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A novel pH-sensitive liposome formulation containing oleyl alcohol

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Abstract

pH-sensitive liposomes are designed to undergo acid-triggered destabilization. First generation pH-sensitive liposomes, based on the cone-shaped lipid dioleoylphosphatidylethanolamine (DOPE), have been shown to lose fusogenicity in the presence of serum. Here, we report the design and evaluation of novel serum-resistant pH-sensitive liposome formulations that are based on the composition of egg phosphatidylcholine (PC), cholesteryl hemisuccinate (CHEMS), oleyl alcohol (OAlc), and Tween-80 (T-80). When loaded with the fluorescent probe calcein, these liposomes exhibited excellent stability at pH 7.4 and underwent rapid destabilization upon acidification as shown by calcein dequenching and particle size increase. Adjusting the mole percentages of T-80 and OAlc in the formulation could regulate the stability and pH-sensitive properties of these liposomes. Liposomes with a higher T-80 content exhibited greater stability but were less sensitive to acid-induced destabilization. Meanwhile, formulations with a higher OAlc content exhibited greater content release in response to low pH. The pH-triggered liposomal destabilization did not produce membrane fusion according to an octadecylrhodamine B chloride (R_{18}) lipid-mixing assay. Compared to DOPE-based pH-sensitive liposomes, the above formulations showed much better retention of their pH-sensitive properties in the presence of 10% serum. These liposomes were then evaluated for intracellular delivery of entrapped cytosine-β-D-arabinofuranoside (araC) in KB human oral cancer cells, which have elevated folate receptor (FR) expression. The FR, which is amplified in many types of human tumors, has been shown to mediate the internalization of folate-derivatized liposomes into an acidic intracellular compartment. FR-targeted OAlc-based pH-sensitive liposomes, entrapping 200 mM araC, showed ~17-times greater FR-dependent cytotoxicity in KB cells compared to araC delivered via FR-targeted non-pH-sensitive liposomes. These data indicated that pH-sensitive liposomes based on OAlc, combined with FR-mediated targeting, are promising delivery vehicles for membrane impermeable therapeutic agents.

Keywords: Folate receptor; Drug targeting; Cytosine arabinoside; pH-sensitive liposome; Drug delivery

1. Introduction

Liposomes are phospholipid bilayer vesicles that have been studied extensively as potential drug carriers. Major obstacles to liposomal drug delivery have been the slow drug release and lack of fusogenic activity of regular liposomes following internalization into the endosomal compartment. These factors contribute to the diminished efficiency of cytosolic delivery [1], especially when the encapsulated molecules are large or highly hydrophilic [2–4]. In order to overcome this obstacle, pH-sensitive liposomes have been developed that are stable at physiological pH but are destabilized upon acidification following cellular internalization, thereby, promoting the release of their contents into the cytosol [5–7].

Most reported formulations of pH-sensitive liposomes are composed of dioleoylphosphatidylethanolamine (DOPE), which has a strong propensity to form a nonbilayer structure due to its cone-shape geometry, and a weakly acidic amphiphile, such as cholesteryl hemisuccinate (CHEMS), which confers stability to the bilayer phase at neutral pH [8–12]. Under acidic conditions, CHEMS becomes partially protonated, thus losing its negative charge and, therefore, its ability to stabilize the bilayer structure, which is based on electrostatic repulsion. This further results in the destabilization and/or fusion of the liposomes. The DOPE-based pH-sensitive liposomes have been shown to improve the cytoplasmic delivery of membrane-impermeable therapeutic agents [7,13,14]. However, the potential application of these

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liposomes as drug carriers *in vivo* is hampered by their relatively poor stability in the presence of serum [15–18]. Attempts to increase the serum stability of the DOPE-containing liposome formulations by incorporating a polyethyleneglycol (PEG)-derivatized lipid resulted in significantly reduced pH-sensitivity [19].

The folate receptor (FR) is known to be overexpressed in a wide variety of solid tumors as well as in myeloid leukemias [20–22]. Folate conjugates of various therapeutic and diagnostic entities, including liposomes, have been shown to be internalized by cells via FR-mediated endocytosis into an acidic endosomal compartment [23–25]. In addition, folate-derivatized liposomes have been shown to selectively increase the delivery of cytotoxic drugs into tumor cells with elevated FR expression [26]. However, highly hydrophilic agents, such as cytosine-β-D-arabinofuranoside (araC), delivered by liposomes might be sequestered in the endosomal compartment resulting in a slow rate of cytosolic release. Combining FR-targeting, which promotes cellular endocytosis, with pH-sensitive liposomes, which promotes endosomal drug release, therefore, presents an attractive approach for improving the cytosolic delivery of araC.

In this study, we report the design and evaluation of novel pH-sensitive liposome formulations based on the use of oleyl alcohol (OAlc) in combination with egg phosphatidylcholine (PC) as the membrane destabilizing components. The stability and pH-sensitivity of the liposomes in buffer and in 10% serum were determined by fluorescence dequenching assays. Furthermore, the OAlc-containing liposomes, targeted to the FR, were evaluated for the intracellular delivery of araC. The possible mechanism for the pH-sensitivity of these liposomes is also discussed.

2. Materials and methods

2.1. Materials

Egg PC and DOPE were purchased from Avanti Polar Lipids (Alabaster, AL). Octadecylrhodamine B chloride (R_{18}) was purchased from Molecular Probes (Eugene, OR). Calcein, CHEMS, araC, folic acid dihydrate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Sepharose CL-4B resin, Tween-80 (T-80), and Triton X-100 were obtained from Sigma Chemical Co. (St. Louis, MO). OAlc was purchased from Aldrich Chemical Co. (St. Louis, MO). Polycarbonate membranes and the handheld LiposoFast™ Extruder were obtained from Avestin Inc. (Ottawa, ON). Tissue culture media were purchased from Life Technologies (Rockville, MD). Folate-PEG-cholesterol (f-PEG-Chol) was synthesized as reported previously [27].

2.2. Cell culture

KB cells, a human oral cancer cell line that expresses amplified FR, were obtained as a gift from Dr. Philip Low at Purdue University (West Lafayette, IN). The cells were cultured at 37 °C as a monolayer in folate-free RPMI 1640 media supplemented with antibiotics and 10% fetal bovine serum in a humidified atmosphere containing 5% CO_2.

2.3. Liposome preparations

Liposomes encapsulating calcein were prepared using a procedure based on polycarbonate membrane extrusion, as described previously [27]. Briefly, a chloroform solution of the lipid mixture with the desired composition was dried into a thin film in a round-bottom flask on a rotary evaporator, and then further dried under vacuum. The lipid mixture was then hydrated in 80 mM calcein. The suspension was subjected to six cycles of freezing and thawing, briefly sonicated, and then extruded through a 0.1 μm pore-size polycarbonate membrane using a handheld LiposoFast™ Extruder. Unentrapped calcein was separated from the liposomes by gel filtration on a Sepharose CL-4B column equilibrated in phosphate-buffered saline (PBS, 136.9 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4). The mean diameter of the extruded liposomes was determined by photon correlation spectroscopy on a NICOMP Particle Sizer Model 370. The final calcein concentrations in the liposome preparations were calculated based on absorbance at 495 nm using a molar extinction coefficient of 80,000 M^{-1} cm^{-1}.

2.4. Membrane fusion assay

The membrane fusion assay was carried out as described previously [28,29]. Briefly, labeled liposomes were prepared by incorporating a lipid soluble probe, R_{18}, at 5.7 mol% of the total lipid, a concentration that results in fluorescence self-quenching. Both labeled and unlabeled liposomes were prepared at a total lipid concentration of 2 mM. These were then mixed at a volume ratio of 1:4 (labeled/unlabeled). The liposome mixture was diluted to 5 μM lipids in the appropriate buffer, followed by a 10-min incubation at 37 °C. R_{18} fluorescence intensity was then measured at the excitation and emission wavelengths of 560 and 590 nm, respectively.

2.5. Fluorescence dequenching assay

Liposome content release was characterized using a calcein dequenching assay, as described previously [19]. All fluorescence measurements were performed using a Perkin-Elmer LS-5B spectrofluorometer operated with an FTWinlab (Morena Valley, CA) computer program. The excitation and emission wavelengths were set at 490 and 520 nm, respectively. Calcein-loaded liposomes containing 40 nmol of lipid were added to 2 ml of PBS (adjusted to pH 7.0 and 7.4) or sodium acetate buffer (100 mM NaCl, 10 mM acetate, pH 5.0, 5.5, 6.0, and 6.5) in a disposable cuvette. After a 10-min incubation at 37 °C, buffer pH was adjusted to 7.4 and calcein fluorescence was measured. The fluorescence was then remeasured following the addition of 0.1% Triton X-

100, which caused 100% calcein leakage from the liposomes. The percent of calcein release was calculated based on the equation:

$$\%\text{Calcein Release} = ((I_{pH} - I_o)/(I_{100} - I_o))100\%$$

where I_o was the fluorescence at pH 7.4, I_{pH} was the fluorescence intensity following incubation at acidic buffer pHs, and I_{100} was the fluorescence after the addition of Triton X-100. In addition, the changes in the mean particle size of the OAlc liposomes in an acidic environment were measured at preset time intervals.

2.6. Determination of liposome stability in the presence of serum

Liposomes entrapping 80 mM calcein and containing 40 nmol of lipid were added to 2 ml buffer at various pH values in the presence or absence of 10% newborn calf serum. After a 10-min incubation at 37 °C, calcein fluorescence was measured and percentage of calcein release calculated, as described above. In addition, liposome particle size measurements were also performed at various time points.

2.7. Cytotoxicity assay

The delivery of araC by OAlc liposomes was evaluated in KB cells. Preparation of araC-containing liposomes was carried out by the same method used for the preparation of calcein-containing liposomes described above, except 200 mM araC was used to hydrate the dried lipid mixture instead of calcein. In addition, 0.5 mol% of f-PEG-Chol was incorporated into the lipid mixture for the preparation of FR-targeted liposomes. The araC concentrations in the liposome preparations were calculated based on absorbance at 280 nm using a molar extinction coefficient of 475 M^{-1} cm^{-1}, after solubilizing the lipid membranes in a methanol/water (2:1) mixture. The encapsulation efficiency of these liposomes was found to be approximately 15%.

The cytotoxicity of various liposomal araC formulations was determined by the MTT assay, as described previously [30]. Briefly, KB cells were seeded in 96-well plates to reach ~25% confluence at the time of the study. The cells were treated in triplicate with 1:4 serial dilutions of the various araC formulations. Following a 2 h incubation at 37 °C, the cells were then washed with PBS. Fresh media, containing 10% fetal bovine serum, were then added and the cells were cultured for another 48 h. MTT was then added to the culture medium at a concentration of 0.6 mg/ml and the cells were incubated at 37 °C for an additional 2 h. The medium was then removed and the cells were dissolved in acidified isopropanol and the absorption at 495 nm was measured using an automatic plate reader, as an indicator of cell viability. The concentration of araC leading to 50% cell killing (IC_{50}) was calculated from a concentration dependent cell viability curve.

3. Results

3.1. Formulation of pH-sensitive liposomes containing OAlc

All liposome compositions are described in molar ratios in this article. The pH-sensitive liposomes were designed using a primary lipid composition of egg PC/CHEMS (50:50) combined with varying amounts of T-80 and OAlc. T-80 was found be necessary since, in its absence, stable liposomes could not form at an OAlc to egg PC molar ratio above 1:2.

3.2. Effects of T-80 and OAlc content on acid-induced liposome destabilization

We predicted that the incorporation of T-80 would confer greater stability and reduced pH-sensitivity to egg PC-based liposomes, whereas increasing OAlc content would have the opposite effect. To evaluate this hypothesis, calcein-containing large unilamellar vesicles (LUV) (~ 100 nm in diameter) composed of egg PC/CHEMS/T-80/OAlc with two different T-80 contents and escalating OAlc-to-PC ratios were prepared and evaluated for pH-dependent calcein release.

First, liposomes with varying amounts of OAlc and a high T-80 content were compared for pH-dependent content release. As shown in Fig. 1A, liposomes composed of egg PC/CHEMS/T-80/OAlc (50:50:5:80) released 33% of their contents after a 10 min incubation at 37 °C at pH 5.0. In contrast, liposomes composed of egg PC/CHEMS/T-80/OAlc (50:50:5:20) showed only 6% calcein release under identical conditions. These findings suggested that elevated OAlc content resulted in increased pH-sensitivity of the liposomes.

Secondly, the above study was repeated using liposomes with a low T-80 content. Once again, the liposomes with higher OAlc content showed greater calcein release at acidic pH, as shown in Fig. 1B. Furthermore, the overall amount of calcein release was much higher compared to the above-described liposomes with a high T-80 content. For example, liposomes composed of egg PC/CHEMS/T-80/OAlc (50:50:2:80) showed 83% calcein release at pH 5. These findings suggested lowering T-80 content increased the pH-responsiveness of the liposomes.

Thirdly, we also examined non-pH-sensitive liposomes composed of PC/CHEMS/T-80 (50:50:2) using the same calcein dequenching assay. As anticipated, these liposomes showed only minimal calcein release ($<5\%$) at low buffer pH (data not shown). All liposome formulations evaluated in this study were also found to be stable (showing $<5\%$ calcein leakage) over a period of 8 weeks in PBS (pH 7.4) when stored at 4 °C.

3.3. Effect of buffer pH on liposome particle size

As shown in Fig. 2, liposomes composed of egg PC/CHEMS/T-80/OAlc at molar ratios of 50:50:2:80 or 50:50:5:80, showed a 10- and a 15-fold increase in mean diameter, respectively, after a 30-min incubation at pH 5.0.

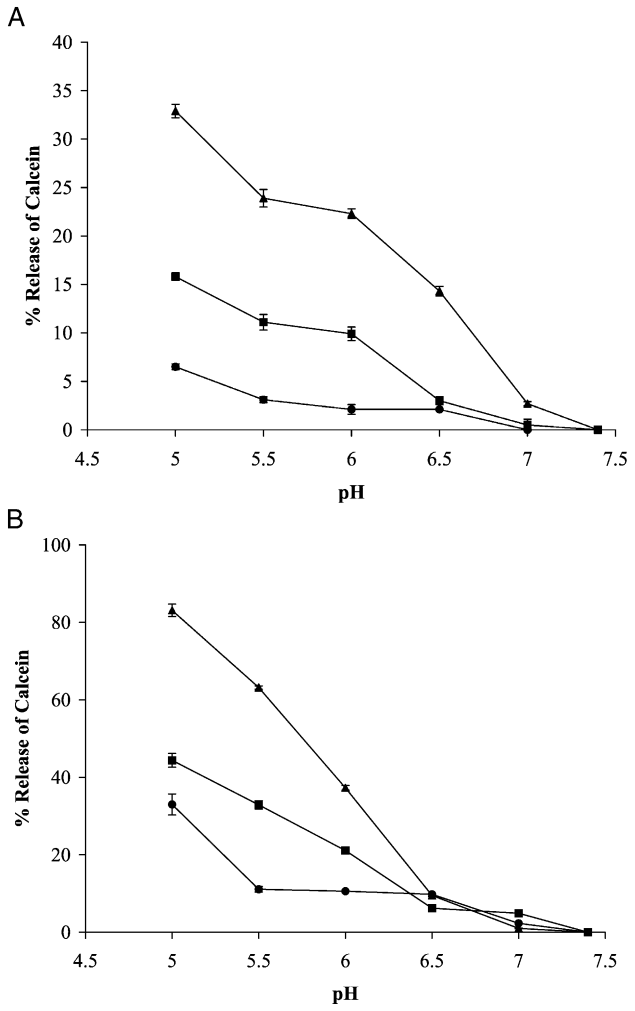

Fig. 1. Acid-induced calcein release from OAlc-containing liposomes with either high (Panel A) or low (Panel B) T-80 contents. Liposomes encapsulating 80 mM calcein were incubated for 10 min at 37 °C in buffers with a series of different pH values. Percent leakage of calcein was calculated based on fluorescence measured before and after addition of Triton X-100, as described in Materials and methods. Each data point represents the mean of three parallel experiments; error bar = 1 S.D. The compositions of the liposomes used were egg PC/CHEMS/T-80/OAlc at molar ratios of: in Panel A, (●) 50:50:5:20; (■) 50:50:5:40; (▲) 50:50:5:80; and in Panel B, (●) 50:50:2:20; (■) 50:50:2:40; (▲) 50:50:2:80.

The increase in particle size was time dependent. No changes in particle sizes were observed when these liposomes were kept in pH 7.4 buffer.

3.4. Membrane fusion assay

To determine the likely mechanism of acid-induced liposomal destabilization, a membrane fusion assay based on lipid mixing was performed using liposomes containing a self-quenching concentration of the lipophilic probe, R_{18}. The composition of the R_{18}-labeled liposomes was egg PC/CHEMS/T-80/OAlc/R_{18} (50:50:2:80:5.7), whereas, the unlabeled liposomes were composed of egg PC/CHEMS/T-80/

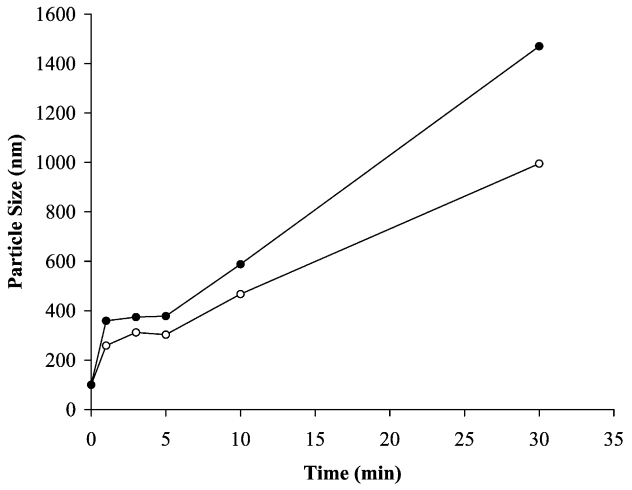

Fig. 2. pH-dependent particle size increase of OAlc liposomes incubated at pH 5.0 buffer. The liposomes were composed of egg PC/CHEMS/T-80/OAlc at molar ratios of (○) 50:50:5:80 or (●) 50:50:2:80.

OAlc (50:50:2:80). Meanwhile, liposomes composed of DOPE/CHEMS/R_{18} (60:40:5.7) and DOPE/CHEMS (60:40) were used as a positive control for the above assay. As shown in Fig. 3, the OAlc-containing liposomes did not show a significant increase in R_{18} fluorescence at any pH, suggesting a lack of membrane fusion. In contrast, R_{18} fluorescence from the DOPE/CHEMS liposomes gradually increased with decreasing pH, suggesting liposomal fusion. The degree of observed R_{18} fluorescence dequenching was

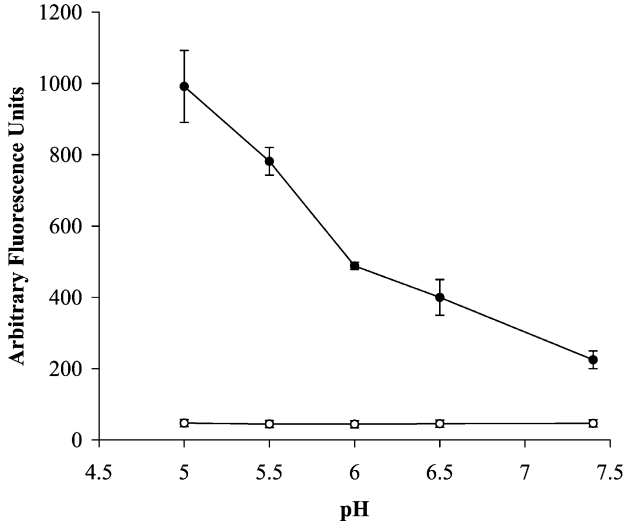

Fig. 3. Acid-induced R_{18} fluorescence dequenching in pH-sensitive liposomes. Mixtures of labeled and unlabeled liposomes (at 1:4 ratio) were incubated for 10 min at 37 °C in buffers with a series of different pH values. The compositions of the liposomes used were (●) DOPE/CHEMS (60:40) and (○) egg PC/CHEMS/T-80/OAlc (50:50:2:80). Each data point represents the mean of three parallel experiments; error bar = 1 S.D.

much greater at pHs below 6, which correspond to the pK_a of CHEMS.

3.5. Stability and pH-sensitivity of OAlc liposomes in the presence of serum

For pH-sensitive liposomes to be utilized for *in vivo* drug delivery, they must retain both stability and pH-sensitive properties in the presence of serum. It has been previously reported that serum protein binding to liposomes negatively affect the acid-triggered destabilization of DOPE-based pH-sensitive liposomes [15–18]. We, therefore, examined the effect of serum on liposomes composed of egg PC/CHEMS/T-80/OAlc (50:50:2:80), using liposomes composed of DOPE/CHEMS (60:40) as a reference control formulation [31]. As shown in Fig. 4, the liposomes containing OAlc retained most of their pH-sensitive properties upon incubation with serum. Calcein release from these liposomes after a 10-min incubation at pH 5 was 56% in the presence of 10% serum. In contrast, liposomes composed of DOPE/CHEMS showed only 20% release under the same conditions. It was noted that for both OAlc and DOPE-based liposomes, serum exposure adversely affected the degree of calcein release in response to low buffer pH. The OAlc-based liposomes, however, were affected to a lesser degree, with a decrease of calcein leakage from 83% to 56%, compared to a decrease from 84% to 19% for the DOPE-based liposomes. Serum exposure at pH 7.4 did not significantly affect liposomal particle size over 24 h (data not shown).

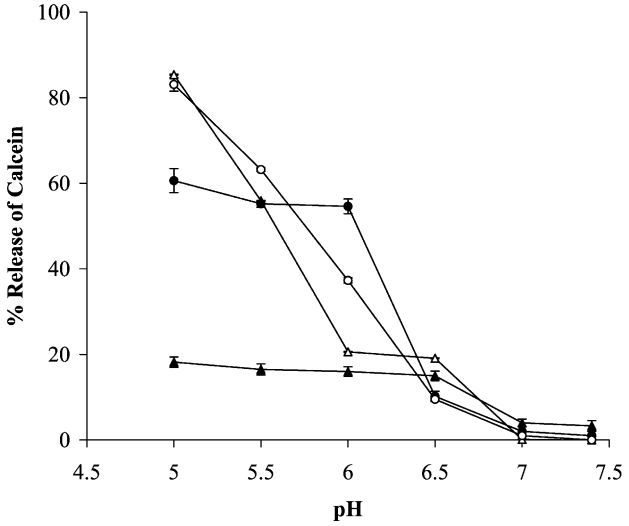

Fig. 4. Effect of serum on the pH sensitivity of OAlc liposomes. Liposomes composed of either egg PC/CHEMS/T-80/OAlc (50:50:2:80) or DOPE/CHEMS (60:40) were compared for pH-dependent calcein release in the presence or absence of 10% serum. Each data point represents the mean of three parallel experiments; error bar = 1 S.D. Conditions used: (▲) DOPE/CHEMS liposomes in the presence of 10% serum; (△) DOPE/CHEMS liposomes in buffer; (●), OAlc liposomes in the presence of 10% serum; (○) OAlc liposomes in buffer.

Table 1
Cytotoxicity of various araC formulations in KB cells

Liposome formulation[a]	IC_{50} (μM)
FR-targeted, OAlc pH-sensitive liposomal araC	78
FR-targeted, OAlc pH-sensitive liposomal araC + 1 mM free folic acid	312
FR-targeted, non-pH-sensitive liposomal araC	1312
Non-targeted, non-pH-sensitive liposomal araC	> 10,000
Free araC + empty FR-targeted, OAlc liposomes	4375

[a] The liposome compositions were: FR-targeted, OAlc pH-sensitive liposomes: egg PC/CHEMS/T-80/OAlc/f-PEG-Chol (50:50:2:80:0.5); FR-targeted, non-pH-sensitive liposomes: egg PC/CHEMS/T-80/f-PEG-Chol (50:50:2:0.5); Nontargeted, non-pH-sensitive liposomes: egg PC/CHEMS/T-80 (50:50:2).

3.6. Delivery of araC to KB cells by FR-targeted OAlc-based pH-sensitive liposomes

To assess the potential utility of OAlc liposomes for increasing cytosolic drug delivery, cytotoxicity studies were carried out using FR-targeted liposomes entrapping araC in cultured KB cells. f-PEG-Chol, an FR-specific targeting ligand, was incorporated in the liposome formulation to facilitate FR-mediated cellular uptake. No significant change in the pH sensitivity of the targeted liposomes, as evaluated by the calcein dequenching assay, was observed with these liposomes (data not shown).

All araC-containing liposome formulations were found to be stable (< 5% leakage) over 6 weeks in PBS (pH 7.4) when stored at 4 °C. As shown in Table 1, FR-targeted pH-sensitive liposomal araC showed significantly elevated cytotoxicity, with an IC_{50} of 78 μM. When 1 mM free folic acid was present during drug incubation, the cytotoxicity was reduced by ~ 4-fold, with an IC_{50} of 312 μM, indicating that the observed araC delivery was FR-dependent. In contrast, FR-targeted non-pH-sensitive liposomal araC showed a much lower cytotoxicity towards KB cells (IC_{50} of 1312 μM). In the absence of receptor targeting, liposomal araC showed very low cytotoxicity (IC_{50}>10,000 μM), even when compared to the free drug. This was presumably due to the lack of a cellular uptake mechanism for the liposome entrapped drug. Empty pH-sensitive liposomes did not contribute significantly to cellular cytotoxicity (data not shown), as demonstrated by the resulting cytotoxicity of the free drug/empty liposome combination. These results indicated that both FR-targeting and a pH-sensitive liposomal composition were required for the efficient intracellular delivery of membrane-impermeable drugs, such as araC.

4. Discussion

In this study, we evaluated novel pH-sensitive liposome formulations that were based on the incorporation of a fatty alcohol, OAlc. This includes a detailed characterization of the pH-sensitivity and serum stability of these liposomes. Furthermore, we have examined the FR-specific intracellu-

lar delivery of the anticancer drug, araC, utilizing this novel liposomal formulation.

Previously reported formulations for pH-sensitive liposomes mostly incorporate the lipid DOPE as the principal component to promote bilayer destabilization. As shown in Fig. 4, these liposomes tend to lose most of their pH-sensitivity in the presence of 10% serum. Alternative compositions with greater resilience to serum are, therefore, needed for *in vivo* drug delivery. It has been suggested that OAlc, an unsaturated fatty alcohol, is capable of forming a hydrogen bond through its hydroxyl to an oxygen atom on the phosphate group on the PC molecule, resulting in the formation of a complex with geometry similar to that of DOPE, as illustrated in Fig. 5 [32]. This in turn could result in a lowering of the energy barrier for the lipid transition from a lamellar phase to a hexagonal II phase, which is required for membrane destabilization. However, a lipid mixing assay failed to show membrane fusion activity for the OAlc-containing liposomes in response to low buffer pH. Therefore, the observed pH-dependent leakage among these liposomes entrapping calcein might primarily be due to membrane destabilization, without inducing bilayer fusion.

Since the OAlc content in the lipid composition determines the extent of OAlc/PC complex formation, it should, therefore, be possible to increase the pH-sensitivity of the liposomes by increasing the OAlc content in the formulation. This concept has been demonstrated by the marked increase in calcein leakage when the OAlc content was increased in the liposomal formulation, as shown in Fig. 1.

The potential *in vivo* use of pH-sensitive liposome formulations is dependent on retention of their stability and pH-sensitivity in the presence of serum. As shown in Fig. 4, OAlc-containing liposomes demonstrated excellent stability and pH-sensitivity in the presence of serum. In addition, the degree of pH-sensitivity could be conveniently tuned by altering OAlc content in the formulation. Incorporation of T-80, which introduced a steric barrier on the surface of the liposomes, resulted in increased liposome stability while reducing its pH-dependent aggregation. Consequently, maintaining a delicate balance between these two factors is very important in creating an effective pH-sensitive liposomal formulation that is optimal for drug delivery.

AraC is a cytosine analogue widely used clinically as an anti-leukemic drug. AraC, which is highly hydrophilic, is

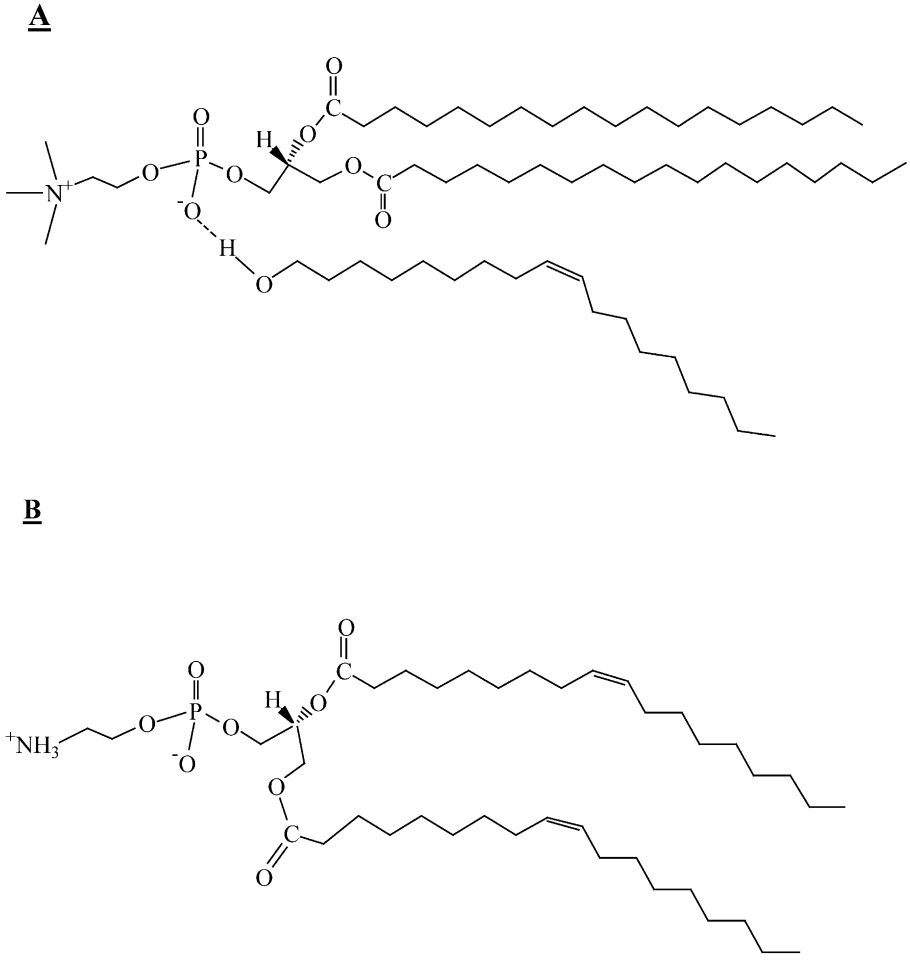

Fig. 5. Structures of (A) the proposed PC/OAlc complex and (B) DOPE. Note that, in both structures, the hydrophilic moieties occupy a relatively small volume compared to the lipophilic region. This should promote the formation of nonbilayer structures and liposome destabilization.

taken up by cells via a nucleoside transporter [33,34]. As a cell cycle-dependent agent, araC showed relatively poor cytotoxicity when added to cultured KB cells as a free drug (IC_{50} of 4375 μM) using an exposure time of 2 h. We, therefore, examined the effect of encapsulation of araC into FR-targeted liposomes, both pH-sensitive and non-pH-sensitive, on the *in vitro* cytotoxicity of this agent. As shown in Table 1, both FR-targeting and the use of a pH-sensitive formulation were found to be critical for maximizing liposomal araC cytotoxicity.

Since folic acid has high affinity for the FR and is stable, small, non-immunogenic, and readily available, it is ideal for exploration as a targeting ligand. Moreover, the FR is frequently overexpressed among human tumors, including ~ 70% of acute myeloid leukemias, while being absent in most normal tissues. FR-targeted liposomes can be readily produced in sufficient quantities for future clinical development. Therefore, FR-targeted OAlc liposomes, such as those entrapping araC, might have potential utility in the treatment of myeloid leukemias. FR-targeted delivery to solid tumors could also be possible given the high serum stability of the OAlc-containing liposomes, if prolonged systemic circulation and efficient endothelial extravasation could be achieved with the liposomes.

In addition to intracellular drug delivery, the pH-sensitive liposomal formulation described here may also constitute a drug delivery vehicle to pathological tissues, such as cancer, inflammation and infection sites, and ischemic areas, in which the pH is known to be lower than normal tissue [35]. Besides low molecular weight drugs such as araC, OAlc liposomes might find utility in the delivery of bioactive polypeptides, antisense oligodeoxyribonucleotides, as well as plasmid DNA in the delivery of gene therapy. Further studies on these liposomes, therefore, are warranted to explore these potential areas of application.

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