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Development of wrapped liposomes: Novel liposomes comprised of polyanion drug and cationic lipid complexes wrapped with neutral lipids

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Abstract

Novel wrapped liposomes comprised of polyanion drug and cationic lipid complexes wrapped with neutral lipids were prepared using an efficient, innovative procedure. In this study, dextran fluorescein anionic (DFA) was used as an example of a polyanionic compound. During the process, neutral lipids accumulated around the complexes and eventually covered the complexes. The resulting liposomes were 120–140 nm in diameter and the encapsulation efficiency was up to 90%. In fetal bovine serum, DFA/cationic lipid complexes degraded rapidly but the wrapped liposomes were considerably more stable. Following intravenous administration to rats, DFA/cationic lipid complexes were rapidly eliminated whereas the wrapped liposomes exhibited a much longer blood half-life. These data suggest that DFA is located on the surface of the complexes, but DFA is present inside the wrapped liposomes. The drug-delivery properties of the wrapped liposomes established in the present study suggests that formulations based on this technology could offer important advantages for the administration of many types of drug including antisense oligonucleotides, plasmids and siRNAs which may therefore lead to improved therapeutic effectiveness of this range of drugs. The method of preparation of the wrapped liposomes is so simple that it should be straightforward to adapt to a manufacturing scale.

Keywords: Liposome; Cationic lipid; Drug delivery; Formulation; DOTAP; PEG

1. Introduction

Liposomes are widely used as carriers for a variety of drugs [1,2]. In general, liposomes protect the drug from metabolism and inactivation in the plasma [3]. Encapsulation into polyethylene glycol (PEG) liposomes increases the efficacy and decreases the side effects of the drugs because the drugs are delivered preferentially to the site of action due to the enhanced permeability and retention effect [4]. A large number of drugs with weak basic or acidic characteristics can be actively concentrated in liposomes in response to a transmembrane pH gradient [5] or ammonium sulfate gradient [6]. However, these methods are restricted to low molecular weight drugs. High molecular weight drugs such as peptides, proteins, antisense oligonucleotides (ODNs), plasmids and siRNAs cannot be efficiently loaded into liposomes using a pH gradient [7]. It may be feasible to incorporate such high molecular weight drugs into liposomes comprised of neutral lipids but a low encapsulation efficiency would limit the practical application of this approach [8,9]. An alternative strategy would be to introduce high molecular weight drugs into cationic liposomes that should result in high encapsulation efficiencies and the presence of cationic lipids would facilitate uptake by cells [9–11]. However, cationic liposomes are very unstable in blood and are rapidly eliminated in vivo after intravenous administration [9,12]. Consequently, cationic liposomes cannot deliver nucleic acids to specific target sites in the body, especially to solid tumors. Furthermore, cationic

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liposomes often cause liver and inflammatory toxicities after systemic administration of cationic liposomes [13,14]. Recently, coated cationic liposomes (CCL) and stabilized antisense lipid particles (SALP) described by Stuart et al. [15,16] and Semple et al. [17,18], respectively, were reported to overcome the problems described above. Both these liposomal formulations have high encapsulation efficiencies, good stability in blood and long duration after intravenous administration. Moreover, they have been studying in vivo activities of their liposomes.

The purpose of this study was to identify alternative liposomal formulations of high molecular weight compounds that are stable in blood and to develop a novel method of preparing such formulations that achieve a high encapsulation efficiency (i.e., different from CCL and SALP). We recognized that the presence of multiple charged groups in nucleic acids and peptides might present challenges to producing an effective formulation; therefore, our investigations were conducted with dextran fluorescein anionic (DFA) as a model multiple-charged compound. The strategy was to produce complexes of DFA with cationic lipids and to wrap these complexes with neutral lipids.

2. Materials and methods

2.1. Materials

Dextran, fluorescein, 3000 MW, anionic (DFA) was purchased from Molecular Probes (Eugene, OR). Egg phosphatidylcholine (EPC) was purchased from Nippon Oil and Fat (Tokyo, Japan). 1,2-Dioleoyl-3-trimethylammonium propane (DOTAP) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(Polyethylene glycol)-2000] (PEG-DSPE) were purchased from Avanti Polar Lipids (Alabaster, AL). Fetal bovine serum (FBS) was purchased from GIBCO BRL (Grand Island, NY). Nuclepore polycarbonate filters were purchased from Corning (Acton, MA). Sepharose CL-4B was purchased from Amersham-Pharmacia Biotech (Uppsala, Sweden). All other chemicals and solvents were of analytical grade quality.

2.2. Animals

Male Crl:CD(SD) rats, 7 to 8 weeks of age (Charles River, Japan, Inc., Yokohama, Japan, 200 to 300 g at the start of the experiments), were housed for about one week under controlled conditions with free access to food and water before use. The Welfare Committee for Experimental Animals in our institute approved all the experiments performed in the study.

2.3. Preparation of DFA/DOTAP/PEG–DSPE complexes

A mixture of 10 mg DFA, 60 mg DOTAP and 24 mg PEG–DSPE (DFA/DOTAP/PEG–DSPE, 1/26/2.6, mol/mol/mol) were hydrated in 3 mL distilled water and extruded 20 times through two stacked Nuclepore polycarbonate membrane filters with pore sizes of 0.4 and 0.1 μm. The resultant complexes were diluted with two volumes of water and centrifuged in an ultracentrifuge (Optima™ XL-A, Beckman, Fullerton, CA) for 1 h at approximately 110,000×*g*. The supernatant containing uncomplexed DFA was removed and the pellet containing the complexes was resuspended in water to provide a preparation containing 30 mg/mL total lipids. The particle sizes and Zeta potentials of the resultant complexes were measured by dynamic light scattering (Zetasizer Nano-ZS, Malvern Instruments, Herrenberg, Germany). Average diameters were evaluated as a Z-average using monomodal method (a cumulant analysis). The volume-based distributions were obtained by Nano-ZS in the Gaussian-fit mode.

2.4. Preparation of wrapped liposomes

Aliquots (0.75 mL) of the complexes obtained after sizing were diluted with 1 mL ethanol. An ethanolic solution of EPC and PEG–DSPE (0.25 mL of a solution containing EPC 240 mg/mL and PEG–DSPE 50 mg/mL, EPC/PEG–DSPE, 17.3/1, mol/mol) was added to the complexes. At this stage, the proportion of ethanol in the mixture was 62.5% (v/v), and the concentrations of DFA, DOTAP and PEG–DSPE in complexes were 1.25, 7.5 and 3 mg/mL (DFA/DOTAP/PEG–DSPE, 1/26/2.6, mol/mol/mol), respectively, and the concentrations of additional EPC and PEG–DSPE were 30 and 6.25 mg/mL, respectively. Distilled water (23 mL) was slowly added whilst stirring the mixture continuously. The suspension was centrifuged for 1 h at approximately 110,000×*g*. The supernatant was discarded thereby eliminating ethanol completely. Less than 10% DFA was detected in the supernatant. The pellet was resuspended in phosphate buffered saline (PBS; pH 7.4) to provide a preparation containing 30 mg/mL total lipids. The particle size of the resultant wrapped liposomes was measured by dynamic light scattering and the content of EPC was determined using the phosphorus assay (Wako, Tokyo, Japan).

As a control, wrapped liposomes without complexes were prepared as follows: An aliquot (0.25 mL) of an ethanolic solution of EPC and PEG–DSPE (EPC 240 mg/PEG–DSPE 50 mg/mL, EPC/PEG–DSPE, 17.3/1, mol/mol) was added to aqueous ethanol (57.1%; 1.75 mL). Distilled water (23 mL) was slowly added to this suspension whilst stirring. The suspension was centrifuged for 1 h at approximately 110,000×*g*. The supernatant was removed and the pellet resuspended in PBS to provide a preparation containing 30 mg/mL total lipids.

2.5. Solubility in aqueous ethanol

The solubility of free DFA and various preparations in aqueous ethanol was determined by monitoring turbidity at 600 nm (U-3210, Hitachi, Tokyo, Japan). Compounds or preparations were added to water containing varying amounts of ethanol and these samples were stirred vigorously for about 1 min at room temperature. The amounts of compounds added to aqueous ethanol were as follows: free DFA 1.25 mg/mL: DOTAP and PEG–DSPE in DOTAP/PEG–DSPE liposomes were 7.5 and 3 mg/mL (DOTAP/PEG–DSPE, 26/2.6, mol/mol), respectively; DFA, DOTAP and PEG–DSPE in DFA/DOTAP/PEG–DSPE complexes were 1.25, 7.5 and 3 mg/mL (DFA/DOTAP/PEG–DSPE, 1/26/2.6, mol/mol/mol), respectively; EPC and PEG–DSPE in EPC/PEG–DSPE were 30 and 6.25 mg/mL (EPC/PEG–DSPE, 17.3/1, mol/mol), respectively.

2.6. Microscopy

Negative-stain electron microscopy was performed using the method of Magin and Weinstein [19] to assist examination of the morphology of the liposomes. Aliquots of wrapped liposomes, naked (unwrapped) complexes and wrapped liposomes in the absence of complexes were added to 1% aqueous solution of ammonium molybdate (adjusted to pH 7.3 using ammonia) to provide solutions containing 0.5 mg/mL total lipids. Several drops of each sample were placed on separate collodion-coated copper grids of mesh size 400 (Nisshin EM Co., Tokyo, Japan). After 1 min, excess water was absorbed onto filter paper. The grids were air dried and then examined under a H-7000 electron microscope (Hitachi, Tokyo, Japan) at 75 kV voltage.

2.7. Effect of NaCl

Changes in turbidity caused by the addition of NaCl solution were monitored by measuring the absorbance at 600 nm before and after addition of 0.1 volumes of NaCl solution to samples of wrapped liposomes and complexes [20]. The final concentration of NaCl in this turbidity test was 0.9 w/v%. The particle sizes of wrapped liposomes and complexes before and after addition of NaCl solution were also measured by dynamic light scattering.

2.8. Stability in FBS

In vitro release experiments were carried out in test tubes by diluting samples 1:99 (v/v) with 100% FBS. The concentration of total lipids in each mixture was

0.3 mg/mL. The mixtures were incubated at 37 °C and aliquots were removed at different times up to 6 h for determination of liposomal and released DFA. Liposomal DFA was separated from released DFA by a Sepharose CL-4B column (1.0×20 cm) equilibrated with PBS (pH 7.4) at 25 °C. An aliquot of each mixture was applied onto the column, which was eluted with PBS at approximately 0.5 mL/min. For analysis of DFA in the eluent, samples were diluted with 9 volumes of 1% triton X-100 solution and these solutions were analyzed fluorometrically using a multiwell fluorescence plate reader, Cytofluor™ II (PE Biosystems, Foster city, CA) with excitation and emission monochrometers set at 485 nm and 530 nm, respectively. Measurement of DFA in the first and second eluant provided quantification of liposomal and released DFA. The retention of DFA in liposomes was calculated as a percentage of total DFA applied onto the column.

2.9. Rat PK

Aliquots of naked complexes and wrapped liposomes were diluted to provide solutions containing 5 mg/mL total lipids. Free DFA was dissolved at 0.2 mg/mL in PBS. Rats were fixed in a dorsal position under urethane anesthesia (1 g/kg, i.p.) then samples of complexes, wrapped liposomes and free DFA were administered rapidly to separate groups of 3 rats via the femoral vein at a volume of 2 mL/kg. The dose of total lipids was 10 mg/kg. For the analysis of the plasma concentrations of DFA, approximately 0.3 mL blood was withdrawn at 1, 3, 10, 30, 60 and 180 min after dosing from the jugular vein using heparinized syringes; the blood samples were centrifuged for 5 min at 10,000×*g* at 4 °C and the plasma was removed. The DFA concentration in each plasma sample was determined by monitoring fluorescence (see Section 2.8). The % of the injected dose of DFA present in the plasma compartment was calculated assuming that the plasma volume of a 250 g rat was 7.8 mL [21]. The elimination half-lives ($t_{1/2}$), the areas under the plasma concentration–time curves ($AUC_{0-3\ h}$), the mean residence times (MRT) in rats were calculated from the plasma concentration–time curves after intravenous injection by a non-compartment model using the software package WinNonlin™ Professional Ver.4.1 (Pharsight Co., Mountain View, CA).

3. Results

3.1. Solubility in ethanol

The degree of turbidity, which provides an indication of solubility, of free DFA and a range of lipid preparations in water containing varying concentrations of ethanol is shown in Fig. 1. The data revealed that DFA was dissolved completely in 0–70% ethanol solution but that the compound came out of solution at the highest concentrations of ethanol. EPC/PEG–DSPE only dissolved when the ethanol concentration was over 60%. DOTAP/PEG–DSPE was slightly insoluble in 0–30% ethanol but was completely dissolved when the proportion of ethanol was greater than 30%. However, DFA/DOTAP/PEG–DSPE complexes did not dissolve completely in aqueous ethanol at any concentration of ethanol.

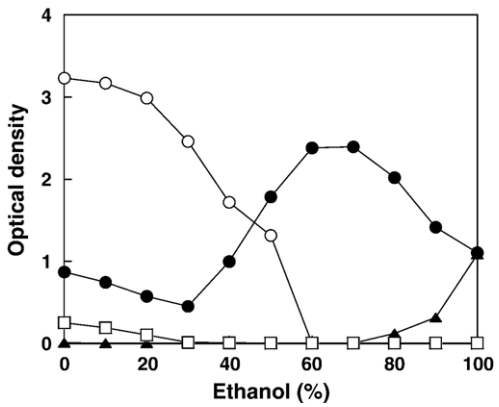

Fig. 1. The solubility of each sample in aqueous ethanol at 25 °C was measured. DOTAP/PEG–DSPE (7.5 mg/3 mg/mL, DOTAP/PEG–DSPE, 26/2.6, mol/mol) (□), DFA/DOTAP/PEG–DSPE (1.25 mg/7.5 mg/3 mg/mL, DFA/DOTAP/PEG–DSPE, 1/26/2.6, mol/mol/mol) (●), DFA (1.25 mg/mL) (▲) and EPC/PEG–DSPE (30 mg/6.25 mg/mL, EPC/PEG–DSPE, 17.3/1, mol/mol) (○).

3.2. Preparation of complexes

Complexes were prepared efficiently by the procedure described in "Materials and methods". The representative particle size and polydispersity of complexes were 104 nm and 0.12, respectively. The representative distribution of complexes is shown in Fig. 2A. Most of the DFA will be complexed to the outer surface of the DOTAP/ PEG–DSPE liposome but some DFA may also be encapsulated inside the liposomes because DFA was mixed with DOTAP and PEG–DSPE before the vesicles were formed. Small complexes (104 nm, polydispersity 0.12) were formed in water whereas large complexes (1417 nm, polydispersity 0.28) were produced in PBS, probably because charged ions associated electrostatically with complexes. The complexes with PEG–DSPE are likely to more stable than complexes composed of DFA and DOTAP, because interaction between complexes would cause aggregation and precipitation in the absence of PEG–DSPE. The preparation of wrapped liposomes was explored using complexes of PEG–DSPE that were formed in water.

3.3. Preparation of wrapped liposomes

In the absence and presence of PEG–DSPE in wrapping lipids (EPC/PEG–DSPE), the wrapped liposomes were formed. As PEG–DSPE is essential for leading to increased circulation times and enhanced localization of the wrapped liposomes in diseased tissues, wrapping lipids composed of EPC and PEG–DSPE were selected. The procedures described in Materials and methods enabled efficient formulation of the complexes wrapped with neutral lipids; the formulation process achieved encapsulation efficiencies of up to 90%. The representative particle size and polydispersity of the wrapped liposomes were 122 nm and 0.07, respectively. The representative distribution of the wrapped liposomes is shown in Fig. 2B.

3.4. Microscopy observation

Wrapped liposomes, naked complexes and wrapped liposomes in the absence of complexes were examined using transmission electron microscopy. However, attempts to observe the complexes failed because the complexes rapidly aggregated when ammonium molybdate was added. Micrographs of the wrapped liposomes revealed small vesicles in the outer membrane and the morphology of these vesicles was different from the morphology of the outer membrane of the wrapped liposomes (Fig. 3A). Micrographs of wrapped liposomes not containing complexes did not show small

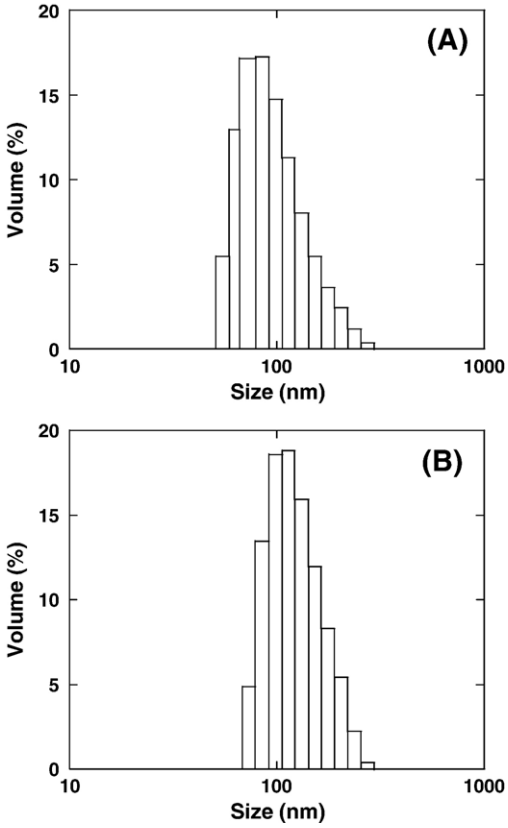

Fig. 2. Size distributions of the complexes (A) and the wrapped liposomes (B) as determined by dynamic light scattering. The size distributions were shown as the volume-based distributions obtained by Nano-ZS in the Gaussian-fit mode.

vesicles in the outer membrane (Fig. 3B). Therefore, the small vesicles observed (Fig. 3A) are likely to be DFA/DOTAP/PEG–DSPE complexes. The wrapped liposomes with no complexes were larger in size than those containing complexes (Fig. 3A). In case of the presence of the complexes, the neutral lipids accumulated around the complexes and deposited. In case of absence of the complexes, however, there was no target object which the neutral lipids should accumulate around. Absence of the complexes may result in the formation of larger liposomes. Using the dynamic light-scattering measurement, the particle sizes (with polydispersity in parentheses) of the complexes, the wrapped liposomes in the absence and presence of the complexes were 105 nm (0.11), 187 nm (0.09) and 132 nm (0.09), respectively. The thickness of the anhydrous bilayer is approximately 4 nm [22]. Microscopic examination suggested that the thickness of the inner aqueous layer of the wrapped liposomes was approximately 5–20 nm. Thus, the combined increase in thickness of the anhydrous bilayer and inner aqueous layer would imply approximately 18–48 nm increase in particle size from the complexes to the wrapped liposomes, which is in agreement with the observed approximate 20–30 nm increase in size. The size of the complexes was slightly smaller than 105 nm when observed by microscopy. The particle size of the complexes may decrease during the preparation of the wrapped liposomes. The structural features of the wrapped liposomes are currently being investigated further.

3.5. Turbidity changes on addition of NaCl

Turbidity increased immediately after addition of NaCl to complexes (Fig. 4A). However, turbidity was unaffected when NaCl was added to the wrapped liposomes (Fig. 4B). The observed changes in turbidity were consistent with the particle sizes determined by dynamic light scattering. The particle sizes of the complexes were 124 nm (polydispersity 0.14) and 480 nm (polydispersity 0.32) before and after the addition of NaCl, respectively, whereas the particle sizes of the wrapped liposomes were 145 nm (polydispersity 0.09) and 164 nm (polydispersity 0.27) before and after the addition of NaCl. The interpretation of these data is that the naked complexes had a charged surface and therefore were affected by the presence of NaCl but that the wrapped liposomes had a neutral surface and were consequently less affected by addition of NaCl. The minimal effect on the particle size of the wrapped liposomes produced by the addition of NaCl implies that neutral lipids covered the complexes extensively. On the other hand, when Zeta potentials were measured directly in NaCl solution, there was no large difference between the Zeta potentials of the complexes (5.1 mV) and the wrapped liposomes (0.1 mV) though the Zeta potentials of the complexes were slightly larger than those of the wrapped liposomes. Thus, the presence of PEG may shield the charges on the surface.

3.6. Stability in FBS

Rapid release of DFA was observed when naked complexes were incubated in FBS (Fig. 5). Electrostatic

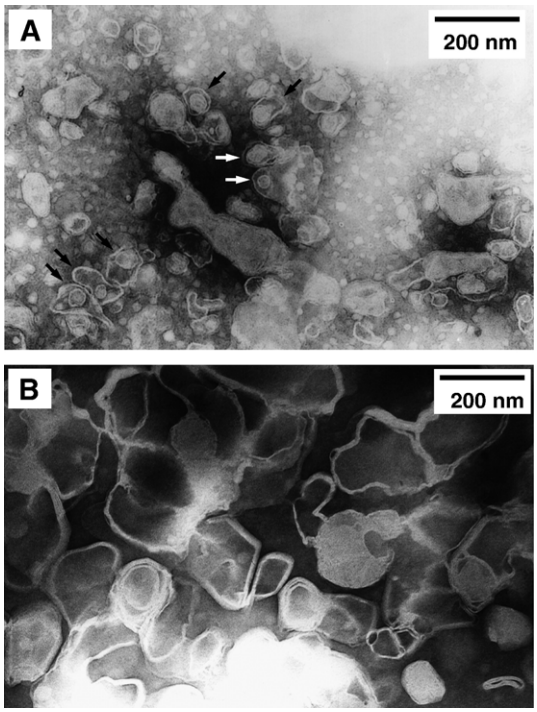

Fig. 3. Negative stain electron micrographs of wrapped liposomes (A) and liposomes without DFA/DOTAP/PEG–DSPE complexes (B). Arrows indicate the vesicles containing particles.

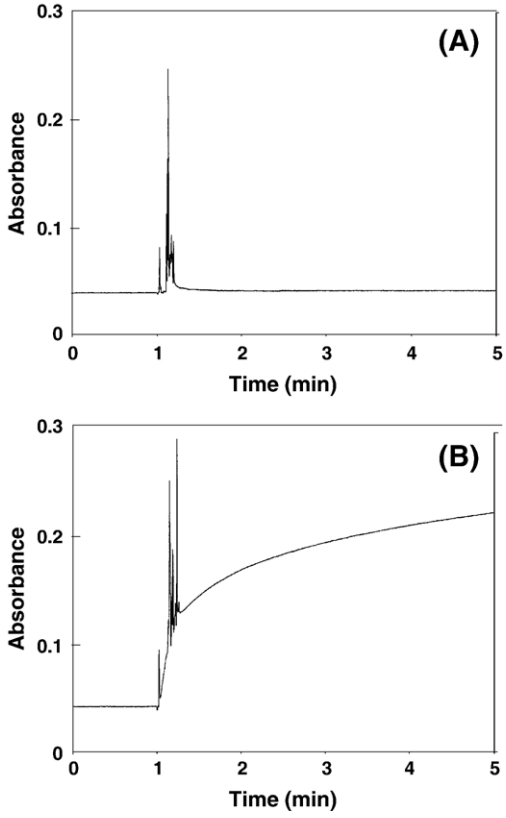

Fig. 4. Change in the relative turbidity of wrapped liposomes (A) and naked complexes (B) following addition of NaCl solution at 25 °C.

interaction between anionic and cationic components of FBS with DFA and DOTAP probably contributed to the rapid release of DFA and DOTAP from complexes. On the other hand, slow release of DFA was observed when the wrapped liposomes were incubated in FBS (Fig. 5), thereby demonstrating the stability afforded by covering the complexes with neutral lipids. Consequently, we anticipate that wrapped liposomes are capable of localizing and releasing their content at the site of action. Some DFA was released from the wrapped liposomes immediately after addition to FBS,

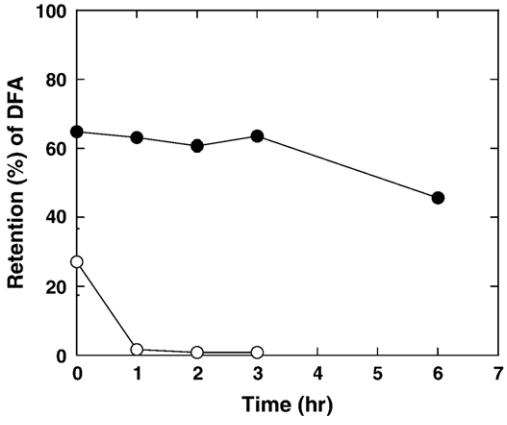

Fig. 5. In vitro retention of DFA in wrapped liposomes (●) and naked complexes (○). Retention was measured after incubation in FBS at 37 °C. [Total Lipids] = 0.3 mg/mL. Data represent the mean ± S.D. from three experiments.

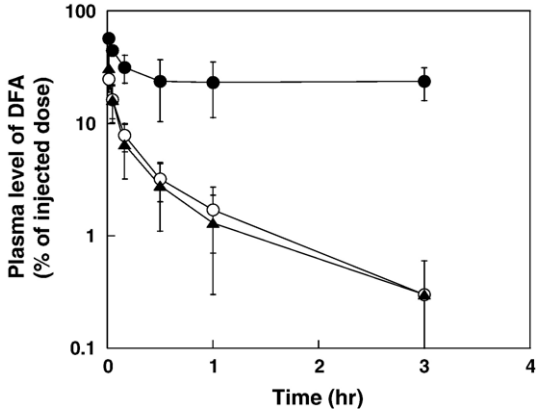

Fig. 6. Plasma levels of DFA following intravenous administration to rats of wrapped liposomes (●), naked complexes (○) and free DFA (▲). Free DFA was administered at a dose of 0.4 mg/kg. Wrapped liposomes and complexes were administered at approximate total lipid doses of 10 mg/kg. Data represent the mean ± S.D. from three animals.

which suggests that some complexes may not have been completely covered by neutral lipids.

3.7. Rat PK

After bolus intravenous administration of DFA solution and complexes to rats, DFA was eliminated rapidly from blood; the blood level of DFA was 31.0% and 24.8% of the injected dose at 1 min after injection of DFA solution and complexes, respectively, and 0.3% and 0.3% at 180 min (Fig. 6). The plasma PK of the complexes was similar to that reported previously [12]. After intravenous administration of the wrapped liposomes, the blood level of DFA was much higher than that after administration of DFA solution and naked complexes; the blood level of DFA was 57.0% of the injected dose at 1 min after injection and 23.6% at 180 min (Fig. 6). The half-life, $AUC_{0-3\ h}$ and MRT of encapsulated DFA in wrapped liposomes (12.9 h, 3.31 h·μg/mL, 18.3 h, respectively) were significantly greater than the respective pharmacokinetic parameters observed for naked DFA (0.76 h, 0.73 h·μg/mL, 0.61 h) and for DFA in the complexes (0.79 h, 0.80 h·μg/mL, 0.71 h) suggesting that DFA in wrapped liposomes was cleared more slowly from the circulation than naked DFA and DFA in the complexes. Thus, the wrapping of DFA/DOTAP/PEG–DSPE complexes with EPC/PEG–DSPE was effective at extending the half-life of DFA. In fact, the wrapped liposomes provided extended circulation times comparable to those achieved with conventional stealth liposomes such as Doxil [23].

4. Discussion

In this study, we developed novel liposomes that we have termed “wrapped liposomes”, which have a small diameter and high encapsulation efficiency. The formulation approach was evaluated using polyionic DFA as a test compound. The wrapped liposomes were more stable in FBS than naked DFA/cationic lipids complexes. Also, higher concentrations of total

DFA were maintained for longer in rat plasma after intravenous administration of wrapped liposomes compared to levels achieved after administration of naked complexes.

The preparation method involved the formation of DFA/ cationic lipids complexes that were subsequently wrapped with neutral lipids using differences of solubility in aqueous ethanol. The solubility of cationic lipids in aqueous ethanol was decreased by complexation with DFA. DOTAP/ PEG–DSPE liposomes were soluble in aqueous ethanol (30–100% v/v). However, DFA/DOTAP/PEG–DSPE complexes were insoluble in aqueous ethanol (0–100% v/v) suggesting that the surfaces of DOTAP/PEG–DSPE liposomes were homogenously covered with DFA. On the other hand, EPC/PEG–DSPE was soluble in aqueous ethanol when the proportion of ethanol was over 50% (v/v). Therefore, in 62.5% ethanol, the complexes were insoluble and neutral lipids were dissolved (Fig. 7). Under the condition, the optical density of the aqueous ethanol solution prepared with the complexes increased more than twice, which could suggest the formation of aggregates. However, the particle size of the resultant wrapped liposomes was 122 nm, as recorded in Section 3.3, implying that aggregates were not present although it is possible that aggregation was a reversible process. It was assumed that when the ratio of ethanol was decreased by addition of distilled water, the neutral lipids gradually deposited around the complexes and finally the complexes were covered with neutral lipids. It was confirmed that the complexes were covered with neutral lipids by microscopic examination, turbidity changes after the addition of NaCl, stability in FBS and PK in rats following intravenous administration. All of the data supported the proposition that the complexes were covered with neutral lipids and that DFA was present inside the liposomes and not on the surface of liposomes.

The presence of PEG–DSPE in the complexes and wrapping lipids (EPC/PEG–DSPE) might make wrapping lipids accumulate around the complexes more efficiently and orderly. Aqueous two-phase partitioning occurs when PEG is mixed with dextran in water. When PEG liposomes were added to this two phase system consisting of PEG and dextran, PEG liposomes partitioned into the PEG phase [24], which may be attributable to the interaction of PEG liposomes with other PEG molecules. The initial step in the process was presumed to involve the accumulation of PEG–DSPE around the complexes caused by this PEG–PEG interaction between PEG–DSPE in the complexes and PEG–DSPE used for wrapping. Secondly, EPC accumulated around the complexes presumably due to the hydrophobic interaction between the acyl chains of EPC and the acyl chains of PEG–DSPE. The mechanisms involved in the formation of the wrapped liposomes are currently being investigated further.

Attempts have been made to develop several ODNs, such as ISIS-3521 and G3139, as anticancer drugs [25,26]. However, some development projects were terminated in Phase III clinical trials because dosing failed to achieve efficacious anti-tumor responses in humans [27,28]. The main problem was likely to be poor delivery of ODNs to the target site. Encapsulation of ODNs in liposomes was proposed by Yu et al. [8] as a possible method of resolving the problem.

When ODNs are encapsulated into neutral liposomes covered with PEG derivatives, the liposomes are not rapidly

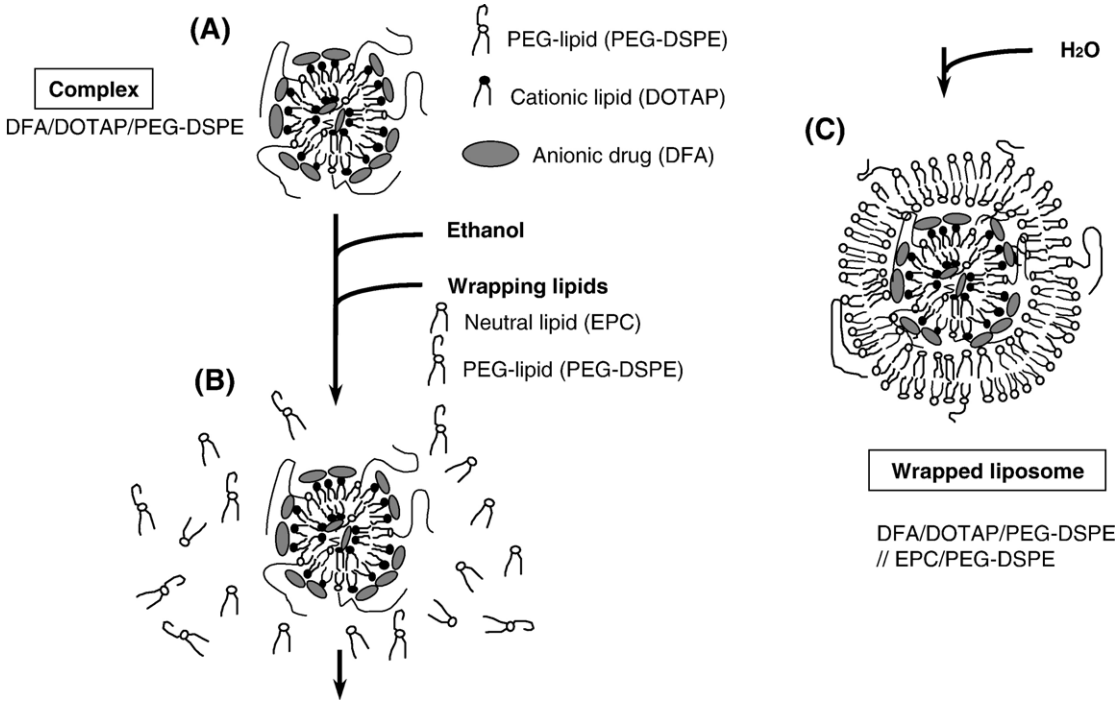

Fig. 7. Schematic showing the steps and hypothesized intermediate structures involved in the preparation of wrapped liposomes. (A) DFA/DOTAP/PEG–DSPE complexes were formed in water. (B) Ethanol was added and then EPC/PEG–DSPE was added. Complexes were insoluble and EPC/PEG–DSPE was soluble in EtOH/ water (62.5/37.5, v/v). (C) Water was added and EPC/PEG–DSPE precipitated around the complexes to produce wrapped liposomes.

taken up into the liver nor spleen and exhibit good stability in plasma; consequently these formulations can provide long circulation half-lives [8]. However, the encapsulation efficiency of neutral liposomes is low (25–30%) which is a disadvantage for practical applications [8]. On the other hand, cationic liposomes are capable of high encapsulation efficiencies and offer enhanced intracellular delivery [9,10]. However, the negatively charged ODNs are only adsorbed on the surface of the positively charged liposomes by electrostatic interaction and, therefore, cationic liposomes are very unstable in plasma due to desorption of ODNs from the liposomes and adsorption onto serum proteins [12]. Cationic liposomes aggregate in blood and the presence of a positive charge results in clearance by the reticuloendothelial system within a few minutes of administration. Thus, only low amounts of ODNs are delivered to target tissues such as tumors. Furthermore, cationic liposomes often cause liver and inflammatory toxicities after systemic administration of cationic liposomes [13,14]. Therefore, cationic liposomes are unlikely to work as a systemic carrier in clinical applications.

CCL and SALP described by Stuart et al. [15,16] and Semple et al. [17,18], respectively, were reported to overcome the problems described above. Both these liposomal formulations have high encapsulation efficiencies, good stability in blood, long duration after intravenous administration and in vivo activities, and, therefore, it is expected that these liposomes may have clinical application. These liposomes have packed bilayers comprised of cholesterol or lipids that have a high gel–liquid crystal phase transition temperature. On the other hand, the wrapped liposomes have loose bilayers comprised of EPCs, which have low phase transition temperature. In general, factors that lead to increased order in the lipid bilayer, such as increased cholesterol content, acyl chain length or acyl chain saturation, result in slower release properties [29]. Therefore, the wrapped liposomes were expected to be more unstable and attacked by high-density lipoproteins (HDL) more effectively than CCL and SALP. However, in spite of the presence of unstable membranes, after intravenous administration the wrapped liposomes exhibited long half-lives that were comparable to the duration of CCL and SALP. The presence of stable membranes has the advantage that the liposomes and their contents are likely to be delivered efficiently to target tissues but the stable membranes may hinder delivery of liposomal contents into the cytoplasm of target tissues [30]. It is feasible that the wrapped liposomes could be more effective than either CCL or SALP at delivering ODNs to the actual site of action and thereby improve the therapeutic effectiveness of antisense ODNs.

Unlike small molecule drugs such as doxorubicine, the transport of macromolecules into cytoplasm appears to be limited by permeation through liposomal membranes and cell membranes. We wished to investigate whether (i) the wrapped liposome technique could be applied to nucleic acids such as ODNs, siRNAs and plasmids, (ii) wrapped liposomes would deliver nucleic acids to target sites, and (iii) wrapped liposomes would improve the therapeutic activity as compared to free nucleic acids. Additional investigations were performed using the wrapped liposomes encapsulating a siRNA that inhibits expression of Krüppel-like zinc-finger 5 (KLF5). KLF5 is a transcription factor that is involved in the processes of cardiovascular remodeling and angiogenesis. It was discovered that wrapped liposomes encapsulating KLF5 siRNA prevented tumor angiogenesis and progression and significantly inhibited tumor growth when administered to mouse via the tail vein (Yagi, N. et al., unpublished data). KLF5 siRNA alone did not exhibit similar efficacy. This finding demonstrates the exciting potential of using the wrapped liposomes to achieve improved clinical effectiveness of siRNAs.

Moreover, similar methodology is likely to be applicable to the formulation of polycationic drugs in the wrapped liposomes. We believe that the use of wrapped liposomes will be applicable to a wide variety of charged compounds, including peptides and proteins. The problem of encapsulating proteins into liposomes has been difficult to overcome [7] but we expect that it will be possible to encapsulate proteins efficiently in the wrapped liposomes.

In summary, we have developed novel liposomes, which we have termed wrapped liposomes, that incorporate important advantages of neutral and cationic liposomes. We believe that the wrapped liposomes offer a safe and practical means of formulating polyanionic and polycationic drugs that can achieve suitable pharmacokinetic profiles thereby overcoming many of the drawbacks associated with the clinical development of these types of drug.

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