriers depended on carriers. The designed amphipathic compounds are called artificial lipid, which has similar properties with phospholipid to form vesicles. Therefore, artificial lipid with a phenyl group, *e.g.*, 3,5-bis(dodecyloxy)benzoic acid, was synthesized and added to the liposome formulation. The PEGylated liposomes incorporating CPT were stable *in vivo*.[19] However, there was not enough information about interaction between CPT and artificial lipid molecules in liposomes.

To develop the therapeutic utilization of CPT, it is necessary to prepare liposomes with high incorporation efficiency and stability of CPT. This study demonstrated that incorporation of 5 mol% of 3,4,5-tris(dodecyloxy)benzoic acid increased incorporation efficiency and stability of CPT in liposomes.

MATERIALS AND METHODS

Materials (*S*)-(+)-Camptothecin (CPT), cholesterol (Ch), high performance liquid chromatography (HPLC) grade methanol and tetrahydrofuran (THF) were purchased from Wako Pure Chemicals (Tokyo, Japan). Hydrogenated soybean phosphatidylcholine (HSPC, >90% phosphatidylcholine), and oleic acid (OA) were purchased from NOF Corporation (Tokyo, Japan). 4-*n*-Dodecyloxy benzoic acid (M12B) was purchased from Tokyo Chemical Industry (Tokyo, Japan). 3,5-Bis(dodecyloxy)benzoic acid (B12B) and 3,4,5-tris(dodecyloxy)benzoic acid (T12B) were synthesized as reported previously[20] (Fig. 1). Other chemicals were of reagent grade.

Preparation of Liposomes Liposomes incorporating CPT were prepared as described elsewhere.[19] Briefly, HSPC, Ch, OA, DB and CPT (molar ratio, HSPC : Ch : OA : DB : CPT = 7 : 3 : 1 : 0—3 : 1; weight ratio, total lipid : CPT = 30 : 1) were dissolved in methanol/chloroform mixture (1/4 (v/v)). The solvent was evaporated in a rotary evaporator at 55 °C under stream of N_2 gas. The lipid film containing the drug was hydrated with 2.5 ml of sodium phosphate-buffered solu-

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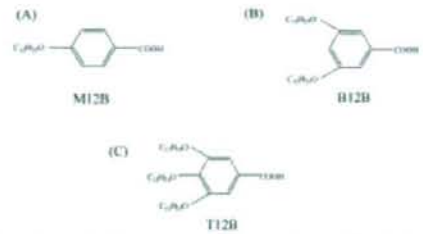

Fig. 1. Chemical Structure of 4-*n*-Dodecyloxy Benzoic Acid (A), 3,5-Bis(dodecyloxy)benzoic Acid (B), and 3,4,5-Tris(dodecyloxy)benzoic Acid (C)

tion (pH 6.04, 2.33% KH_2PO_4: 1.44% $NaHCO_3$=4 : 1 volume ratio) to protect conversion from the lactone of CPT to carboxylate. The suspension was sonicated for 8 min and was concentrated by centrifugation at 3900 rpm for 10 min to remove the big particles including unentrapped CPT aggregate. Size distribution of liposomes was monitored using a dynamic light scattering particle size analyzer (ELS-800, Otsuka Electronics, Osaka, Japan) at 25 °C by diluting liposome suspensions to an appropriate volume with water.

Determination of CPT and DB Content in Liposomes Drug incorporation efficiency was determined using the ultra-centrifugation method. Liposomes incorporating CPT were centrifuged at 52000 ***g*** for 1 h at 4 °C to separate free CPT. Then, the incorporation efficiency was obtained using two methods: determination of the CPT concentration of the supernatant containing free CPT, and determination of the amount of CPT entrapped in the precipitate, which was disrupted using chloroform. The incorporation efficiencies estimated using both methods were similar. The former method was used in the following experiment. The total drug concentrations in liposomes before centrifugation (liposome A) and in the supernatant after centrifugation (supernatant B) were determined using a F-4010 fluorescence spectrophotometer (Hitachi Electronics, Tokyo, Japan) with the excitation and emission wavelengths of 369 and 437 nm, respectively as described previously.[16]

Incorporation efficiency of DB was determined by HPLC (wavelength at 254 nm). A Shimadzu LC-10AT (Shimadzu Co., Ltd., Japan) apparatus equipped with a Shimadzu RF-10AXL fluorescence detector in which the wavelengths were set at 254 nm. Separation was performed with an YMC-Pack ODS-AA-302 column (150×4.6 mm I.D., YMC Co., Ltd., Kyoto, Japan). For M12B and B12B, the mobile phase was composed of 19 : 1 or 99 : 1 methanol–phosphate-buffered solution (pH 3.02), respectively, and the flow rate was set at 1.0 ml/min. For T12B, 17 : 3 (v/v) methanol–THF. The incorporation efficiency of drug or DB in the liposomes was calculated as follows.

$$\text{incorporation efficiency of drug or DB (\%, w/w)} = \frac{\text{drug (DB)}_{\text{liposome A}} - \text{drug (DB)}_{\text{supernatant B}}}{\text{drug (DB)}_{\text{total}}} \times 100$$

***In Vitro* Drug Release** *In vitro* release of CPT from the liposomal formulation was analyzed by membrane dialysis against phosphate-buffered saline (PBS, pH 7.4) at 37 °C. Briefly, 1 ml of CPT liposomes was placed in a dialysis tube (Spectra/Por CE (MWCO 12000—14000, Spectrum Laboratories, Inc., Rancho Dominguez, CA, U.S.A.)) and then suspended in a temperature-controlled, jacketed flask containing 100 ml of PBS. After various time intervals, aliquots were withdrawn and assayed for CPT content by fluorophotometry. Drug release profiles (percent release *versus* time) were plotted.

Fluorescence Polarization Measurements To evaluate distribution of CPT in the liposomes, we examined mobility of CPT using intrinsic fluorescence of CPT by fluorescence polarization measurements. We prepared about 10 μM of lipid concentration of M12B-, B12B- and T12B-liposome (HSPC : Ch : OA : M12B : CPT=7 : 3 : 1 : 1 : x, HSPC : Ch : OA : B12B : CPT=7 : 3 : 1 : 1 : x, and HSPC : Ch : OA : T12B : CPT=7 : 3 : 1 : 1 : x, molar ratio) aqueous suspension with CPT concentration varied from 5.7×10^{-1} μg/ml to 0.1 mg/ml (corresponding on x molar ratio). Steady-state fluorescence polarization measurements were performed on F-4500 fluorescence spectrophotometer (HITACHI, Electronics). One-centimeter rectangular quartz fluorometer cell was used, and the excitation and emission wavelengths were set at 369 and 437 nm, respectively. The fluorescence polarization of liposomes in sodium phosphate-buffered solution (pH 6.04) was measured at room temperature (24 °C). $I_{\parallel}$ ($I_{\perp}$) is the intensity of photons with electric vectors parallel (perpendicular) to the beam direction.

$$\text{polarization degree} = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$$

Statistical Analysis The statistical significance of the data was evaluated with Student's t test. A p value of 0.05 or less was considered significant.

RESULTS AND DISCUSSION

Preparation and Characterization of CPT Incorporated in Liposomes The incorporation stability of CPT in polymeric micelles *in vitro* and *in vivo* was increased by benzyl esterification of hydrophobic segment of block copolymer, supposed to be π–π interaction of phenyl group with CPT.[16—19] Therefore, the artificial lipids with a phenyl group, DBs were synthesized and added to liposome formulation to obtain stable liposomes incorporating CPT (DB-liposome). Basic formulation, HSPC : Ch : OA : CPT=7 : 3 : 1 : 1 (molar ratio) was decided as HSPC, Ch and OA since liposomes with OA showed about five-fold higher incorporation efficiency of CPT compared with ones without OA.[19] Here we prepared three kinds of liposomes incorporating M12B, B12B and T12B, referred as M12B-, B12B-, and T12B-liposome, respectively. The particle size was not significantly different among all the DB-liposome formulations, and was about 150 nm, when the liposomes were prepared at a feeding ratio of 1/30 CPT/total lipid.

Effect of DB/lipid Ratio on Incorporation to Liposomes CPT and DB contents in liposomes were determined at liposome formulation as HSPC : Ch : OA : DB : CPT=7 : 3 : 1 : 0—3 : 1 (mol), as shown in Figs. 2 and 3. When the ratio of each DB to starting total lipid of M12B-, B12B- and T12B-liposomes was increased to 15.3 mol%, the incorporation efficiency of M12B, B12B and T12B was increased to 84, 61

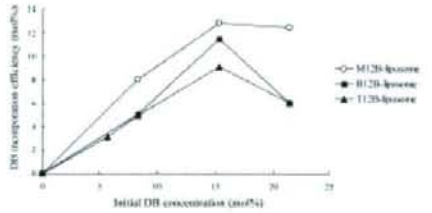

Fig. 2. Effect of Initial DB Concentration on Final DB Incorporation Levels in DB-Liposomes Composed of HSPC : Ch : OA : DB : CPT = 7 : 3 : 1 : 0—3 : 1 (Molar Ratio)

Data are average of 2 independent experiments.

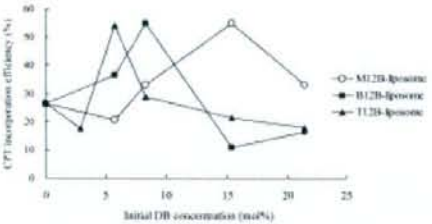

Fig. 3. Effect of Initial DB Concentration on Final CPT Incorporation Efficiency in DB-Liposomes Composed of HSPC : Ch : OA : DB : CPT = 7 : 3 : 1 : 0—3 : 1 (Molar Ratio)

Data are average of 2 independent experiments.

and 54%, respectively (Fig. 2). Also, incorporation efficiency of CPT up to 55% in all liposomes was obtained (Fig. 3). However, in the optimal formulation, DB (mol%) was different. Without DB, 26.3% of CPT was incorporated in liposomes (Control-L). The maximum CPT incorporation efficiency was obtained at the formulations of DB-liposomes: HSPC : Ch : OA : M12B : CPT = 7 : 3 : 1 : 2 : 1 (molar ratio, M12B-L), HSPC : Ch : OA : B12B : CPT = 7 : 3 : 1 : 1 : 1 (B12B-L), and HSPC : Ch : OA : T12B : CPT = 7 : 3 : 1 : 0.67 : 1 (T12B-L), as reflected about 2-fold increase (55% for M12B-L and B12B-L, and 54% for T12B-L, final drug to lipid molar ration up to 0.07) compared with Control-L. Among DB-liposomes, T12B seemed most effective to incorporate CPT in liposomes since it worked at the smallest addition amount. The excess amount of T12B decreased incorporation of CPT in liposomes, suggesting that the capacity to contain the complex of T12B with CPT might be limited in liposomes. When increase of initial CPT amount in T12B-liposomes, the incorporation efficiency of CPT was decreased (data not shown). The incorporation of DBs in liposomes seemed to decide incorporation of CPT in liposomes. Because CPT could not complex with B12B (data not shown), DBs may distribute in liposomes, and then CPT may interact with DBs and could be incorporated into the interior of the bilayers. CPT molecules may be accumulated in hydrophobic region of membranes. The presence of DB contributed increased 30% of incorporation efficiency. These findings suggested that increased CPT incorporation efficiency by DBs might be due to interaction between phenyl and dodecyloxy

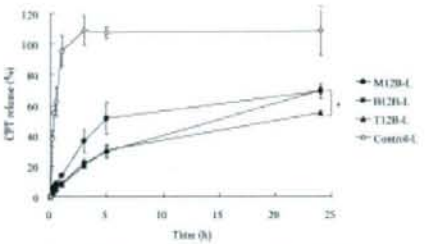

Fig. 4. Percent CPT Released as a Function of Time at 37 °C

CPT release from the liposomes of M12B-L (○), B12B-L (■), T12B-L (▲) and Control-L (◇) at the initial CPT entrapped in liposomes concentration of 200 μg/ml. CPT was monitored by membrane dialysis as described in Materials and Method using PBS as sink solution at pH = 7.4. Data are expressed in the mean ± standard deviation of 3 independent experiments. * $p<0.05$.

group of DBs and lactone ring of CPT over increase of acidity by carboxyl group of DBs.

***In Vitro* Drug Release** The incorporation stability of CPT in M12B-L, B12B-L and T12B-L was examined from drug release test by incubation in PBS at 37 °C, as shown in Fig. 4. The CPT released from Control-L was 100% for 3 h while that from M12B-L, B12B-L and T12B-L were 36.3, 22.3 and 20.5%, respectively. The CPT released from M12B-L was significantly higher than that from B12B-L and T12B-L for 5 h. CPT incorporated in M12B-L may be distributed at the surface of liposomes more than that in B12B-L and T12B-L, therefore CPT was released highly. During 24-h-period, M12B-L, B12B-L and T12B-L released 68.8, 69.0 and 54.5%, respectively. Release of CPT among DB-liposomes was higher T12B<B12B<M12B, and T12B-L showed significantly lower release than B12B and M12B at 24 h ($p<0.05$). This result indicated that DBs increased incorporation stability of CPT, and incorporation stability of CPT was increased with increase of the numbers of dodecyloxy-group of DBs. Dodecyloxy-group of DBs might induce drug's lactone ring to penetrate into lipid bilayers.

Fluorescence Measurements Anisotropy measurements were worthwhile as a strong indication of incorporation stability. Polarization degree of intrinsic fluorescence of CPT incorporated in the liposomes was evaluated. Figure 5 revealed that the polarization degree was CPT concentration-dependant. The polarization degree values were directly related to the kind of environment where the CPT was distributed. Free rotations in DMSO solution were related to the smallest values of polarization degree, compared to the state in liposomes, indicating that CPT molecule can move freely. M12B-, B12B- and T12B-liposomes at 0.11—0.13 μM of CPT concentration, exhibited higher polarization degree (0.47, 0.45, 0.53, respectively) compared with Control-liposomes. These results reflected that CPT molecules were inserted deeply into the lipid bilayer of liposomes. A pronounced decrease in polarization was observed below about 1 μM CPT in liposomes, revealed disordering of the lipid bilayer in the presence of CPT. Among DB-liposomes, T12B-liposome seemed to have high incorporation stability, resulting from protection of disordering property of CPT in liposomes. This result corresponded with that of release test;

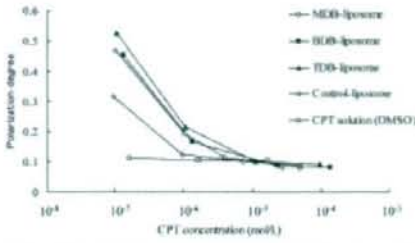

Fig. 5. Polarization Degree *versus* CPT Concentration Incorporated in M12B-Liposome (○), B12B-Liposome (■), T12B-Liposome (▲) Control-Liposome (◇) and CPT DMSO Solution at Room Temperature (24 °C)

M12B-, B12B- and T12B-liposome (HSPC : Ch : OA : M12B : CPT = 7 : 3 : 1 : 1 : x, HSPC : Ch : OA : B12B : CPT = 7 : 3 : 1 : 1 : x and HSPC : Ch : OA : T12B : CPT = 7 : 3 : 1 : 1 : x, molar ratio, respectively) aqueous suspension with CPT concentration varied from 5.7×10^{-5} μg/ml to 0.1 mg/ml.

CPT in T12B-L showed the lowest release among DB-liposomes. These findings suggested that CPT in T12B-L would be incorporated into the interior of the bilayers by interaction of dodecyloxy-group of T12B with drug's lactone ring, and release slowly, while CPT in Control-L would be in contact with the water/lipid interface, be changed into the ionized form, and be released quickly.

Liposomal CPT delivery systems may be promising to cancer therapy. We have reported presently to apply this PE-Gylated formulation *in vivo* to evaluate anticancer effect.[19]

CONCLUSIONS

By incorporating various amounts of artificial lipid, DB, incorporation efficiency of CPT in liposomes increased. Additionally, we demonstrated, 4,5-tris(dodecyloxy)benzoic acid (T12B) stabilized significantly CPT in liposomes at about one third of M12B amount compared with other DBs as measured by CPT release. These findings suggested that incorporation stability of CPT in liposomes was increased, likely due to the interaction between lactone ring of CPT and dodecyloxy group more than phenyl group of DB, resulting in CPT incorporated into the interior of the bilayers.

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In vivo antitumor activity of camptothecin incorporated in liposomes formulated with an artificial lipid and human serum albumin

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Abstract

Camptothecin (CPT) is a strong antitumor agent, but its use limited by its low solubility and the instability of the active lactone form. To overcome these difficulties, liposomes incorporating CPT (CPT liposomes) were designed and tested. CPT liposomes were formulated by the addition of 3,5-bis(dodecyloxy)benzoic acid (DB) to polyethylene glycol-containing liposomes, and by coating the surface of the liposomes with human serum albumin (HSA, HSA-DB-L). HSA-DB-L successfully entrapped CPT with about 80% efficiency and with a particle size of about 150 nm. HSA-DB-L showed attenuated drug release and storage stability. Pharmacokinetics studies in mice showed that i.v. injection of HSA-DB-L (2.5 mg/kg) led to prolonged circulation in the plasma; the area under the curve was 22-fold higher than that of CPT solution. The tumor growth in mice with subcutaneous transplantation of colon 26 tumor cells was significantly inhibited after a single i.v. injection of HSA-DB-L at a dose of 15 mg/kg without any significant body weight loss. HSA-DB-L increased the accumulation of CPT in tumor tissue significantly (9.6-fold) more efficiently than CPT solution 24 h after i.v. injection. These findings suggest that HSA-DB-L could increase the stability and the antitumor effect of CPT. CPT delivery by novel liposome formulations is a potential approach for effective treatment of cancer.

Keywords: Camptothecin; Liposome; Antitumor effect; HSA; Colon 26 tumor

1. Introduction

Camptothecin (CPT) is a naturally occurring cytotoxic alkaloid isolated from the Chinese plant *Camptotheca accuminata* [1]. CPT and some of its analogs have shown a broad spectrum of antitumor activity against many solid tumors in xenografts, including colorectal cancer [2,3]. CPT inhibits the enzyme DNA topoisomerase I, initially by noncovalent binding and subsequently by stabilization of the complex through a nucleophilic attack by the enzyme at the acyl position of the CPT lactone ring [4]. Under physiological conditions, i.e., at pH 7 or above, the lactone ring readily opens to yield the inactive carboxylate form of the drug, and this conversion is in pH-dependent equilibrium [5] (Fig. 1(A)). Moreover, the presence of human serum albumin (HSA) in the blood or serum showed rapid CPT lactone ring opening. This was probably due to preferential HSA binding to the carboxylate form resulting in a change in the lactone-carboxylate equilibrium. Thus shifts the equilibrium toward the pharmacologically ineffective carboxylate form [6]. Only the lactone form of CPT is biologically active; however, this form exhibits poor aqueous solubility.

To overcome these stability and solubility problems of CPT, several approaches have been investigated. In addition to the synthesis of new derivatives and pro-drug products [7,8], the development of adequate drug carriers is gaining increasing attention. There are many reports about effective formulation and utilization of CPT in cancer therapy by using drug delivery technologies such as incorporation in liposomes [9,10], polymer micelles[11,12], microemulsions [10], and microspheres [13,14]. About liposomes, the protection of the lactone form of CPT by liposomes has been known [9]. Low solubility of CPT in water or lipids, however, was limited to develop liposomal CPT. Safer and more water-soluble CPT derivatives, therefore,

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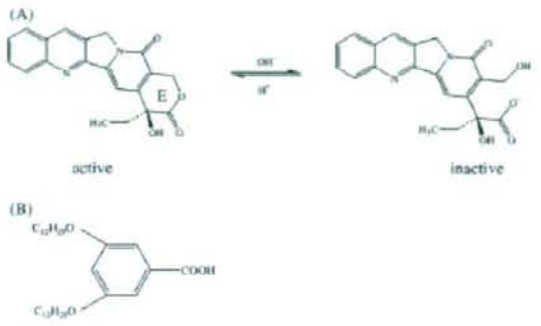

Fig. 1. Structure of camptothecin (CPT) and equilibrium reaction between the active and inactive form (A) and structure of 3,5-bis(dodecyloxy)benzoic acid (DB).

were used to be entrapped in the liposomal water phase [15,16]. CPT has higher activity than these derivatives [17]. If stable CPT liposomal formulations in vivo can be developed, high antitumor effect will be obtained.

To this purpose, novel CPT liposomes were formulated by the addition of an artificial lipid with a phenyl group to polyethylene glycol-modified liposomes, and by coating the surface of the liposomes with HSA. Because we reported that stable CPT-loaded polymer micelles in vivo were produced using benzyl polymer [11,12,18,19], presumably due to be π–π interaction of the phenyl group with CPT, lipids with a phenyl group in liposomes might interact with CPT and could incorporate CPT effectively. An artificial lipid with a phenyl group, 3,5-bis (dodecyloxy) benzoic acid (DB), therefore, was synthesized (Fig. 1(B)) and added to the liposome formulation. We also modified liposomes with polyethylene glycol (PEG) for long circulating, because they can passively deliver chemotherapeutic agents to tumor sites via the enhanced permeation and retention (EPR) effect [20–22]. Furthermore, we coated the liposomes by HSA because pre-coating polystyrene particles with HSA enhanced their stability in blood [23].

In the present study, we demonstrated that CPT could be efficiently incorporated into the pegylated liposomes by the addition of DB, combined with coating HSA on the surface of the liposomes. The drug release from the liposomes, the storage stability, pharmacokinetics, and in vivo antitumor activity of HSA-DB-L were examined. The liposomal form of CPT displayed antitumor activity against mice bearing colon adenocarcinoma 26 when administered by a single i.v. injection.

2. Materials and methods

2.1. Materials

(*S*)-(+)-Camptothecin (CPT), cholesterol (Ch), high performance liquid chromatography (HPLC) grade acetonitrile and triethylamine acetate were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Hydrogenated soybean phosphatidylcholine (HSPC, >90% phosphatidylcholine), distearoylphosphatidylethanolamine-*n*-[methoxy(polyethylene glycol)-2000] (PEG2000), distearoylphosphatidylethanolamine-*n*-[methoxy(polyethylene glycol)-5000] (PEG5000) and oleic acid (OA) were purchased from NOF Corporation (Tokyo, Japan). HSA was purchased from Sigma Chemical Co. (St. Louis, MO, USA). DB was synthesized as reported previously [24]. Other chemicals were of reagent grade.

2.2. Preparation of liposomes

CPT-incorporating liposomes (CPT liposomes) composed of the formulations summarized in Table 1 were prepared by a lipid-film hydration method. Briefly, CPT (1 mg) and lipids (e.g., 30 mg of total lipids for DB-L, HSPC/Ch/OA/DB/PEG2000=7:3:1:1:0.4 (molar ratio)=19.3/4.1/1.0/1.7/3.9 (mg)) were dissolved in a mixture of chloroform: methanol (4:1, volume ratio), and the solvent was removed by evaporation under nitrogen gas flow. The lipid film was hydrated with 2 mL of phosphate-buffered solution (pH 6.0, 2.33% KH_2PO_4: 1.44% $NaHCO_3$=4:1, volume ratio). The lipid mixture was sonicated for 30 min using an ultrasonic bath. Excess CPT was precipitated and could be separated from the liposomes by centrifugation at 1400 ×g for 10 min. Liposomes with average sizes ranging from 150 to 200 nm with low polydispersity index (<0.3) were obtained, as determined using a dynamic light scattering particle size analyzer, and surface potentials of them were determined by the electrophoresis light scattering method (ELS-800, Otsuka Electronics Co., Ltd. Osaka, Japan) at 25 °C by diluting liposome suspensions to an appropriate volume with water.

Good and reproducible recovery of liposomes in the supernatant (>80%) was obtained, as determined by an enzymatic assay using a Phospholipid C-test Wako (Wako Pure Chemical Industries, Ltd.).

Control liposomes (Control-L) were composed of HSPC, Ch, OA and PEG2000. Control-L containing 8 mol% and 15 mol% DB are hereafter designed as DB-L and 2DB-L, respectively. DB-L5000 had a similar composition as DB-L, but contained PEG5000 instead of PEG2000. For coating of DB-L with HSA (HSA-DB-L), the DB-L suspension (e.g., 7.5 mg total lipids/mL)

Table 1
Characterization of CPT liposomes

Code	Formulation (molar ratio)	Particle size (nm)	Incorporation efficiency [a] (%)	% Injected dose in plasma after 4 h [b]
CPT solution		–	–	0.02±0.01
Control-L	HSPC/Ch/OA/PEG2000 (7:3:1:0.4)	137.4±15.4	75.6± 11.4	0.3±0.1
DB-L	HSPC/Ch/OA/DB/PEG2000 (7:3:1:1.0.4)	148.4±0.9	80.7± 8.4	1.0±0.2
2DB-L	HSPC/Ch/OA/DB/PEG2000 (7:3:1:2.0.4)	177.2±50.5	76.9± 16.9	0.04
DB-L5000	HSPC/Ch/OA/DB/PEG5000 (7:3:1:1.0.4)	148.4±0.9	85.8± 4.9	0.2±0.2
HSA-DB-L	HSPC/Ch/OA/DB/PEG2000 (7:3:1:1.0.4)+HSA	161.3±9.2	85.8± 4.9	2.5±0.8

Each value represents the mean±SD. ($n=3$).
*$P<0.05$, **$P<0.01$, ***$P<0.001$.
[a] Prepared at a feeding ratio of 1/30 (CPT/total lipid, w/w).
[b] Results at a dose of 2.5 mg CPT/kg in ddY mice.

was incubated with 4% HSA aqueous solution at room temperature (20–25 °C) for 1 h. HSA-DB-L was used without separation of free HSA in in vivo experiment.

The CPT solution was prepared by dissolving CPT (13.0 mg) in 50 mL of polyethylene glycol 400, propylene glycol and polysorbate 80 (40:50:2, volume ratio) [25].

2.3. Determination of HSA amount associated with HSA-DB-L

The adsorbed HSA was measured using bicinchoninic acid (BCA) protein assay reagent (Pierce, Rockford, IL, USA) and analyzed by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) after separation of liposomes by ultracentrifugation (100,000×g, 1 h, 4 °C). The gel was stained with Coomassie brilliant blue (Quick-CBB, Wako Pure Chemical Industries, Ltd., Osaka, Japan).

2.4. Cytotoxicity of liposomes containing DB

Mouse colon adenocarcinoma 26 (C26) cells (5.0×10^3 cell/well) were plated into 96-well culture plates 1 day before the experiment. The cells were incubated for 48 h at 37 °C with liposomes. The cytotoxicity was then determined using a WST-8 assay (Cell Counting Kit-8, Dojindo Laboratories, Kumamoto, Japan). The number of viable cells was determined by measuring the absorbance at 450 nm on an automated plate reader.

2.5. Determination of efficiency of CPT incorporation into liposomes

Drug incorporation efficiency was determined using the ultracentrifugation method. CPT liposomes were centrifuged at 52,000×g for 1 h at 4 °C to separate free CPT from liposomal CPT. Then, the incorporation efficiency was obtained using two methods: determination of the CPT concentration of the supernatant containing free CPT, and determination of the amount of CPT entrapped in the precipitate, which was disrupted using chloroform. The incorporation efficiencies estimated using both methods were similar. The former method was used in the following experiment. The total drug concentrations in liposomes before centrifugation (drug initial) and in the supernatant after centrifugation (drug supernatant) were determined using a fluorescence spectrophotometer (F-4010, Hitachi Electronics, Tokyo, Japan) with excitation and emission wavelengths of 369 and 437 nm, respectively. The percentage of the drug entrapped in the liposomes was calculated as follows:

$$\text{Incorporation efficiency (\%)} = \left(\text{drug}_{\text{initial}} - \text{drug}_{\text{supernatant}}\right)/\text{drug}_{\text{initial}} \times 100.$$

2.6. In vitro drug release of CPT liposomes

In vitro release of CPT from the liposomal formulation was analyzed by membrane dialysis against phosphate-buffered saline (PBS, pH 7.4) at 37 °C under sink condition. Briefly, 1 mL of CPT liposomes (0.4–0.5 mg CPT/mL) was placed in a dialysis tube (Spectra/Por CE, MWCO 300,000, Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA) and then suspended in a temperature-controlled, jacketed flask containing 100 mL of PBS. After various time intervals, aliquots of the medium were withdrawn and assayed for CPT content by fluorophotometry.

2.7. Storage stability study

The lactone form of CPT in the HSA-DB-L was evaluated after storage at room temperature (20–25 °C) for an extended period of time. The lactone and carboxylate forms of CPT liposomes were then immediately measured by reverse-phase HPLC analysis.

The HPLC analysis was performed at room temperature. A Shimadzu LC-10AT (Shimadzu Co., Ltd., Japan) apparatus equipped with a Shimadzu RF-10AXL fluorescence detector in which the excitation and emission wavelengths were set at 369 and 437 nm, respectively, was used. Separation was performed with a Tosoh TSK-gel ODS-80Ts column (150×4.6 mm I.D.).

Table 2
Cytotoxicity of CPT liposomes to colon 26 cells

Code (formulation)	IC50 (μg/mL)
Control-L not containing CPT (HSPC/Ch/OA/PEG2000)	1.1 mg/mL*
DB-L not containing CPT (HSPC/Ch/OA/PEG2000)	1.1 mg/mL*
CPT solution	0.077
DB-L (HSPC/Ch/OA/DB/PEG2000)	0.048
HSA-DB-L (HSPC/Ch/OA/DB/PEG2000+HSA)	0.042

* Concentration of lipids.

The mobile phase was composed of 23:77 (v/v) acetonitrile-triethylamine acetate buffer (1% (v/v), adjusted to pH 5.5 with glacial acetic acid), and the flow rate was set at 1.0 mL/min [26].

2.8. Measurement of CPT concentration in plasma in mice

CPT liposomes or CPT solution was intravenously (i.v.) administered to male ddY mice (weighing 18–20 g, Tokyo Laboratory Animal Science Co., Ltd. Tokyo, Japan) via the lateral tail veins at a dose of 2.5 mg/kg (0.1–0.2 mL/10 g body weight). At various times after the administration, blood was withdrawn using a heparinized syringe and centrifuged at 15,300 ×g for 4 min to obtain the plasma. Plasma was added to 0.1 mL of 0.15 M aqueous phosphoric acid followed by mixing [27] and CPT was extracted with 0.8 mL of chloroform: methanol (4:1, volume ratio). This operation gave the total concentration of free CPT and CPT incorporated into liposomes as a lactone form. After centrifugation of the mixture at 15,300 ×g for 4 min, 25 μL of the organic solvent layer was directly injected into the HPLC system to determine the concentration of CPT. The area under the concentration curve (from 0 h to 24 h: AUC) and clearance were calculated using the bootstrap method [28].

2.9. Biodistribution studies in tumor-bearing mice

At 1 week after transplantation of 1.0×10^5 cells, when tumor size reached approximately 100 mm^3, C26-bearing CDF1 female mice (6 weeks old) were injected via a lateral tail vein with HSA-DB-L or CPT solution at a dose of 2.5 mg/kg as CPT. After 24 h, the mice were anesthetized with diethyl ether. Blood samples were collected and then the major organs and tumor were excised, rinsed in physiologic saline, weighed, and frozen at −20 °C. The tissues were homogenized in 5.0 mL of PBS. CPT was extracted from tissue homogenate or plasma as described above and the CPT concentration was determined by HPLC.

2.10. In vivo antitumor activity

The antitumor activity against a solid tumor was evaluated with C26. C26 cells (1.0×10^5 cells in 0.2 mL) were transplanted subcutaneously into the backs of CDF1 female mice (5 weeks old, Sankyo Labo Service Corporation, Tokyo, Japan). Drug injection was started 2 weeks after tumor transplantation, when the tumor volume reached approximately 100 mm^3 (Day 0), by i.v. injection via a lateral tail vein. CPT solution was used as a single injection at a dose of 1.5 mg/kg, and HSA-DB-L (e.g., 2.5 mg CPT and 25 mg total lipid/mL) as a single injection at a dose of 10 or 15 mg/kg, and a repeated injection of 10 mg/kg. The control group was injected with 0.9% NaCl solution (0.1 mL/10 g body weight). Tumor volumes and body weights were measured at intervals of a few days. Tumor volume was calculated as follows: volume $= 1/2LW^2$; L is the long diameter and W is the short diameter of a tumor. Percent tumor growth inhibition (T/C%) was calculated from the relative tumor volume at day 8 following the equation: $T/C\% = 100\times$(mean relative tumor volume of treated group)/(mean relative tumor volume of control group). The animal experiments were done with ethical approval from our Institutional Animal Care and Use Committee.

2.11. Statistical analysis

The results were analyzed statistically using Student's t-test. A P-value of 0.05 or less was considered significant.

3. Results

3.1. Characterization of CPT liposomes

To obtain CPT liposomes that were stable in vivo, liposomes were prepared using various formulations by adding DB as a lipid containing a phenyl group and by coating with HSA. The use of DB as a liposome component had not previously been reported, so first the cytotoxicity of DB was evaluated in cell culture. The 50% cell growth inhibitory concentration (IC_{50}) of DB-L and Control-L not containing CPT was 1.1 mg lipid/mL, indicating that DB in liposomes was not toxic (Table 2). Furthermore, the cytotoxicity of CPT-loaded liposomes and CPT solution in DMSO was examined. IC_{50} of CPT solution, DB-L and HSA-DB-L showed 0.077, 0.048 and 0.042 μg CPT/mL. Despite of the existence of PEG2000 in liposomes, IC_{50} values of DB-L and HSA-DB-L were lower than that of CPT solution. The CPT lactone ring opened at about 20 min in medium, resulting in biological inactivity [6]. Incorporation of CPT in liposomes could maintain active lactone form even in the presence of serum, indicating that liposome formulations could keep the antitumor effect of CPT.

About Control-L, addition of PEG2000 to the liposomes increased incorporated efficiency of CPT from 30% to 75% (data not shown). The particle size and incorporation efficiency were not significantly different among all the CPT liposome formulations, and were about 150 nm and 80%, respectively.

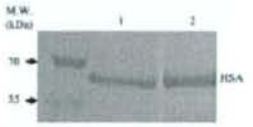

Fig. 2. SDS-PAGE of HSA associated on the liposomes. Lane 1: HSA-DB-L (HSPC/Ch/OA/DB/PEG2000+HSA). Lane 2: HSA-DB-L without PEG2000 (HSPC/Ch/OA/DB+HSA).