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pH gradient loading of anthracyclines into cholesterol-free liposomes: enhancing drug loading rates through use of ethanol

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Abstract

Application of cholesterol-free liposomes as carriers for anticancer drugs is hampered, in part, because of standard pH gradient based loading methods that rely on incubation temperatures above the phase transition temperature (*Tc*) of the bulk phospholipid to promote drug loading. In the absence of cholesterol, liposome permeability is enhanced at these temperatures which, in turn, can result in the collapse of the pH gradient and/or unstable loading. Doxorubicin loading studies, for example, indicate that the drug could not be loaded efficiently into cholesterol-free DSPC liposomes. We demonstrated that this problem could be circumvented by the addition of ethanol as a permeability enhancer. Doxorubicin loading rates in cholesterol-free DSPC liposomes were 6.6-fold higher in the presence of ethanol. In addition, greater than 90% of the added doxorubicin was encapsulated within 2 h at 37 °C, an efficiency that was 2.3-fold greater than that observed in the absence of ethanol. Optimal ethanol concentrations ranged from 10% to 15% (v/v) and these concentrations did not significantly affect liposome size, retention of an aqueous trap marker (lactose) or, most importantly, the stability of the imposed pH gradient. Cryo-transmission electron micrographs of liposomes exposed to increasing concentrations of ethanol indicated that at 30% (v/v) perturbations to the lipid bilayer were present as evidenced by the appearance of open liposomes and bilayer sheets. Ethanol-induced increased drug loading was temperature-, lipid composition- and lipid concentration-dependent. Collectively, these results suggest that ethanol addition to preformed liposomes is an effective method to achieve efficient pH gradient-dependent loading of cholesterol-free liposomes at temperatures below the *Tc* of the bulk phospholipid.

Keywords: Liposome; Ethanol; Doxorubicin; pH gradient; Anthracycline

Abbreviations: AUC, area under the curve; CH, cholesterol; CHE, cholesteryl hexadecyl ether; cryo-TEM, cryo-transmission electron microscopy; DAPC, 1,2-diarachidoyl-*sn*-glycero-3-phosphatidylcholine; dox, doxorubicin; dpm, disintegrations per minute; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphatidylcholine; DSPE, 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine; PEG, poly(ethylene glycol); ^{3}[H], tritium radiolabel; H_2SO_4, sulfuric acid; HEPES, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]; HBS, HEPES buffered saline; LUV, large unilamellar vesicle; MLV, multilamellar vesicle; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PK, pharmacokinetic; QELS, quasielastic light scattering; SDS, sodium dodecyl sulfate; Tc, phase transition temperature; v/v, volume to volume ratio

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1. Introduction

Within the last decade, significant improvements in liposome technology have generated agents suitable for human use. Liposomal anti-cancer agents currently on the market include Doxil® (doxorubicin), DaunoXome® (daunorubicin) and DepoCyt® (cytosine arabinoside) [1–3]. These agents have demonstrated enhanced therapeutic activity and/or reduced toxicity relative to the free drug form, indicating that encapsulation of drugs in lipid based carriers is beneficial. Unfortunately, designing liposome carriers has proven to be an empirical process. Not only is it important to ensure that drug uptake and stability is achieved in a given liposome formulation, but also that the encapsulated drug is bioavailable over a time frame that is appropriate to achieve maximum therapeutic effects [4]. This delicate balance has been difficult to achieve. It is evident that not all drugs are amenable to encapsulation in lipid based carriers with current technologies. Thus development of novel methods to improve drug loading and, in turn, to investigate appropriate release rates for a given drug remains a fundamental hurdle in the development of other anticancer drug formulations.

Recent work from this laboratory has shown that liposomes composed of distearoylphosphatidylcholine (DSPC) and low amounts of distearoylphosphatidylethanolamine conjugated poly(ethylene glycol) (DSPE-PEG) retained idarubicin better than conventional cholesterol-containing liposomes in vivo [5]. Incorporating hydrophilic polymers, such as PEG, was sufficient to improve the stability of the liposomes, as judged by in vivo pharmacokinetic (PK) assessments. In liposomes composed of phospholipids, the absence of cholesterol results in a formulation that can be exquisitely sensitive to changes in temperature and osmotic gradients. These attributes are controlled in part by the presence of grain boundaries, lipid domains and disc formation [6–8], all of which are absent in liposomes containing > 30% cholesterol.

The studies described in this report investigated anthracycline encapsulation within DSPC/DSPE-PEG lipid mixtures through the use of transmembrane pH gradient-based loading methods. Although anthracyclines such as doxorubicin and idarubicin have a similar structure, they have significantly different octanol/buffer concentration ratios [9]. Increased idarubicin uptake in liposomes may be directly attributed to idarubicin's higher relative hydrophobicity [10], aiding in its transbilayer movement which, in turn, results in increased loading rates as well as efficient loading at lower temperatures when compared to doxorubicin. Although our rationale to characterize cholesterol-free liposomes composed of gel phase DSPC was to effectively improve retention of drugs that exhibit increased permeability across the bilayer, there is also an interest in assessing drugs that may exhibit decreased bilayer permeability. An approach to improve the loading efficiency of such drugs is to enhance the drug partitioning and/or permeability of the lipid bilayer.

Several methods have been shown to increase membrane permeability including incorporation of cholesterol below the *T*c of the bulk phospholipid [11], PEG-modified lipids [12,13], and lysophospholipids [14]. Simpler methods, such as the addition of short-chain alcohols [15–17] or surfactants, such as detergents [18], have also been applied. Considering that the interaction of short-chain alcohols, such as ethanol, with lipid bilayers is well documented and it is easily removed from samples, this was identified as a preferred method. Many groups have investigated the specific interaction of ethanol with lipid bilayers [19–22]. Addition of ethanol to lipid membranes results in an increase in the dielectric constant [23], dehydration of the phospholipid head groups [24], and an increase in ion permeability [17]. It should also be noted that ethanol has been extensively used in the preparation of liposomes for improving transdermal liposomal drug delivery [25], improving encapsulation of proteins [26] and gene-based agents [27,28], increasing trapped volume [29], and ensuring compositional homogeneity [30].

In the studies described herein, doxorubicin's low permeability made it difficult to effectively encapsulate the drug in cholesterol-free liposomes. Therefore, ethanol was utilized at concentrations well below that required to collapse the imposed pH gradient in order to increase drug loading rates. The results suggest that ethanol can be used as an effective strategy to overcome difficulties with drug loading, especially when considering the potential of cholesterol-free formulations for drug delivery.

2. Materials and methods

2.1. Materials

Dipalmitoylphosphatidylcholine (DPPC), DSPC and diarachidoylphosphatidylcholine (DAPC) lipids and DSPE-PEG lipids (molecular weight 2000) were obtained from Avanti Polar Lipids, (Alabaster, AL, USA). Ethyl alcohol (99.9% v/v) was manufactured by Commercial Alcohols (Chatham, ON, Canada). HEPES, citric acid, sephadex G-50 (medium), ^{3}[H]-cholesteryl hexadecyl ether (CHE), cholesterol (CH) and ^{14}[C]-lactose were obtained from NEN Life Science Products (Oakville, ON, Canada). ^{14}[C]-Methylamine hydrochloride was obtained from Amersham Pharmacia Biotech (Oakville, ON, Canada). Picofluoro-15 scintillation fluid was obtained from Packard Bioscience (Groningen, The Netherlands). The anthracyclines idarubicin hydrochloride and epirubicin hydrochloride (Pharmacia and Upjohn, Boston, MA, USA), doxorubicin hydrochloride (Faulding, Montreal, QC, Canada), and daunorubicin hydrochloride (Novopharm, Toronto, ON, Canada) were manufactured by the indicated companies and obtained from British Columbia Cancer Agency (Vancouver, BC, Canada).

2.2. Preparation of liposomes

DSPC/DSPE-PEG_{2000} (95:5 mole ratio) and DSPC/CH/DSPE-PEG_{2000} (50:45:5 mole ratio) liposomes were prepared by the extrusion technique [31]. Briefly, lipids were dissolved in chloroform and mixed together in a test tube at appropriate molar ratios. ^{3}[H]-CHE was added as a non-exchangeable, non-metabolizable lipid marker [32,33]. The chloroform was evaporated under a stream of nitrogen gas and the sample was placed in a high vacuum overnight. The lipid films were rehydrated in citrate (300 mM citric acid, pH 4.0) by gentle mixing and heating. The newly formed multilamellar vesicles (MLVs) were passed 10 times through an extruding apparatus (Northern Lipids, Vancouver, BC, Canada) containing two stacked 100-nm polycarbonate filters. The mean diameter and size distribution of each liposome preparation (prior to addition of ethanol or drugs), analyzed by a NICOMP model 270 submicron particle sizer (Pacific Scientific, Santa Barbara, CA, USA) operating at 632.8 nm, was typically 100 ± 30 nm.

2.3. Remote loading of anthracyclines

The remote loading procedure has been well characterized for weak bases such as anthracyclines [34]. Following hydration of lipid films in citrate (300 mM citric acid; pH 4.0), extrusion and size determination, liposomes were passed down a sephadex G-50 column (10×1.5 cm) equilibrated with HEPES buffered saline (HBS; 20 mM HEPES, 150 mM NaCl, pH 7.4) to exchange the external buffer. The eluted liposomes had a transmembrane pH gradient, pH 4.0 inside and pH 7.4 outside. Ethanol and drugs were added to the liposome preparation (5 mM total lipid concentration) at a 0.2 drug-to-lipid mole ratio at varying incubation temperatures. Note that in all cases, ethanol was added following drug addition to prevent exposure of liposomes to excessively high ethanol concentrations.

For drug loading rate determination, 100 μl aliquots were added to mini spin columns at 1, 2, 5, 10, 15, 30, 60 and 120 min following remote loading. Spin columns were prepared by adding glass wool to a 1-cc syringe and sephadex G-50 beads packed by centrifugation ($680 \times g$, 2 min). Following addition of the sample to the column, the liposome fraction was collected in the void volume (centrifugation $680 \times g$, 2 min) and both lipid and drug content were analyzed. The lipid concentration was measured by ^{3}[H]-CHE radioactive counts and drug concentration was determined by measuring the absorbance at 480 nm (HP 8453 UV–visible spectroscopy system, Agilent Technologies Canada, Mississauga, ON, Canada) in a 1% Triton X-100 solution and compared to a standard curve.

2.4. Drug and liposomal membrane association studies

The amount of drug associated with liposomes prepared without a pH gradient was determined. These studies were performed to ascertain the effect of drug hydrophobicity and the presence of ethanol on drug association with lipid membranes. Based on the experimental design, the drug concentration is a collective measurement of drug that has equilibrated across the lipid membrane in the aqueous space, drug associated with the lipid membrane through partitioning, head group and electrostatic interactions. In the absence of a pH gradient, it is believed that most of the drug is associated with the membrane, although drug precipitation in the aqueous space cannot be disregarded. DSPC/DSPE-PEG_{2000} (95:5 mole ratio) and DSPC/CH/DSPE-PEG_{2000} (50:45:5 mole ratio) liposomes were prepared as described in Section 2.2, hydrated in HBS, pH 7.4, and extruded. Drugs (idarubicin, daunorubicin, doxorubicin or epirubicin) and/or ethanol was combined with liposomes (5 mM total lipid concentration) at a 0.2 μmol drug/μmol phospholipid ratio and incubated at 40 °C for 60 min. One-hundred-microliter aliquots were passed down mini spin columns ($680 \times g$, 2 min) and both lipid and drug concentrations were measured by radioactive counts (TriCarb® Model 1900TR liquid scintillation analyzer, Meriden, CT, USA) and an anthracycline extraction assay (Section 2.8) followed by fluorescence spectrometer detection, respectively.

2.5. Determination of pH gradient and percent ^{14}[C]-lactose retention

To measure the transmembrane pH gradient, ^{14}[C]-methylamine was added to liposomes and incubated with increasing concentrations of ethanol (0%, 5%, 10%, 20%, and 30% v/v) for 60 min at 37 °C. Samples were passed down sephadex G-50 mini spin columns to separate liposome encapsulated ^{14}[C]-methylamine. The pH gradient was calculated as previously determined [35] by the following equation:

$$\Delta\mathrm{pH} = -\frac{\log[\mathrm{H}]^{+}in}{[\mathrm{H}]^{+}out} = -\log\frac{(\mathrm{CH_3NH_3})^{+}in}{(\mathrm{CH_3NH_3})^{+}out} \quad (1)$$

To determine the percent lactose retention, lipid films were hydrated in HBS (pH 7.4) containing tracer quantities of ^{14}[C]-lactose. Following extrusion, liposomes were incubated at 40 °C with increasing ethanol concentrations (0–30%) for 60 min and 100-μl aliquots were passed down sephadex G-50 mini spin columns. Both lipid and lactose concentrations were determined using specific activity counts of ^{3}[H]-CHE and ^{14}[C]-lactose.

2.6. Cryo-transmission electron microscopy (cryo-TEM)

Cholesterol-free liposomes were analyzed by cryo-TEM, the method employed and interpretation of liposome images has been previously described [36]. Briefly, in a climate chamber, a drop of the liposomes solution was placed on a copper grid containing a polymer film and

blotted, forming a thin aqueous layer on the membrane. The sample was flash frozen in ethane allowing the film to vitrify, an essential step to prevent crystal formation. The copper grid containing the sample was transferred to an electron microscope at liquid nitrogen temperature where it was analyzed.

2.7. Plasma elimination of liposomes

In vivo drug release of anthracyclines encapsulated in liposomes in the absence and presence of 10% (v/v) ethanol was evaluated. For PK analysis, samples were prepared and remote loaded as mentioned and subsequently concentrated to 16.5 mM lipid concentration with cross-flow cartridges (500,000 MWCO) manufactured by A/G Technology Corp. (Needham, MA, USA).

Female Balb/c mice, 20–22 g, breeders were purchased from Charles River Laboratories (St. Constant, QC, Canada) and bred in house. Mice were housed in microisolator cages and given free access to food and water. All animal studies were conducted according to procedures approved by the University of British Columbia's Animal Care Committtee and in accordance with the current guidelines established by the Canadian Council of Animal Care.

^{3}[H]-CHE radiolabeled liposome samples were administered intravenously into the lateral tail vein of female Balb/c mice. Mice were injected with 33 μmol/kg drug and 165 μmol/kg lipid (0.2 μmol drug/μmol phospholipid). At various time points post drug administration, blood was collected by tail nick or cardiac puncture. Plasma lipid and drug concentrations were quantified by liquid scintillation counting and an anthracycline extraction assay (Section 2.8) followed by fluorescence spectrometer detection, respectively.

2.8. Anthracycline extraction assay

Doxorubicin, daunorubicin, epirubicin or idarubicin was extracted from plasma or buffer samples with a standard extraction assay [37]. Briefly, an aliquot of plasma was added to a 16×100 mm test tube made up to 800 μl with distilled water. Subsequently, 100 μl of both SDS and 10 mM H_2SO_4 was added, vortexed and followed by the addition of 2 ml of 1:1 isopropranol/chloroform. Samples were placed in -80 °C for 1 h. All tubes were equilibrated to room temperature, vortexed and centrifuged at $1000 \times g$ for 10 min. The bottom organic phase was carefully transferred into a clean test tube and samples measured on an LS 50B luminescence spectrometer (Perkin-Elmer, Beaconsfield, Buckinghamshire, England) using an excitation wavelength of 480 (5-nm bandpass) and an emission wavelength of 550 (10-nm bandpass). The doxorubicin extraction efficiency for plasma samples was determined to be > 90% and slightly lower for tissue samples.

2.9. Pharmacokinetic modeling and statistical analysis

The plasma elimination data was modeled using Win NONLIN (version 1.5) pharmacokinetic software (Pharsight Corporation, Mountain View, CA, USA) to calculate PK parameters of liposomal doxorubicin. The mean plasma area under the curve (AUC) for a defined time interval was determined by the standard trapezoidal rule. All data values are reported as mean ± standard deviation (S.D.). A standard one-way analysis of variance (ANOVA) was used to determine statistically significant differences of the means. For multiple comparisons, post-hoc analysis using the Tukey–Kramer test was used. $P < 0.05$ was considered significant.

3. Results

3.1. Drug uptake studies of anthracyclines in cholesterol-free liposomes

Previous studies demonstrated that encapsulation of idarubicin in DSPC/DSPE-PEG$_{2000}$ liposomes (prepared in pH 4.0 citrate buffer and exchanged into a pH 7.4 HBS buffer) was optimal between 37 and 40 °C, a temperature range below the phase transition temperature of DSPC [5]. In contrast to idarubicin, doxorubicin could not be efficiently loaded under the same conditions (Fig. 1A, open circles). In fact, less than 25% of the added doxorubicin accumulated in DSPC/DSPE-PEG$_{2000}$ liposomes over the 2-h time course at 40 °C. The rate of daunorubicin loading (open triangles) was faster than doxorubicin, but slower than idarubicin. It should be noted that the rate of drug loading in the cholesterol-free liposomes increased when the loading temperature was elevated (data not shown). For idarubicin, daunorubicin and doxorubicin, 100% loading was achieved in DSPC/DSPE-PEG$_{2000}$ liposomes within 2 min when the incubation temperature was higher than the *T*c of DSPC (55 °C). For idarubicin the loading efficiency at 60 °C is > 95% after a 2-min incubation, but less the > 30% after 1 h, suggesting a collapse in the pH gradient used to promote loading and/or re-equilibration of drug across a leaky membrane.

3.2. Plasma elimination studies of anthracyclines encapsulated in cholesterol-free liposomes

Lipid membranes are selectively permeable and permit the bidirectional flow of solutes, such as drugs, and thus we investigated whether the observed differences in drug loading rates at 40 °C would be an indication of drug elimination rates in vivo. Idarubicin, daunorubicin and doxorubicin were remotely loaded into DSPC/DSPE-PEG$_{2000}$ (95:5 mole ratio) liposomes and injected into the lateral tail vein of female Balb/c mice at 33 μmol/kg drug and 165 μmol/kg lipid doses (0.2 μmol drug/μmol lipid ratio). Drug and lipid

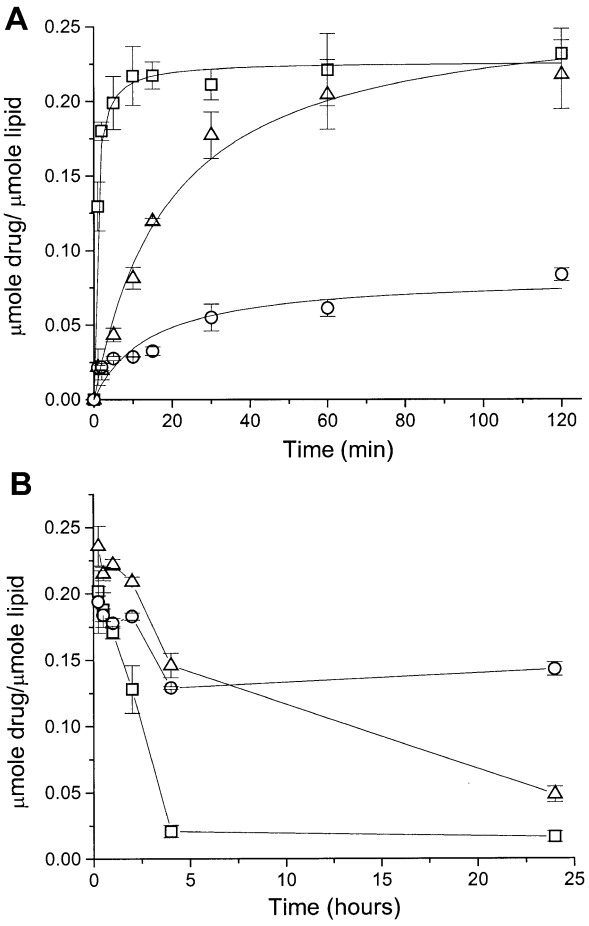

Fig. 1. (A) Time course of uptake of anthracyclines in cholesterol-free liposomes. DSPC/DSPE-PEG$_{2000}$ (95:5 mole ratio) liposomes (with transmembrane pH gradient, pH 4 inside, 7.4 outside) were incubated with idarubicin (□), daunorubicin (△) or doxorubicin (○) at 40 °C (drug-to-lipid mole ratio = 0.2). At various time points, 100-μl aliquots of sample were passed down mini spin columns and subsequently analyzed for drug and lipid concentrations as described in Section 2. Lipid concentration for studies was 5 mM. (B) Plasma elimination of anthracyclines encapsulated in DSPC/DSPE-PEG$_{2000}$ liposomes. Large unilamellar vesicles radiolabeled with ^{3}[H]-CHE were encapsulated with idarubicin (□), daunorubicin (△) or doxorubicin (○) by remote loading. Liposomal drugs were administered intravenously via the dorsal tail vein of Balb/c mice at a dose of 33 μmol/kg drug and 165 μmol/kg total lipid (0.2 drug-to-lipid mole ratio). Blood was collected at various time points following administration. Plasma was assayed for lipid and doxorubicin concentration as described in Section 2.

plasma concentrations were measured by standard procedures and described in methods (Sections 2.7 and 2.8), and plotted as the μmol drug/μmol lipid ratio versus time post administration (Fig. 1B). Plasma lipid elimination profiles were similar for all samples (results not shown) and therefore the calculated drug-to-lipid mole ratio provides an indication of the amount of drug released from the liposomes over time after injection. Significant differences ($P < 0.05$) in plasma drug-to-lipid mole ratio were observed at 24 h post drug administration. Values of 0.02, 0.05 and 0.14 μmol drug/μmol lipid were measured for idarubicin, daunorubicin and doxorubicin encapsulated in cholesterol-free liposomes at this time point. As predicted, the release of anthracyclines from liposomes could be related to drug loading rates.

3.3. Drug and liposomal membrane association of anthracyclines

To this point, we have established a connection between anthracycline drug loading rates at 40 °C, a temperature below the *Tc* of the bulk phospholipid, and the retention of anthracyclines encapsulated in cholesterol-free liposomes in vivo. Doxorubicin's lower drug loading rate and increased retention in cholesterol-free liposomes as compared to both daunorubicin and idarubicin is consistent with doxorubicin's lower partition coefficient relative to idarubicin [10], as illustrated in Fig. 2. We measured the amount of drug associated with cholesterol-free (open bars) and cholesterol-containing (filled bars) liposomes prepared without a pH gradient; the measured values shown should be taken to represent liposomal membrane association and it should not be viewed as a direct measurement of drug partitioning. Based on the experimental conditions, the amount of drug measured is a collective measurement of drug that has equilibrated across the lipid membrane in the aqueous space (or precipitated), drug associated with the lipid membrane through partitioning, hydrophobic and electrostatic interac-

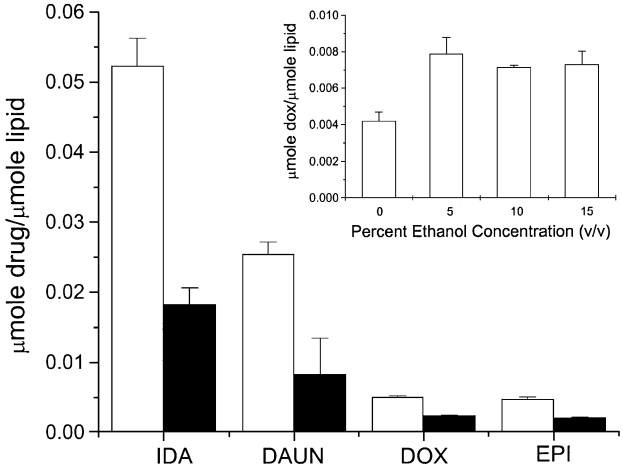

Fig. 2. Influence of drug hydrophobicity on liposomal membrane association in cholesterol-free and cholesterol-containing liposomes. DSPC/DSPE-PEG$_{2000}$ (95:5 mole ratio, open bars) and DSPC/CH/DSPE-PEG$_{2000}$ (50:45:5 mole ratio, filled bars) liposomes exhibiting no pH gradient were incubated with the anthracyclines idarubicin, daunorubicin, doxorubicin and epirubicin at 40 °C for 60 min (lipid concentration was 5 mM). One-hundred-microliter aliquots were passed down mini spin columns and analyzed for lipid and drug concentrations by liquid scintillation counting and an anthracycline extraction assay (Section 2.8) followed by luminescence spectrometer detection. Inset: The influence of ethanol on doxorubicin liposomal membrane association. DSPC/DSPE-PEG$_{2000}$ (95:5 mole ratio, open bars) liposomes exhibiting no pH gradient were incubated with doxorubicin and increasing concentrations of ethanol (0–15% v/v) at 40 °C for 60 min. Lipid and drug concentrations were measured as detailed above and in Section 2.

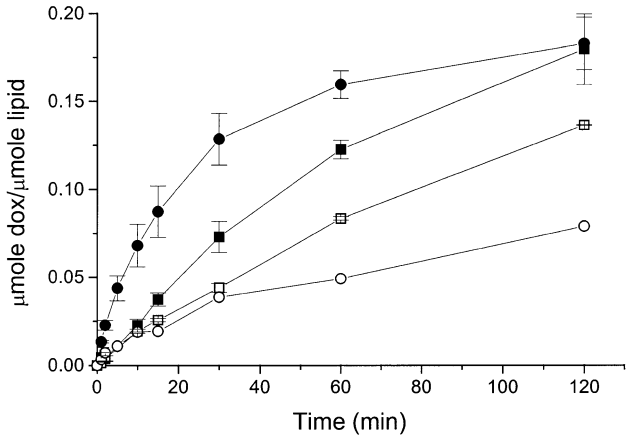

Fig. 3. Ethanol-induced increases in drug loading rates in liposomes. DSPC/CH/DSPE-PEG_{2000} (50:45:5 mole ratio, squares) and DSPC/DSPE-PEG_{2000} (95:5 mole ratio, circles) liposomes (with transmembrane pH gradient, pH 4 inside, 7.4 outside) were incubated at 37 °C with doxorubicin (0.2 drug-to-lipid mole ratio) in the absence (open symbols) and presence (closed symbols) of 10% (v/v) ethanol. At various time points, 100-µl aliquots of sample were passed down mini spin columns and subsequently analyzed for drug and lipid concentrations as described in Section 2.

tions. In the absence of a pH gradient, it is believed that most of the drug is associated with the membrane, although drug equilibration into the aqueous space cannot be disregarded. The results in Fig. 2 demonstrate that the anthracycline liposomal membrane association is reduced by a factor of more than 2 when the DSPC/DSPE-PEG_{2000} liposomes are prepared with 45 mol% cholesterol. This result is consistent with the understanding that cholesterol decreases the partitioning of drugs into bilayers, an effect that has been shown by others to be greater at lower temperatures [38]. For liposomes prepared without cholesterol, the drug liposomal membrane association data (shown as the amount of drug associated per micromole of liposomal lipid) suggests that the level of idarubicin associated was almost 10-fold greater than that observed for doxorubicin or epirubicin, the most hydrophilic anthracyclines evaluated. The liposomal membrane association behaviour of daunorubicin is intermediate between idarubicin and doxorubicin.

These results have a number of interesting implications. We have focused here on one aspect that is built on the suggestion that enhancing doxorubicin membrane association and/or enhancing membrane permeability of the lipid bilayer, under conditions that do not affect the stability of the pH gradient, could increase drug loading rates of doxorubicin in cholesterol-free liposomes. As the results from our drug liposomal membrane association assay are consistent with relative hydrophobicities of the anthracyclines [9,10], this assay was utilized to evaluate whether doxorubicin membrane association could be enhanced with the addition of ethanol at 40 °C. The results are summarized in the inset graph of Fig. 2. In the presence of ethanol (0–15% v/v), the drug-to-lipid ratio was between 0.007 and 0.008 µmol dox/µmol lipid, approximately two-fold higher than in the absence of ethanol ($P<0.01$). Note that calculations based on the equilibration of drug concentration across lipid membranes at 1 mM doxorubicin, assuming no membrane partitioning, were expected to yield 0.002 µmol dox/µmol lipid; thus, it can be suggested that the majority of the associated drug is membrane-bound. There were no significant differences in doxorubicin membrane association as the ethanol concentrations increased from 5% to 15% v/v. It needs to be noted that the methods used do not account for membrane association at equilibrium. Non-equilibrium conditions are introduced during the separation of lipid-associated and free drug on the G-50 sephadex spin columns. If membrane-associated ethanol is removed while being passed down the column, this may result in a loss in membrane-associated doxorubicin and an underestimation of the level of membrane association.

3.4. Influence of ethanol on doxorubicin loading in liposomes

As summarized in Fig. 3, the rate and extent of doxorubicin loading at 37 °C was significantly increased by addition of ethanol (10%, v/v) to DSPC/DSPE-PEG_{2000} liposomes. Greater than 90% encapsulation efficiency was achieved following a 2-h incubation at 37 °C, a value that was 2.3-fold higher than that observed in the absence of ethanol. For DSPC/DSPE-PEG_{2000} liposomes, initial drug loading rates were 6.40 (nmol dox/µmol lipid) min^{-1} as compared to 1.40 (nmol dox/µmol lipid) min^{-1} in the presence and absence of ethanol, respectively. In the absence of ethanol, liposomes composed of DSPC/CH/DSPE-PEG_{2000} exhibited improved encapsulation efficiencies

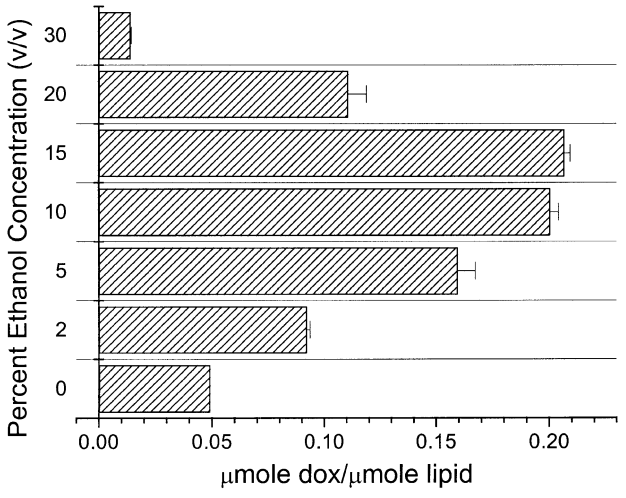

Fig. 4. Influence of ethanol concentration on the accumulation of doxorubicin in cholesterol-free liposomes. DSPC/DSPE-PEG_{2000} (95:5 mole ratio) liposomes (with transmembrane pH gradient, pH 4 inside, 7.4 outside) were incubated at 37 °C with doxorubicin and increasing concentrations of ethanol. At 1 h post drug loading, aliquots were passed down mini spin columns and the eluted fraction was analyzed for drug and lipid concentration by methods outlined in Section 2.

Table 1
The influence of ethanol concentration on size, percent lactose retained and pH gradient in DSPC/DSPE-PEG$_{2000}$ (95:5 mole ratio) liposomes

Ethanol concentration	Liposome size (nm)[a]		Lactose retention (% of control)[b]	pH gradient[c]
(%)	Precolumn	Postcolumn		
0	107	109	100 ± 7	2.77 ± 0.23
5	113	112	103 ± 9	2.80 ± 0.24
10	116	112	97 ± 7	3.44 ± 0.50
20	120	116	100 ± 6	1.89 ± 0.04
30	144	133	74 ± 6	0.86 ± 0.01

[a] Liposome size determined by QELS before and after size exclusion chromatography.
[b] Measurements determined by trapped ^{14}[C]-lactose added during sample rehydration.
[c] Measurements determined by internal and external concentrations of ^{14}[C]-methylamine after 1-h incubation at 37 °C.

(72% at 2 h) and faster initial drug loading rates (1.5 (nmol dox/μmol lipid) min^{-1}) as compared to liposomes prepared without cholesterol. It should be noted that previous studies have shown that cholesterol decreases the partitioning of ethanol in membranes, predominantly at lower temperatures [39] and thus addition of ethanol to liposomes prepared with 45 mol% cholesterol had a minimal effect on doxorubicin loading rates. In the presence of ethanol (10%, v/v), DSPC/CH/DSPE-PEG$_{2000}$ liposomes exhibited an initial drug loading rate of 2.4 (nmol dox/μmol lipid) min^{-1}, a rate that was three-fold lower than that observed for DSPC/DSPE-PEG$_{2000}$ liposomes.

3.5. Optimal ethanol concentration for drug loading in liposomes

In order to determine the optimal ethanol concentration for doxorubicin loading into DSPC/DSPE-PEG$_{2000}$ liposomes, the effect of increasing ethanol concentrations was investigated by measuring doxorubicin loading efficiency after a 1-h incubation at 37 °C (Fig. 4). The highest encapsulation efficiencies were observed when the liposomes were incubated in 10% to 15% (v/v) ethanol. As shown in Fig. 4, the encapsulation efficiency was reduced significantly when the concentration of ethanol was ≥20% (v/v). When the DSPC/DSPE-PEG$_{2000}$ liposomes were incubated in the presence of 40% and 50% ethanol, there was an observed increase in solution viscosity or "gelling" of the sample.

Since more subtle changes in liposome structure may occur in the presence of ≤20% (v/v) ethanol, we assessed the effect of ethanol addition on liposome size, ^{14}[C]-lactose retention and the pH gradient used to engender drug loading and promote drug retention (Table 1). DSPC/DSPE-PEG$_{2000}$ liposomes were exposed to various concentrations of ethanol for 1 h prior to measuring liposome size by quasielastic light scattering (QELS). DSPC/DSPE-PEG$_{2000}$ liposomes exposed to increasing ethanol concentrations all exhibited minimal increases in mean diameter and polydispersity as judged by standard deviations. At ethanol concentrations ≤20% (v/v), there was less than a 12% increase in liposome size. The particle size analysis data suggested that even at ethanol concentrations ≥20% (v/v), the liposomes

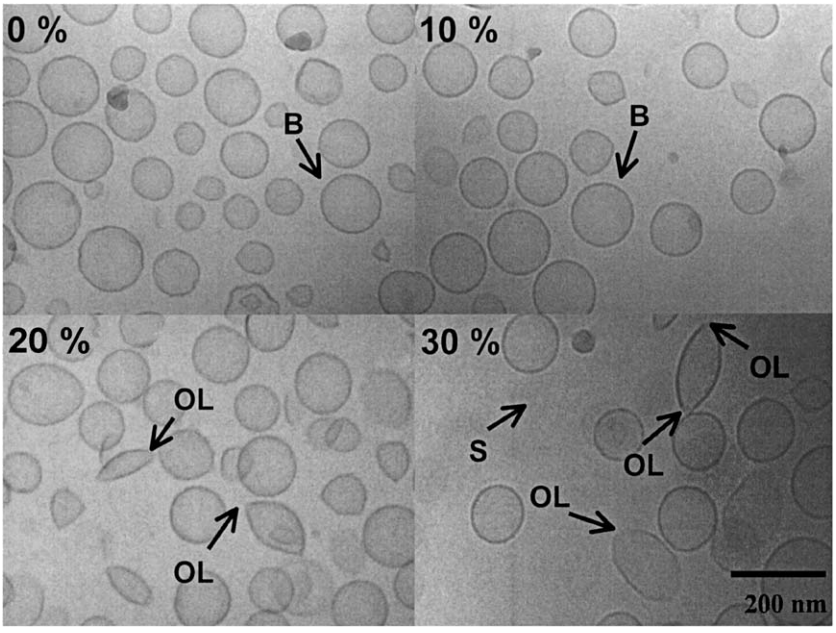

Fig. 5. Influence of ethanol on liposomes structure. Cryo-TEM electron micrographs were obtained of DSPC/DSPE-PEG$_{2000}$ (95:5 mole ratio) liposomes after establishment of a transmembrane pH gradient (pH 4 inside, 7.4 outside). In the presence of 0% and 10% ethanol (v/v), lipid bilayers (B), described as intact with polyhedron shapes, were observed. At 20% ethanol, some open liposomes (OL) were present, however, at 30%, open liposomes (OL) and bilayer sheets (S) were observed. Bar represents 200 nm.

remained as a single population exhibiting a Gaussian distribution. However, a 35% increase in liposome size was observed at ethanol concentrations of 30% (v/v). Samples passed down sephadex G-50 columns to remove residual ethanol exhibited a size that was not significantly different from the sample prior to chromatography.

The permeability of DSPC/DSPE-PEG_{2000} lipid membranes in the presence of ethanol was determined using a radiolabeled ^{14}[C]-lactose trapped marker (Table 1). Lipid films were rehydrated with HBS (pH 7.4) containing trace quantities of the radiolabeled lactose and extruded. Liposome samples were incubated with increasing concentrations of ethanol (0–30% v/v) for 60 min and passed down mini columns to separate retained and free lactose. When incubated with 30% ethanol (v/v), the percent lactose retained decreased significantly ($P<0.05$), consistent with the notion that ethanol at this concentration affected liposome permeability sufficiently to promote release of the entrapped marker. Radiolabeled markers, such as lactose, are also used to indicate liposome trap volumes. In the absence of ethanol, DSPC/DSPE-PEG_{2000} liposomes prepared by the extrusion technique through 100-nm pore size filters exhibited a trapped volume of 1.94 ± 0.11 μl/μmol. This value was comparable to previously published trapped volumes for liposomes prepared by extrusion through 100-nm pore size filters [40].

Another indication of ethanol-induced increases in liposome permeability was provided by measuring the stability of an imposed transmembrane pH gradient. The DSPC/DSPE-PEG_{2000} liposomes used in these studies were prepared in a pH 4.0 citrate buffer and were subsequently exchanged into HBS at pH 7.4. The estimated pH gradient of >3 units can be measured using methylamine as a probe [41]. A measured pH gradient of >2.7 units was observed when ethanol concentrations were ≤ 10% (v/v); however, at higher ethanol concentration (≥20% v/v), there was a significant ($P<0.05$) reduction in the measured transmembrane pH gradient. Previously published data have suggested that the magnitude of the pH gradient is important in terms of maximizing the efficiency of doxorubicin loading as well as playing a critical role in governing drug retention [42]. The decreased loading efficiencies noted in Fig. 4 at ethanol concentrations ≤ 20% (v/v) are likely due to ethanol's effect on collapsing the pH gradient.

Ethanol-induced changes in DSPC/DSPE-PEG_{2000} liposomes were also assessed by cryo-TEM. The representative photomicrographs shown in Fig. 5 suggest that the integrity of liposome structure was maintained in the presence of 10% ethanol (v/v). However, the measured decreases in percent lactose retention and pH gradient at higher ethanol concentrations (≥20%, v/v) could be directly attributed to a breakdown of liposomes structure as observed by cryo-TEM. This was evidenced by the presence of open liposomes (OL) seen when the liposomes were in 20% (v/v) ethanol as well as OL and bilayer sheets (S) observed when the ethanol concentration was increased to 30% (v/v).

3.6. Influence of temperature, lipid concentration and phospholipid acyl chain length on ethanol-enhanced drug loading rates

The results thus far indicate that increases in the rate of anthracycline loading into DSPC/DSPE-PEG_{2000} liposomes parallel the increases in membrane association. Further, it is demonstrated that ethanol can be used to enhance the loading efficiency of doxorubicin, one of the anthracyclines that exhibit the lowest level of membrane partitioning. This effect is presumably the result of ethanol-mediated increases in doxorubicin partitioning. Ethanol-enhanced doxorubicin loading is temperature- (Fig. 6A) and lipid concentration-

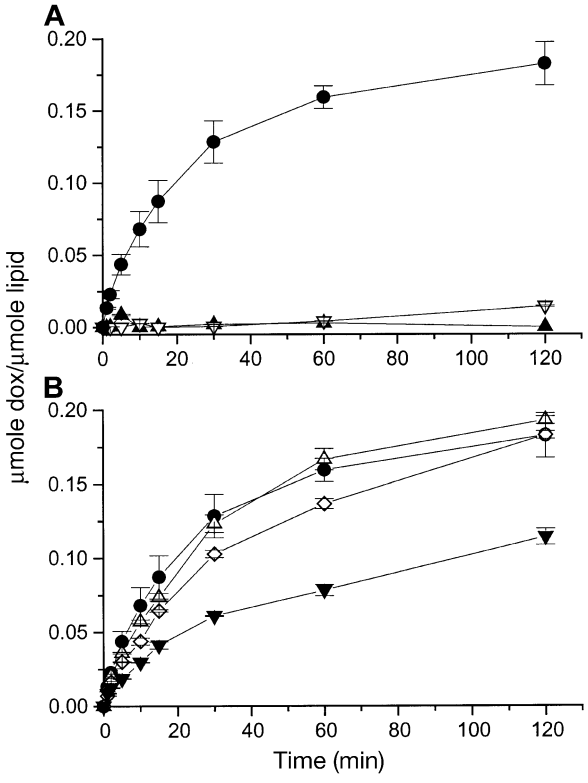

Fig. 6. (A) Influence of temperature on ethanol-enhanced loading of doxorubicin in cholesterol-free liposomes. DSPC/DSPE-PEG_{2000} (95:5 mole ratio) liposomes (with transmembrane pH gradient, pH 4 inside, 7.4 outside) and doxorubicin (0.2 drug-to-lipid mole ratio) were incubated at various temperatures; 37 °C (●), 23 °C (▽), 4 °C (▲). At various time points, 100-μl aliquots of sample were passed down mini spin columns and subsequently analyzed for drug and lipid concentrations as described in Section 2. (B) Influence of lipid concentration on ethanol-enhanced loading of doxorubicin in cholesterol-free liposomes. DSPC/DSPE-PEG_{2000} (95:5 mole ratio) liposomes (with transmembrane pH gradient, pH 4 inside, 7.5 outside) were prepared at various lipid concentrations, 5 mM (●), 10 mM (△), 15 mM (◇) and 20 mM (▼), and incubated with doxorubicin at 37 °C. At various time points, 100-μl aliquots of sample were passed down mini spin columns and subsequently analyzed for drug and lipid concentrations as described in Section 2.

(Fig. 6B) dependent. Interestingly, the rate of drug loading was only enhanced when the temperature was higher than 37 °C. No measurable drug uptake could be observed when the samples were incubated at 23 or 4 °C (Fig. 6A). Increased lipid concentration resulted in significantly reduced drug loading rates. Further studies indicated that increasing ethanol concentration at high lipid concentrations (20 mM) did not significantly improve drug loading rates (data not shown), a result which suggests the importance of maintaining optimal ethanol concentrations as well as ethanol-to-lipid ratios.

Additional studies, shown in Fig. 7, demonstrated that doxorubicin could be loaded in cholesterol-free liposomes prepared with phospholipids of varying acyl chain lengths. These results illustrate three important points. First, as indicated in Section 3.1, doxorubicin loading efficiencies in the absence of ethanol increase as the loading temperature increases. Thus, for DSPC/DSPE-PEG_{2000} and DAPC/DSPE-PEG_{2000} liposomes, doxorubicin encapsulation efficiencies of >95% were achieved when the incubation temperature is held at 60 °C (filled bars). For DAPC/DSPE-PEG_{2000} liposomes, doxorubicin encapsulation efficiencies of 58% and 98% were achieved at 40 °C (grey bars) and 60 °C (filled bars), respectively. These temperatures are well below the *Tc* of DAPC and again illustrate the importance of drug partitioning behaviour in determining drug loading attributes. Second, doxorubicin loading into DPPC/DSPE-PEG_{2000} (*Tc* ~ 41 °C), DSPC/DSPE-PEG_{2000} (*Tc* ~ 55 °C) and DAPC/DSPE-PEG_{2000} (*Tc* ~ 66 °C) liposomes increased as temperature was increased, however, decreased at temperatures above the respective *Tc* of each liposome formulation. When loading was completed in the presence of 10% ethanol, encapsulation efficiencies of all three formulations significantly improved (two- to three-fold) at 40 °C (grey bars). Both DSPC and DAPC formulations achieved greater than 98% trapping efficiencies. Third, the loading efficiencies of doxorubicin into DPPC/DSPE-PEG_{2000} liposomes were in general poor. The addition of 10% (v/v) ethanol to the DPPC/DSPE-PEG_{2000} liposomes, however, did increase the encapsulated efficiencies more than twofold at both 20 and 40 °C. It is likely that improvements in doxorubicin loading into DPPC/DSPE-PEG_{2000} liposomes could be achieved if the loading temperature and ethanol concentration are carefully selected.

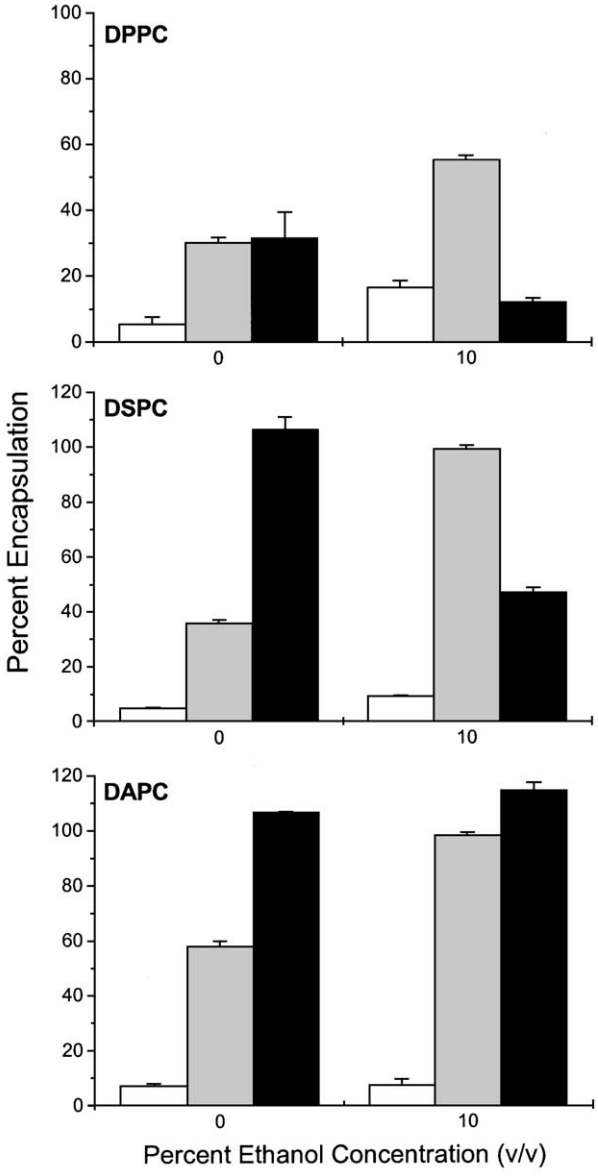

Fig. 7. Effect of phospholipid acyl chain length on ethanol-enhanced loading of doxorubicin in cholesterol-free liposomes. Liposomes exhibiting a transmembrane pH gradient (pH 4 inside, pH 7.4 outside) composed of 95% mole ratio of DPPC, DSPC and DAPC and 5% mole ratio of DSPE-PEG_{2000} incubated at 20 °C (white bars), 40 °C (grey bars) and 60 °C (black bars) with doxorubicin (0.2 drug-to-lipid ratio). At various time points, 100-μl aliquots of sample were passed down mini spin columns and subsequently analyzed for drug and lipid concentrations as described in Section 2.

3.7. Influence of ethanol on release of entrapped doxorubicin in vivo

The use of ethanol to enhance doxorubicin loading into DSPC/DSPE-PEG_{2000} liposomes may be of limited interest if residual ethanol incorporated in the lipid bilayers adversely effects the release of entrapped agents in vivo. Thus, a PK study was completed to determine whether in vivo release of doxorubicin was altered if drug loading was completed in the presence of 10% (v/v) ethanol. Liposomes were prepared as described in Section 2 and loaded to achieve a 0.2 drug-to-lipid mole ratio. Prior to injection, the outside buffer was exchanged using tangential flow dialysis in an effort to remove as much of the residual ethanol as possible. Subsequently, the liposomes were injected intravenously in the lateral tail vein of female Balb/c mice at a dose of 165 μmol/kg lipid and 33 μmol/kg (20 mg/kg) doxorubicin. The plasma elimination profile of doxorubicin and lipid, as well as the calculated drug-to-lipid mole ratio in the plasma compart-

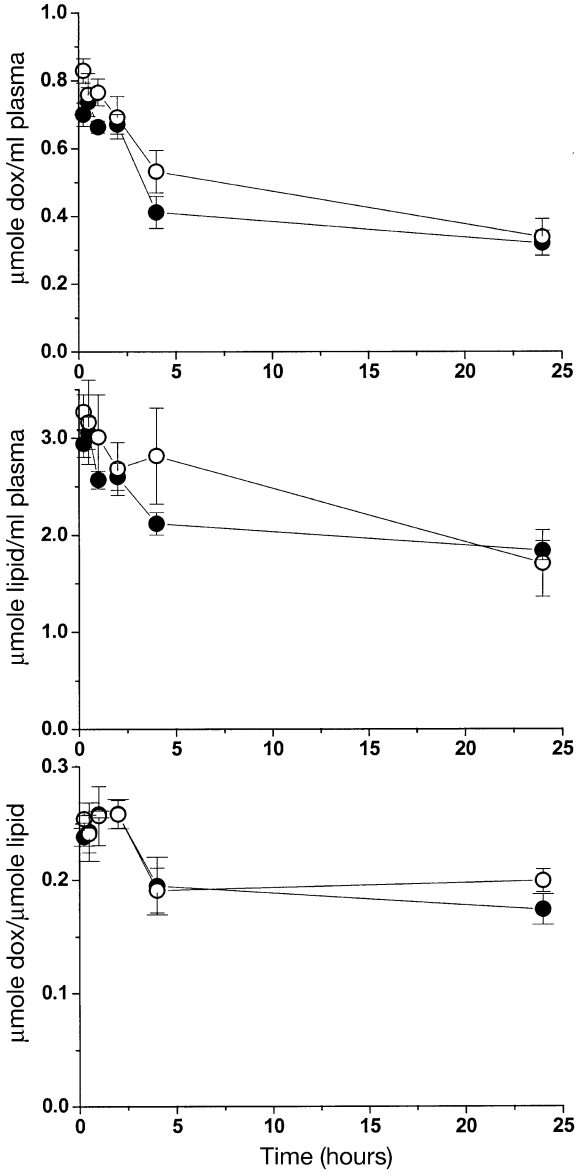

Fig. 8. Plasma elimination of liposomal doxorubicin: comparison of drug release from samples prepared in the absence (○) and presence (●) of 10% (v/v) ethanol. DSPC/DSPE-PEG$_{2000}$ (95:5 male ratio) liposomes radiolabeled with ^{3}[H]-CHE were administered intravenously via the dorsal tail vein of female Balb/c mice at an approximate dose of 33 µmol/kg doxorubicin and 165 µmol/kg total lipid (0.2 drug-to-lipid mole ratio). Blood was collected at 0.25, 0.5, 1, 2, 4 and 24 h. Plasma was prepared and aliquots were assayed for lipid and doxorubicin concentration as described in Section 2. Each data point represents standard deviations of three mice.

ment are shown in Fig. 8. Importantly, minimal differences were observed in the elimination profiles of the liposomal drugs prepared in the absence (open symbols) or presence (filled symbols) of ethanol. Calculated mean plasma $AUC_{0-24\ h}$ for doxorubicin encapsulated in liposomes prepared with and without ethanol was 9.8 and 11.4 µmol h ml^{-1}, respectively. Approximately 39% of the total injected doxorubicin remained in circulation 24 h post drug administration. Further, the measured drug-to-lipid mole ratios were not significantly different at any time points evaluated. These results clearly demonstrate that the use of ethanol to enhance loading of doxorubicin below the phase transition of the bulk phospholipid is a potentially useful method that will not compromise in vivo drug release attributes.

4. Discussion

The delicate balance between retention and release of therapeutic agents entrapped in liposomes is established as an important factor governing the therapeutic and toxic effects of liposomal drugs. Altering lipid membrane composition with the specific goal of optimizing permeability to achieve enhanced drug bioavailability following administration has been extensively explored. For example, liposomes have been engineered to undergo changes affecting drug release in response to pH [43], phospholipase exposure [44,45] and temperature [46] in an effort to achieve improved local drug bioavailability. The behaviour of many of these formulations is dependent on use of liposomes with little or no cholesterol. Removal of cholesterol has introduced problems related to drug loading, liposome stability, liposome–protein binding, liposome elimination and in vivo drug release following i.v. injection. The incorporation of PEG-modified lipids into pure PC liposomes effectively overcomes problems associated with liposome elimination [47–49]. We have also shown that certain drugs are actually retained better in liposomes that lack cholesterol [5]. The general utility of such liposomes will depend, however, on defining methods which facilitate drug loading and manufacturing, particularly since the stability of these liposomes is much more dependent on temperature. If pH gradient-based drug loading methods are being considered, one obvious approach is to select a drug that loads efficiently into cholesterol-free liposomes at incubation temperatures below the *Tc* of the bulk phospholipids. For example, idarubicin loads efficiently into DSPC/DSPE-PEG$_{2000}$ liposomes, which exhibit a transmembrane pH gradient, at 40 °C (see Fig. 1A). In contrast, doxorubicin, an anthracycline that partitions less efficiently into DSPC/DSPE-PEG$_{2000}$ membranes, loads very slowly at 40 °C. Loading of doxorubicin is improved as the incubation temperature increases, however, the stability of the cholesterol-free liposomes is compromised at temperatures above the *Tc* of the bulk phospholipids.

If effective drug loading at temperatures below the *Tc* of the bulk phospholipids is dependent in part on drug partitioning, then we anticipated that improved loading could be achieved through use of agents that could enhance drug partitioning and/or membrane permeability, provided that this did not adversely affect liposome stability. In this study, we have increased doxorubicin liposomal membrane association with ethanol to effectively

improve doxorubicin uptake in DSPC liposomes stabilized with 5 mol% PEG-conjugated DSPE. Ethanol has previously been shown to increase the disorder of lipid bilayers [50,51], and decrease hydration of phospholipids, thereby enhancing permeability. The rationale for using ethanol (or other short-chain alcohols) during drug loading is threefold. First, to improve the loading rates of drugs that are not sufficiently hydrophobic to permeate the bilayer; second, to increase permeability of lipid membranes composed of long acyl chains (greater than C:18); third, to increase the total drug encapsulation levels within liposomes (for example, DPPC cholesterol-free liposomes) loaded at fixed temperatures well below the *T*c of the bulk phospholipid. The studies performed in this report confirmed that drug loading rates below the *T*c of the bulk phospholipid were correlated with the hydrophobicity of the drug. Idarubicin, for example, was the most hydrophobic anthracycline and optimal loading of this drug could be achieved at 40 °C in DSPC/DSPE-PEG_{2000} liposomes. Less hydrophobic agents, such as doxorubicin, required higher temperatures to increase drug loading rates. The results presented (see Figs. 2–4) clearly demonstrated that ethanol addition could improve doxorubicin loading efficiencies at temperatures below the *T*c of the bulk phospholipids. Liposomes composed of long acyl phospholipids are not commonly considered for drug delivery purposes, in part because of difficulties in both preparation and drug loading. Based on our studies, the addition of ethanol will provide opportunities to investigate the applicability of novel drugs encapsulated within formulations containing long acyl chain phospholipids.

Our studies indicated that DPPC cholesterol-free liposomes exhibited a lower capacity for doxorubicin encapsulation. Others have shown that the thermosensitive liposomal formulation, DPPC/DSPE-PEG_{2000} with small amounts of lyso PC (developed by Needham and associates), also has a low drug encapsulation capacity. Loading efficiencies of > 98% can only be achieved for 0.05 drug-to-lipid weight ratios when encapsulated below the *T*c of the membrane and this formulation cannot be effectively loaded above the phase transition temperature (Ref. [52], Ickenstein L, unpublished results). We believe that methods relying on use of ethanol to improve drug loading efficiencies may solve some of the problems that have been encountered when developing these drug-loaded thermosensitive liposomal formulations.

Concerns regarding the use of ethanol to improve pH gradient-based loading of more hydrophilic drugs into DSPC/DSPE-PEG_{2000} have been addressed. At concentrations $\leq$ 15% (v/v) ethanol, liposome size, retention of a trapped aqueous marker, and stability of an imposed pH gradient were not significantly changed (see Table 1). At concentrations $\geq$ 20% (v/v), the presence of open liposomes and bilayer sheets were evident by cryo-TEM (see Fig. 5) and there was a significant reduction in the magnitude of an imposed 3.5 unit pH gradient. The reduction of $[H^+]$ gradient at high ethanol concentrations may be due to either an overall change in the membrane permeability of all liposomes or attributed to a decrease in the number of liposomes available to maintain a pH gradient due to dissolution of the lipid bilayer. The most obvious change in DSPC/DSPE-PEG_{2000} liposomes occurred at ethanol concentrations > 30% (v/v).

Our studies indicated that both drug loading rates and liposomal membrane association could predict the in vivo stability of a drug encapsulated in a particular liposome formulation. The relationships observed are qualified when drugs of a similar structure or family are compared and examined in the same lipid composition, however there are some inconsistencies. For example, increased drug partitioning of idarubicin in cholesterol-free as compared to cholesterol-containing liposomes (Fig. 2) would suggest that there would be increased drug loading rates and drug release in vivo for cholesterol-free liposomes, which is not the case. Faster drug loading rates were observed in cholesterol-containing liposomes (Fig. 3) while enhanced retention of idarubicin in vivo was observed in cholesterol-free liposomes [5]. These particular inconsistencies can be explained by differences in membrane order and fluidity between DSPC cholesterol-free and cholesterol-containing liposomes. Furthermore, Madden et al. [53] performed a survey of many drugs and analyzed drug uptake rates in egg PC vesicles, and determined that drug uptake could not be predicted on log octanol/water partition coefficients alone. Taken together, these observations highlight the importance of other parameters involved in both drug uptake into and release from liposomes including lipid membrane order, drug solubility (aqueous and membrane), drug membrane partitioning, drug electrostatic and hydrophobic interactions. There should be a concerted effort to tease out each of these factors and their contribution to both drug loading and release from liposomes. It would be quite valuable if one could establish high throughput assays capable of predicting the stability of drug liposomes formulations in an effort to decrease the trial and error approach currently used.

One of the principal questions arising from these studies is how does ethanol enhance doxorubicin loading rates, allowing the transfer of doxorubicin from the external medium into the aqueous core of the membrane, while maintaining the proton gradient? One potential mechanism involves increasing the interaction of doxorubicin with liposomal membranes, thereby improving drug loading rates. As the elimination profiles of doxorubicin loaded with and without ethanol were not significantly different, it suggests that most of the residual ethanol was removed. Therefore, increased doxorubicin liposomal membrane association was preferentially introduced during drug loading but reduced (by the removal of ethanol) prior to PK analysis. Ethanol–lipid membrane interactions have been extensively studied. There is still a debate on where ethanol resides in the membrane, and the nature of the interaction,

binding or partitioning [54]. Studies completed to date indicated that ethanol resides at the lipid/water interface near the head groups, with a small amount partitioned in the bilayer core [17,20]. Regarding lipid interdigitation, an increase in the incorporation of ethanol can induce this polymorphic change in membrane structure [29,55–57]. Interdigitation is described as a consequence of the displacement of water from the interfacial region [24], resulting in a disordering effect on lipid packing [50] and intercalation of phospholipids acyl chains from opposing leaflets [58]. The presence of interdigitation domains would likely increase bilayer permeability in both directions and does not explain how the incorporation of low levels of ethanol in lipid membranes resulted in increased doxorubicin drug loading rates while maintaining the pH gradient. A more plausible explanation for the effects of ethanol on selective increases in drug permeability pertains to asymmetric distribution of ethanol in lipid bilayers. Most studies have clearly indicated that short-chain alcohols are positioned at the lipid/water interface of the membrane [59]. Studies performed by Heerklotz [60] demonstrated that membrane stress and permeabilization of bilayers from solutes were induced by asymmetric incorporation of compounds. Asymmetric ethanol incorporation may explain why doxorubicin could permeate into the aqueous space of the liposomes, while proton permeability was not increased substantially. Furthermore, if the majority of ethanol partitioned within the outer leaflet of the bilayer, ethanol would be relatively easily removed and would not affect doxorubicin release from the liposomes.

In summary, the addition of ethanol to preformed liposomes is an effective strategy to increase drug membrane association and membrane permeability, allowing loading of drugs that are not sufficiently hydrophobic to cross lipid membranes on a practical time scale. At low ethanol concentrations, initial drug loading rates were significantly improved without affecting the in vivo behaviour of the resulting liposomes. Ethanol enhanced drug loading will be of particular interest when utilizing thermosensitive liposomal formulations, heat-labile drugs or conditions (such as acidic pH) that promote rapid phospholipid degradation at high temperatures. The studies reported here highlight a few advantages of this method. Importantly, we anticipate that similar approaches may be used to improve loading of other anticancer drugs into cholesterol-free liposomes.

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