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Improved retention of idarubicin after intravenous injection obtained for cholesterol-free liposomes

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Abstract

To date there has been a focus on the application of sterically stabilized liposomes, composed of saturated diacylphospholipid, polyethylene glycol (PEG) conjugated lipids (5–10 mole%) and cholesterol (CH) (>30 mole%), for the systemic delivery of drugs. However, we are now exploring the utility of liposome formulations composed of diacylphospholipid conjugated PEG mixtures prepared in the absence of added cholesterol, with the primary objective of developing formulations that retain encapsulated drug better than comparable formulations prepared with cholesterol. In this report the stability of cholesterol-free distearoylphosphatidylcholine (DSPC):distearoylphosphatidylethanolamine (DSPE)-PEG_{2000} (95:5 mol/mol) liposomes was characterized in comparison to cholesterol-containing formulations DSPC:CH (55:45 mol/mol) and DSPC:CH:DSPE-PEG_{2000} (50:45:5 mol/mol/mol), in vivo. Circulation longevity of these formulations was determined in consideration of variables that included varying phospholipid acyl chain length, PEG content and molecular weight. The application of cholesterol-free liposomes as carriers for the hydrophobic anthracycline antibiotic, idarubicin (IDA), was assessed. IDA was encapsulated using a transmembrane pH gradient driven process. To determine stability in vivo, pharmacokinetic studies were performed using 'empty' and drug-loaded [^{3}H]cholesteryl hexadecyl ether radiolabeled liposomes administered intravenously to Balb/c mice. Inclusion of 5 mole% of DSPE-PEG_{2000} or 45 mole% cholesterol to DSPC liposomes increased the mean plasma area under the curve (AUC_{0-24h}) 19-fold and 10-fold, respectively. Cryo-transmission electron micrographs of IDA loaded liposomes indicated that the drug formed a precipitate within liposomes. The mean AUC_{0-4h} for free IDA was 0.030 μmole h/ml as compared to 1.38 μmole h/ml determined for the DSPC:DSPE-PEG_{2000} formulation, a 45-fold increase, demonstrating that IDA was retained better in cholesterol-free compared to cholesterol-containing liposomes.

Keywords: Polyethylene glycol; Liposome; Drug delivery; Idarubicin; Cholesterol

Abbreviations: AUC, area under the curve; CH, cholesterol; CHE, cholesteryl hexadecyl ether; cryo-TEM, cryo-transmission electron microscopy; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine; 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, polyethylene glycol; MPS, mononuclear phagocytic system; HBS, HEPES buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; T_c, phase transition temperature

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1. Introduction

Many drugs have shown improved therapeutic properties when administered in a liposome encapsulated form. This is a consequence of liposome mediated changes in drug pharmacokinetics and biodistribution that can (i) decrease toxicity and drug metabolism and/or (ii) enhance cellular or tumor drug delivery which, in turn, engenders improved antitumor activity. The progressive success of lipid carrier technology is reflected by the approval of various liposomal formulations of doxorubicin (Myocet, Doxil) and daunorubicin (DaunoXome) for human use [1–3]. The development of liposomes as effective drug delivery systems was achieved, in part, as a consequence of improved properties obtained following incorporation of membrane rigidifying agents such as cholesterol. In fact, some of the early research on liposomes as delivery systems for i.v. applications demonstrated that the presence of cholesterol (i) enhanced retention of entrapped solutes [4–8], (ii) diminished interaction with serum proteins [9,10], (iii) reduced phospholipid loss by phospholipases and lipoproteins [11–13], (iv) reduced macrophage digestion [14] and (v) maintained membrane fluidity over a wide temperature range. It is believed that a combination of these factors collectively yielded lipid carriers that were resilient within the biological milieu in terms of both liposome structure stability and retention of entrapped solutes.

Although neutral liposomes prepared of phosphatidylcholine species and cholesterol were effective as drug carriers, there remained perceived drawbacks to the technology. In particular, the rapid elimination of the liposomes following i.v. injection, an observation associated with liposome accumulation in the mononuclear phagocytic system (MPS), was not fully optimized. Although appropriately designed formulations prepared of phospholipids and cholesterol did provide substantial improvements in drug delivery to diseased sites residing in non-MPS organs [15], it was proposed that additional benefits would be achieved if the rate of liposome elimination could be reduced. These conventional formulations, therefore, were superseded by second generation liposomes termed 'sterically stabilized' or Stealth, that incorporate a surface coating consisting of either a ganglioside G_{M1} or synthetic neutral polymer polyethylene glycol (PEG) [16,17]. These novel formulations significantly advanced and broadened the application of cholesterol-containing liposome formulations. In addition to previously mentioned attributes of conventional cholesterol-containing liposomes, sterically stabilized liposomes exhibit significantly greater circulation half-lives [18–21] and the elimination behavior of these liposomes was no longer as sensitive to liposomal lipid dose [22,23]. Subsequent studies illustrated that a longer circulation half-life could facilitate higher drug accumulation within sites of tumor growth [24–26] and this was associated with greater antitumor activity [27–29]. Furthermore, incorporation of PEG modified lipids has provided flexibility in altering the liposome composition, while maintaining the pharmacokinetic behavior of lipid carriers and therefore we investigated the properties of liposomes prepared in the absence of cholesterol for the intended purpose of retaining entrapped drugs.

Given the progress in liposome technology as delivery systems for anticancer drugs, some may find it surprising that this simple methodology is not applied more generically to other cancer drugs. In principle, liposomes must be developed with desirable and controlled release properties that are selected on the basis of the drug being entrapped. More specifically, we argue that liposomal lipid composition cannot be viewed in generic terms, where one liposome formulation is suitable for all drugs. Rather it is critical for the liposome to be designed around the drug of interest. In our laboratory we determine the suitability of a liposome formulation by an iterative process which correlates pharmacodynamic behavior of the encapsulated drug with the plasma elimination and biodistribution behavior of the liposomal carrier. A critical measured parameter obtained in these studies is in vivo drug release rate which, in turn, is critically dependent on lipid composition. It is well understood that liposomal formulations prepared using zwitterionic lipids, such as diacylphosphatidylcholine, and cholesterol are effective as drug carriers, but the benefits for a given anticancer drug depend on their ability to retain drug following i.v. administration. Further, simple changes in phospholipid acyl chain length can have dramatic effects on in vivo drug release rates [30]. In general, as acyl chain length increases, drug retention increases [31]. There

are some drugs, however, which are simply not retained well in these phospholipid/cholesterol formulations, even when the acyl chain length increases to C22 and exhibit gel to liquid crystal transitions above 100°C. Another solution to improve drug retention is based on preparation of liposomes without cholesterol. This has not been explored thoroughly, but there are now compelling reasons to consider the potential of cholesterol-free liposomes as carriers. As a direct consequence of cholesterol's interactions with phospholipids, the permeability of a liposome increases at temperatures below the phase transition temperature (T_c) of the bulk lipid component [32,33]. Thus, membranes (absent of cholesterol) consisting of gel phase lipids ($T_c > 40$°C) will form a more rigid membrane capable of retaining contents for in vivo administration. Additional incorporation of surface stabilizing PEGs will further prevent surface–surface interactions to facilitate prolonged circulation lifetimes. To date little is known about the application of cholesterol-free liposomes for retention of entrapped solutes and these reasons, alone, were sufficient to propose that cholesterol-free liposomes may be relevant carriers for agents that are not currently retained in conventional formulations.

2. Materials and methods

2.1. Lipids and chemicals

Dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC) and distearoylphosphatidylcholine (DSPC) lipids were purchased from Northern Lipids (Vancouver, BC, Canada). Phosphatidylethanolamine (dimyristoylphosphatidylethanolamine (DMPE), dipalmitoylphosphatidylethanolamine (DPPE) and distearoylphosphatidylethanolamine (DSPE) conjugated polyethylene glycol lipids were obtained from Avanti Polar Lipids (Alabaster, AL, USA). HEPES, citric acid, Sephadex G-50, cholesterol (CH), [^{14}C]lactose and [^{3}H]-cholesteryl hexadecyl ether (CHE) were obtained from NEN Life Science Products (Oakville, ON, Canada). Idamycin (idarubicin hydrochloride for injection) is manufactured by Pharmacia and Upjohn (Boston, MA, USA) and obtained from the British Columbia Cancer Agency.

2.2. Preparation of liposomes

Liposome samples were composed mainly of DSPC:DSPE-PEG$_{2000}$ (95:5 mol/mol), DSPC:CH (55:45 mol/mol) or DSPC:CH:DSPE-PEG$_{2000}$ (50:45:5 mol/mol/mol). In some studies variations of these compositions were prepared by altering the phosphatidylcholine acyl chain length (DPPC or DMPC), PEG molecular weight (750 and 5000) or content (2, 10, 15 molar percent). All liposome samples were prepared by the extrusion technique [34]. Briefly, lipids were initially dissolved in chloroform and mixed together in a test tube at the appropriate mole ratios. [^{3}H]CHE was added as a non-exchangeable, non-metabolizable lipid marker [35,36]. 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 by gentle mixing and heating in HEPES buffered saline (HBS; 20 mM HEPES, 150 mM NaCl, pH 7.45). The newly formed multilamellar vesicles (MLVs) were passed 10 times through an extruding apparatus (Northern Lipids) containing two stacked 100 nm polycarbonate filters. The mean diameter and size distribution of each liposome preparation, analyzed by a NICOMP Model 270 Submicron particle sizer (Pacific Scientific, Santa Barbara, CA, USA) operating at 632.8 nm, was typically 110 ± 30 nm.

2.3. Lactose trapping

To determine the trapped volume, liposomes were prepared as mentioned and hydrated in HBS (pH 7.4) containing [^{14}C]lactose (NEN Life Science Products). Following extrusion and sizing, 100 μl aliquots were passed down mini Sephadex G-50 spin columns. Trapped volume was determined by the following equation:

$$\text{Trapped volume} = \frac{(A/B)}{(C/D)} \times (\text{sample volume}) \qquad (1)$$

A = [^{14}C]lactose dpm eluted from spin column; B = [^{14}C]lactose dpm of initial suspension prior to gel filtration/100 μl; C = [^{3}H]CHE dpm eluted; D = specific activity of lipid stock (dpm/μmole total lipid).

Analysis of [^{14}C]lactose release was determined by aliquoting 10 mM extruded liposome sample in a

dialysis membrane (Spectrum Laboratories, Rancho Dominguez, CA, USA) placed in HBS buffer (pH 7.4) solution at 37°C for 48 h. Aliquots were removed at various time points and run down mini Sephadex G-50 spin columns.

2.4. Remote loading of idarubicin

The remote loading procedure has been well characterized for weak bases such as anthracyclines, especially doxorubicin [37]. We have also employed this technique for the active entrapment of idarubicin. Liposomes were prepared following hydration of lipid films in citrate (300 mM citric acid; pH 4.0). Following extrusion and size determination, liposomes were passed down a Sephadex G-50 column equilibrated with HBS (pH 7.45) to exchange the external buffer. The resulting liposomes have a transmembrane pH gradient, pH 4.0 inside and pH 7.45 outside, and were subsequently stored at 4°C. On the day of the experiment liposomes and drug were incubated at defined temperatures in separate test tubes and subsequently mixed together.

2.5. Time dependent analysis of drug loading

To determine the optimal conditions for drug loading of IDA in cholesterol-free liposomes at a 0.2 drug to lipid ratio, IDA and liposomes (with an established pH gradient) were heated at various temperatures (37°C and 65°C) for 5 min, mixed together and placed at the same temperature. At 1, 2, 5, 10, 15, 30, and 60 min post mixing, 100 μl aliquots were added to G-50 spin columns (1 ml). Sephadex G-50 spin columns were prepared by adding glass wool to 1 ml syringe and Sephadex G-50 beads packed by centrifugation ($720\times g$, 1 min). Following addition of the sample to the column, the liposome fraction was collected in the void volume (centrifugation $720\times g$, 1 min) and both lipid and drug content were analyzed. The lipid concentration was measured by [^{3}H]CHE radioactive counts and IDA was determined by a spectrophotometric assay at 480 nm. The spectrophotometric assay consisted of an aliquot of the eluted sample made up to 100 μl with deionized water and 900 μl 1% Triton X-100. Samples were heated in boiling water to the cloud point of the detergent and cooled to room temperature, absorbance was measured (Du-64 spectrophotometer, Beckman) and compared to a standard curve.

2.6. Cryo-transmission electron microscopy

Cholesterol-free liposomes were analyzed by cryo-transmission electron microscopy (cryo-TEM) and performed in the laboratory of Dr. Katarina Edwards in Uppsala, Sweden. The method employed and interpretation of liposome images have been previously described [38]. Briefly, in a climate chamber a drop of the liposome 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. Mice

Female Balb/c mice (6–8 weeks), 20–22 g, breeders were purchased from Charles River Laboratories (St. Constant, PC) and bred in house. Mice were housed in microisolator cages and given free access to water and food. The room environment was maintained according to standard operating procedures established at the BC Cancer Agency Joint Animal Facility. All animal studies were conducted according to procedures approved by the University of British Columbia's Animal Care Committee and these studies were performed in accordance with the current guidelines established by the Canadian Council of Animal Care.

2.8. Plasma elimination of liposomes

To determine in vivo drug retention behavior and circulation longevity, pharmacokinetic studies were performed. 'Empty' or drug loaded [^{3}H]CHE radiolabeled liposomes were administered intravenously to the lateral tail vein of Balb/c female mice. Studies comparing [^{3}H]CHE with radiolabeled [^{3}H]DPPC were also completed (data not shown) and the results for the two lipid labels were comparable for cholesterol-free formulations. The doses of liposomes and IDA were 165 μmoles/kg lipid and 33 μmoles/kg

IDA, respectively. At various time points post drug administration, blood was collected by tail nick or cardiac puncture. The plasma was prepared and a standard liquid scintillation counting and a standard extraction assay (see below) determined both lipid and IDA content, respectively.

The mean area under the curve (AUC) for a defined time interval was determined from the concentration–time curves and subsequent calculation by the standard trapezoidal rule. The plasma data were modeled using the WinNONLIN Version 1.5 pharmacokinetic software (Pharsight, CA, USA).

2.9. Idarubicin extraction assay

IDA was isolated from plasma in the following manner. An aliquot of plasma sample was added to the 16×100 mm test tube and made up to 800 μl with distilled water. Subsequently 100 μl of both 10% SDS and 10 mM H_2SO_4 were added, vortexed, and followed by the addition of 2 ml of 1:1 isopropanol/chloroform. Samples were placed in −80°C for 1 h. All tubes were equilibrated to room temperature, vortexed and centrifuged at 1000×*g* for 10 min. The bottom organic phase was carefully pipetted into a clean test tube and samples were measured on an LS 50B luminescence spectrometer (Perkin-Elmer, UK) using an excitation wavelength of 485 (5 nm bandpass) and an emission wavelength of 535 (10 nm bandpass) within 30–45 min.

3. Results

3.1. Circulation longevity of cholesterol-free liposomes

Given that the pharmacokinetic behavior of an encapsulated drug will be dependent on the pharmacokinetic characteristics of the drug carrier, studies were completed to assess circulation longevity of various liposome formulations. The cholesterol-free liposome formulation composed of DSPC:DSPE-PEG_{2000} (95:5 molar ratio) was compared with cholesterol-containing formulations consisting of DSPC:CH (55:45) and DSPC:CH:DSPE-PEG_{2000} (50:45:5). Each liposome formulation was injected intravenously into the lateral tail vein of mice and at various time points aliquots of EDTA prepared plasma were analyzed to determine total lipid/ml plasma. As shown in Fig. 1, DSPC liposomes were rapidly eliminated from the circulation with less than 6% of the injected dose (<0.2 μmole/ml plasma) present at 1 h post administration and an estimated mean area under the curve (AUC_{0-24h}) of 2.38 μmole h/ml. Inclusion of cholesterol into the membrane resulted in a 10-fold increase in the mean AUC_{0-24h}; however, at 24 h post administration less than 1% of the injected dose remained in the plasma compartment. Inclusion of 5 mole% DSPE-PEG_{2000} into the DSPC:CH formulation resulted in a 2-fold increase in the mean AUC in comparison to DSPC:CH (55:45), a result which is consistent with many other reports demonstrating that incorporation of PEG modified lipids can enhance the circulation lifetime of liposomes [23]. Inclusion of 5 mole% DSPE-PEG_{2000} into DSPC liposomes without cholesterol engendered significant increases in circulation lifetimes when compared to DSPC formulations without stabilizing lipids. There was a 19-fold increase in mean AUC_{0-24h}, and 29% of the injected dose was present in the circulation after 24 h. This result demonstrated that polyethylene glycol conjugated lipids are an essential component of cholesterol-free lipo-

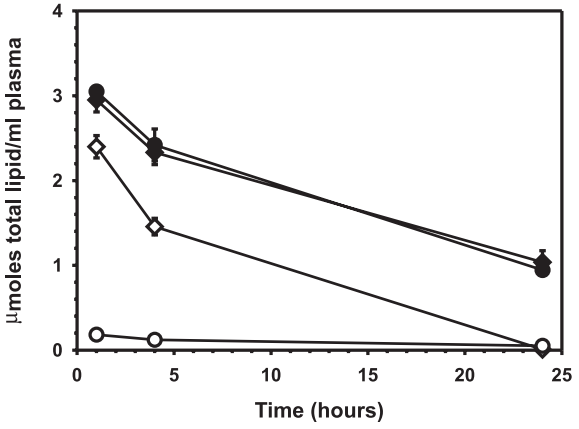

Fig. 1. Elimination of cholesterol-free liposomes from the circulation. Large unilamellar liposomes radiolabeled with [^{3}H]CHE were administered intravenously via the dorsal tail vein of female Balb/c mice at an approximate dose of 150 μmoles/kg total lipid. Blood was collected at 1, 4 and 24 h by tail nick and cardiac puncture procedures, respectively. An aliquot of plasma was used to determine liposomal lipid content as described in Section 2. ●, DSPC:DSPE-PEG_{2000} (95:5 molar ratio); ◆, DSPC:CH:DSPE-PEG_{2000} (50:45:5); ◇, DSPC:CH (55:45); ○, DSPC (100). Each data point represents the average lipid plasma concentration ±S.D. for four mice.

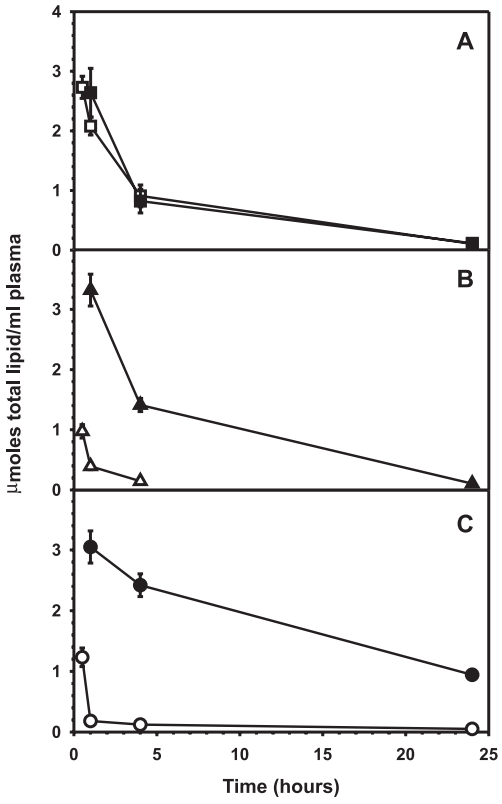

Fig. 2. Elimination of liposomes prepared using phosphatidylcholine species with varying acyl chain lengths in the absence and presence of PE-PEG_{2000}. Mice were injected with liposomes as described in Fig. 1. Liposomes of varying acyl chain lengths DSPC (Panel C), DPPC (Panel B) and DMPC (Panel A) both in the absence (open symbols) and the presence (closed symbols) of 5 mole% of PE-PEG_{2000} were evaluated. Each data point represents the average lipid plasma concentration ± S.D. for four mice.

somes if they are to be used as systemically viable drug carriers.

While characterizing cholesterol-free liposome formulations it was of importance to decide which formulation would be optimal for application as a delivery system for a relatively hydrophobic anticancer agent, such as IDA. The influence of 5 mole% phosphatidylethanolamine (PE)-PEG on the plasma elimination of liposomes containing various acyl chain lengths of phosphatidylcholine, DSPC (C:18), DPPC (C:16) and DMPC (C:14), was evaluated in Fig. 2. In these studies the acyl chain length of the corresponding PE conjugated to PEG contained the same number of carbons to ensure optimal mixing conditions; however, it is now well established that the short chain (C:14 and less) PEG modified PEs are rapidly exchanged out of liposomal membranes after i.v. administration [39,40]. As the acyl chain length of the phospholipid increased, a more significant difference in circulation longevity between phosphatidylcholine (PC) and PC:PE-PEG_{2000} (95:5) liposomes was observed. As suggested above, differences in PEG-lipid induced effects on the liposomal systems may be attributed to the phase state of the liposomes whereby a more liquid–crystalline phase lipid may facilitate rapid exchange of lipid components out of and into the liposomal membrane following i.v. injection. Although DMPC liposomes appear to be eliminated at the same rate as DMPC:DMPE-PEG_{2000} (95:5) liposomes, upon closer examination we have resolved that DMPC liposomes are not stable, a result that is consistent with previous observations [41]. Studies evaluating the retention of encapsulated [^{14}C]lactose indicated that both DMPC and DPPC liposomes lost encapsulated contents rapidly following injection but DSPC retained entrapped lactose over 48 h (data not shown).

3.2. *Influence of polyethylene glycol content and molecular weight on cholesterol-free liposome circulation longevity*

Previous studies have demonstrated that the elimination rate of cholesterol-containing systems increases following PEG incorporation, a result that is dependent on both PEG content and molecular weight [42–44]. As shown in Figs. 3 and 4, variations in PEG-lipid content from 2 to 15 mole%, and PEG molecular weight, from PEG_{750} to PEG_{5000}, had no significant impact on altering the plasma elimination circulation longevity of DSPC liposomes. It should be noted that as PEG concentration was increased to levels in excess of 15 mole% in DSPC liposomes, the solution became clear indicative of non-liposomal micelles or bilayer disc formation [45], a result which is consistent with other reports [46,47]. Furthermore, in the studies reported here it was established that the DSPC:PEG_{2000} formulations with PEG-lipid content up to 15 mole% existed as a single liposome population, but the 20 mole% PEG-lipid formulation existed as two distinct populations as judged by cryo-TEM analysis and fractionation on Sepharose CL-4B (data not shown). When using DPPC, incorporation of 15 mole% PEG resulted in a formulation of a

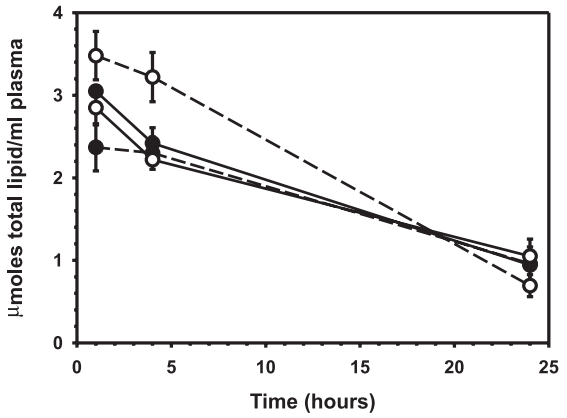

Fig. 3. Elimination of DSPC liposomes containing increasing mole% PE-PEG_{2000}. Mice were injected with liposomes as described in Fig. 1. DSPC liposomes containing 2 (– – –●– – –), 5 (——●——), 10 (——○——), and 15 (– – –○– – –) mole% PEG were evaluated. Each data point represents the average total lipid plasma concentration ± S.D. for four mice.

single population of bilayer disks. As suggested by the data in Fig. 3, even levels of PEG lipid as low as 2 mole% are adequate to maximize the circulation lifetime of these cholesterol-free formulations.

3.3. Optimal drug loading conditions for idarubicin

The primary purpose of evaluating the pharmacokinetic behavior of the cholesterol-free liposomes was to determine whether in the absence of cholesterol, the in vivo retention of drugs poorly retained by cholesterol-containing formulations could be improved. Since the drug retention attributes of anthracycline derivatives may be correlated to their hydrophobicity, we chose to assess the use of DSPC:PEG_{2000} liposomes to enhance the drug retention of one of the most hydrophobic anthracyclines, idarubicin [48]. A liposomal formulation of IDA displaying enhanced drug circulation lifetimes has not been obtained to date, presumably because the IDA is rapidly released from cholesterol-containing systems. The first step required evaluating the influence of cholesterol on IDA release involved preparation of drug loaded formulations. The studies described here have used the well established pH gradient based loading technique for anthracyclines. In partic-

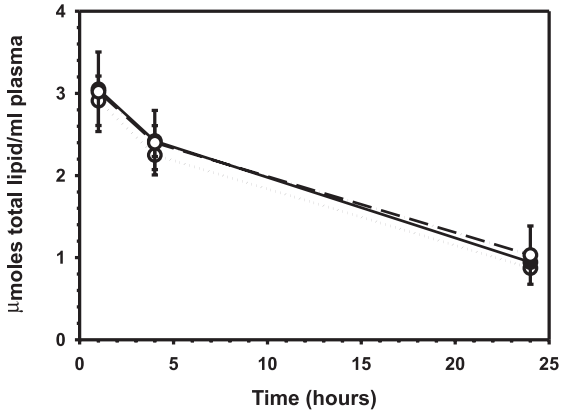

Fig. 4. Elimination of DSPC liposomes containing varying molecular weights of 5 mole% DSPE-PEG. Mice were injected with liposomes as described in Fig. 1. DSPC liposomes containing 5 mole% DSPE-PEG where the PEG molecular weight was 750 (···○···), 2000 (——●——) and 5000 (– – –○– – –). Each data point represents the average total lipid plasma concentration ± S.D. for four mice.

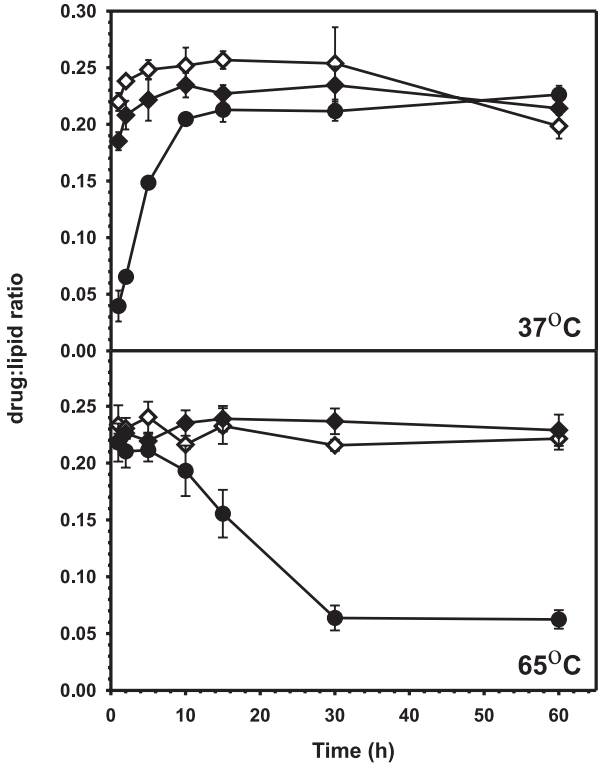

Fig. 5. Idarubicin loading into liposomes. Liposomes (5 mM) were incubated with 1 mM idarubicin (drug:lipid ratio 0.2) at 37°C and 65°C. At various time points, 100 μl of the incubating mixture were run down mini Sephadex G-50 spin columns and subsequently analyzed for lipid and drug concentrations by liquid scintillation counting and a standard anthracycline absorbance assay as described in Section 2. Drug loading was compared between DSPC:DSPE-PEG_{2000} (95:5 mol/mol) (●), DSPC:CH (55:45 mol/mol) (◇) and DSPC:CH:DSPE PEG_{2000} (50:45:5 mol/mol/mol) (♦) liposomes.

ular IDA was loaded at 37°C and 65°C into liposomes exhibiting an approx. 3.5 unit transmembrane pH gradient. As indicated in Fig. 5, IDA displayed optimal loading in cholesterol-free liposomes at 37°C. The accumulation of drug into the liposomes was rapid, with $>95\%$ encapsulation observed in 15 min, after drug addition. In comparison, no difference was observed in DSPC:CH liposomes loading at 37°C and 65°C.

3.4. Evaluation of liposomal idarubicin by cryo-transmission electron microscopy

Previous studies have explored the structure of doxorubicin within liposomes, linking the formation of citrate doxorubicin precipitates to improved retention [49]. To assess the physical state of encapsulated IDA, cryo-electron microscopy was used. 'Empty' and drug loaded DSPC:DSPE-PEG_{2000} (95:5 mol/mol) liposomes were analyzed and the resulting cryo-TEM images have been summarized in Fig. 6. As shown by the representative micrographs in Fig. 6 panels A and C, there was an observed difference in structure between 'empty' cholesterol-free and cholesterol-containing liposomes. Several DSPC:DSPE-PEG_{2000} (95:5 mol/mol) liposomes within the field of view have angular surface features whereas DSPC:CH:DSPE-PEG_{2000} (50:45:5 mol/mol/mol) liposomes consisted primarily as smooth and rounded membranes. In both cholesterol-free (Fig. 6B) and cholesterol-containing liposomes (Fig. 6D) with encapsulated IDA, a precipitate was evident inside the liposomes, resulting in the 'coffee bean' structure observed by others for liposomal formulations of doxorubicin. Although these cryo-TEM images do not eliminate the possibility of IDA interaction with the lipid membrane, it can be concluded that both cholesterol-containing and cholesterol-free formulations have some of the entrapped IDA present as a precipitate in the aqueous core of the liposome.

3.5. Pharmacokinetic analysis of liposomal idarubicin

Pharmacokinetic studies were performed to determine the IDA retention attributes of the cholesterol-

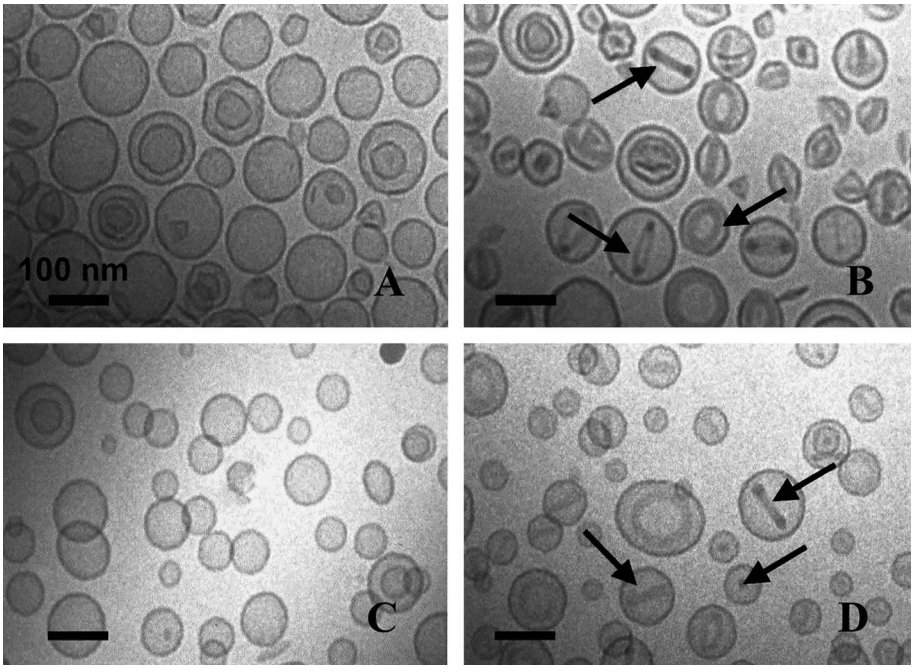

Fig. 6. Cryo-transmission electron micrographs of 'empty' and idarubicin-containing cholesterol-free and cholesterol-containing liposomes. DSPC:DSPE-PEG_{2000} (95:5 mol/mol) (A) and DSPC:CH:DSPE-PEG_{2000} (50:45:5 mol/mol/mol) (C) liposomes were prepared and cryo-TEM were obtained after establishment of a transmembrane pH gradient, but prior to drug loading. Cryo-electron micrographs of IDA loaded liposomes demonstrated precipitation (see arrows) of idarubicin in cholesterol-free (B) and cholesterol-containing liposomes (D). Bar represents 100 nm.

free formulation in vivo. Liposomes were prepared at a 0.2 drug to lipid ratio and subsequently injected i.v. into mice at a dose of 165 μmoles/kg lipid and 33 μmoles/kg IDA. The plasma elimination profile of IDA and lipid, as well as the calculated drug to lipid ratio in the plasma compartment are shown in Fig. 7. In the absence of a drug carrier, idarubicin is rapidly eliminated with $<3\%$ of the injected dose present after 15 min. This is in sharp contrast to the results obtained when IDA is administered encapsulated in DSPC:DSPE PEG_{2000} (95:5 mol/mol ratio). The AUC_{0-4h} for free IDA was 0.03 μmole h/ml in comparison to 1.38 μmole h/ml for cholesterol-free liposomes. The greatest retention of IDA was achieved using cholesterol-free liposomes, resulting in an AUC_{0-4h} that was 45-fold higher than for free IDA. The lipid elimination profiles are consistent with the data shown in Figs. 1 and 2, suggesting that unlike vincristine and doxorubicin [49,50], IDA encapsulation does not cause a significant change in liposome elimination. The calculated changes in drug to lipid ratios indicate that the drug retention attributes of the cholesterol-free formulation are the best and the data support our contention that cholesterol-free liposomes provide a format to be used to deliver drugs not well retained in cholesterol-containing liposomes.

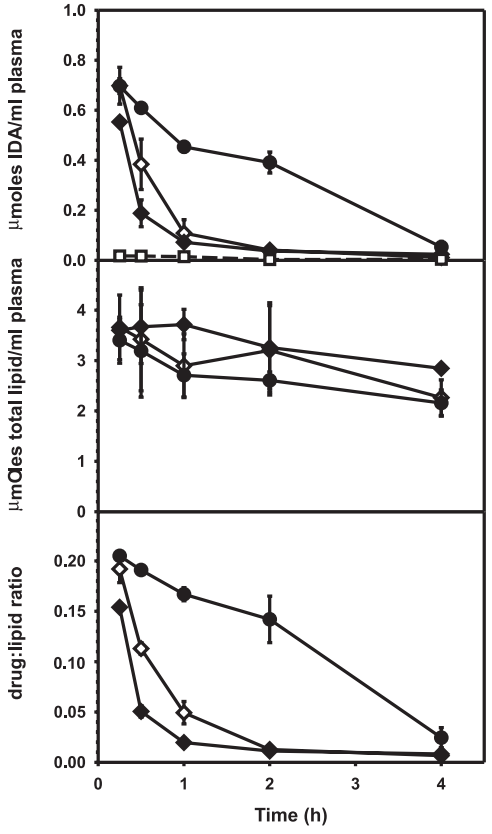

Fig. 7. Plasma elimination of liposomal idarubicin following i.v. injection of cholesterol-free and cholesterol-containing liposomes. Large unilamellar liposomes radiolabeled with [^{3}H]CHE were administered intravenously via the dorsal tail vein of female Balb/c mice at an approximate dose of 165 μmoles/kg total lipid and 33 μmoles/kg IDA. Blood was collected at 0.25, 0.5, 1, 2 and 4 h. Plasma was prepared and aliquots were assayed for lipid and IDA concentration as described in Section 2. Prolonged circulation longevity of IDA was observed in DSPC:DSPE-PEG_{2000} (95:5) (●) in comparison to DSPC:CH:DSPE-PEG_{2000} (50:45:5) (◆), DSPC:CH (55:45) (◇), and free IDA (□). Each data point represents the average total lipid plasma concentration ± S.D. for four mice.

4. Discussion

These studies have focused on cholesterol-free liposome delivery systems, with the aim of establishing their utility for delivery of hydrophobic drugs, such as idarubicin. Prior to establishment of liposome formulations designed for drug delivery applications [7,13,51,52], cholesterol-free vesicles were the standard model for biological membranes. Thus there is a great deal of existing literature on the in vitro physical and chemical properties of liposomes prepared without cholesterol. We do believe that this existing literature provides a solid foundation on which to support the development of cholesterol-free liposomes as intravenous delivery systems. Despite extensive studies of cholesterol-free formulations, there has been little emphasis on their application as drug carriers other than DPPC formulations being considered as thermosensitive formulations [53–55]. Others have focused on the physico-chemical and biological attributes of cholesterol-free liposomes including phase transition temperature determination by differential scanning calorimetry [47], X-ray diffraction [56], and protein binding [57,58], permeability [59] and pharmacokinetic studies [19,42]. Collectively these studies provide conclusive evidence that cholesterol-free liposomes have distinctive properties that may be beneficial for drug carriers. However, when this information has been applied to drugs, such as doxorubicin, the cholesterol-free formulation, even when stabilized by PEG-lipid incor-

poration, exhibit poor drug retention when compared to cholesterol-containing formulations [55]. We believe that this is due, in part, to the chemical attributes of the drug used and the phase transition temperature of DPPC (T_c ~39°C). For a temperature sensitive carrier, it may be advantageous to select lipids that have a characteristic T_c just above body temperature, considering that lipids become more permeable near their phase transition temperature. Therefore selection of lipids with a higher T_c than 40°C may facilitate greater retention of entrapped solutes. We believe that this report provides, for the first time, evidence that cholesterol-free liposomes can exhibit improved drug retention attributes, thus providing the opportunity to develop such formulations for drugs that are poorly retained in cholesterol-containing liposomes.

Within the context of this paper, a direct comparison of cholesterol-free DSPC:DSPE-PEG_{2000} (95:5 mol/mol) liposomes with other successful drug carrier formulations including conventional DSPC:CH (55:45 mol/mol) and sterically stabilized DSPC:CH:DSPE-PEG_{2000} (50:45:5 mol/mol/mol) liposomes is provided. There are two important observations pertaining to cholesterol-free liposomes that warrant further discussion. First and foremost, PEG is an essential component of cholesterol-free liposomes. Its presence engenders enhanced circulation longevity, apparently in a manner that is independent of PEG concentration and molecular weight. Secondly, idarubicin encapsulated in cholesterol-free liposomes demonstrated greater retention in vivo, independent of the formation of a precipitate structure within liposomes.

Our results indicate that DSPC:DSPE-PEG_{2000} (95:5 mol/mol) liposomes exhibit circulation lifetimes comparable to sterically stabilized liposome formulations (DSPC:CH:DSPE-PEG_{2000}; 50:45:5 mol/mol/mol). Woodle et al. have also demonstrated that PEG-PE:PC:CH (1:10:5 molar ratio) and PEG-PE:PC (0.15:0.85 molar ratio) liposomes exhibited similar circulation lifetimes, with circulation half-lives of 15.8 h and 14.9 h, respectively [23]. In the absence of surface grafted PEGs, cholesterol-free DSPC liposomes were rapidly eliminated, an observation that is likely due to either protein binding or liposome aggregation. As noted during the course of these studies, both DPPC and DSPC liposomes aggregate rapidly when cooled to a temperature below the T_c of the acyl chain. In fact, in order to investigate the properties of pure PC systems, at least 0.5 mole% PEG_{2000}-PE is required. Importantly, the circulation longevity of cholesterol-free DSPC:DSPE-PEG_{2000} liposomes following i.v. administration was not influenced by the amount of PEG modified lipid (2–15 mole%), or the PEG molecular weight (750–5000). This is in contrast to previous studies investigating cholesterol-containing liposomes where PEG content and molecular weight are important considerations when optimizing the circulation lifetime of these liposomes [44,60].

In view of the unique observations obtained when using cholesterol-free liposomes, we postulate that the incorporation of PEG modified lipid is primarily responsible for minimizing liposome–liposome aggregation, although we cannot be certain that the surface grafted PEGs inhibit protein binding and this effect may be different when considering their behavior in cholesterol-free as compared to cholesterol-containing liposomes. Others have demonstrated that cholesterol is required to maintain stability of liposomes in the plasma compartment [7,13,51,61]. Many of these studies were completed with small unilamellar liposomes prepared using lipids that exhibited T_c below 37°C, and thus were likely in a fluid phase when injected into mammals. Bedu-Addo et al. and others have demonstrated that cholesterol-free liposomes exhibit a phase transition temperature [47], but this transition broadens and becomes difficult to measure when the cholesterol level increases [62]. When using liposomes prepared of defined acyl length PCs, heating them above the phase transition temperature and subsequent cooling them below the T_c causes membrane defects (grain boundaries) to form. The appearance of defects is clearly evident in the cryo-TEM shown in Fig. 6 and these membrane defects are believed to be the source of non-specific protein binding [61] which, in turn, may define whether the carrier is recognized by the cells of the MPS system. Addition of surface stabilizing compounds such as PEG may shield these defects from recognition by serum proteins, rendering them more stable than liposomes composed exclusively of saturated phospholipids. An inherent attribute of such a conclusion is that pure PC liposomes may display reduced protein binding attributes provided that the

membrane defects are shielded by PEGs. Our results suggest that this can be achieved for a broad range of PEG molecular weight species and surface grafting densities.

The entrapment of idarubicin into cholesterol-free DSPC:DSPE-PEG_{2000} liposomes with an established pH gradient proved to be as effective with cholesterol-free liposomes when compared to cholesterol-containing formulations. As expected, however, the drug loading attributes of the cholesterol-free formulation were more dependent on the temperature used for loading and IDA could not be encapsulated at 65°C in cholesterol-free liposomes, a temperature higher than its phase transition temperature. Similarly, Unezaki et al. loaded thermosensitive (cholesterol-free) DSPC:DPPC:DSPE-PEG (1:9:0.61 molar ratio) liposomes with doxorubicin by the remote loading procedure at 60°C for 10 min and only achieved 65% encapsulation efficiency. Reduced loading may be a consequence of membrane destabilization at temperatures above the T_c of the bulk membrane component. Our studies demonstrated that $>95\%$ IDA was loaded into the PEG-PE stabilized DSPC liposomes at 37°C. The ability to load IDA into liposomes at a lower temperature than the phase transition temperature may be directly attributed to IDA's hydrophobicity [63]. Consistent with cholesterol-containing liposomes, drug loading for the cholesterol-free liposomes is dependent on liposome composition as well as the specific physico-chemical properties of the drug being used. Importantly, the cholesterol-free formulations may be particularly well suited for the more hydrophobic drugs. Woodle et al. hypothesized that by adding PEG to a membrane, one could eliminate the requirement of lipids with high phase transition temperature to allow greater control of leakage rates and other important bilayer properties [23]. Removing cholesterol from the membrane may facilitate even greater flexibility and control of drug leakage rates, when combined with the stabilization effects of PEGs.

Upon drug loading cryo-TEM images indicated that IDA was present in a precipitated form. This observation is consistent with other anthracyclines that have been encapsulated in liposomes by loading methods relying on the use of encapsulated citrate or ammonium sulfate [64]. It is interesting that Gallois et al. have specifically studied IDA's interaction with phospholipid membranes concluding that IDA embeds within the bilayer forming a complex with phosphatidic acid and cholesterol [65]. Considering that neither cholesterol nor phosphatidic acid are present within our liposomes, the remote loading procedure allows IDA to be present at concentrations high enough for the anthracyclines to stack and self-associate [49]. Self-association may be more energetically favorable than interactions dependent on membrane partitioning, although the membrane partitioning behavior of IDA may play a direct role in enhancing the drug retention attributes observed here for DSPC:PEG-PE formulations. As shown clearly in Fig. 6, IDA was present in a precipitated form within cholesterol-containing liposomes, as well as cholesterol-free liposomes. This result suggests that enhanced retention within cholesterol-free liposomes was not solely a consequence of precipitate formation. Our studies demonstrated that membrane composition also governs drug release kinetics, and we continue to believe that the most important factor governing the release characteristics of a liposomally encapsulated drug is lipid composition. As modeled in Fig. 8, the release of entrapped idarubicin (present in both free and precipitated forms) from the aqueous core of a liposome to the external environment is dependent on the partitioning behavior of the drug. This, in turn, is dependent on pH, membrane surface charge and the chemical attributes of the lipid acyl chains. Although our initial rationale for employing cholesterol-free liposomes for retention was simple, our model suggests that release due to interaction of the drug with components of the liposome is complex. For example, the chemical reaction used to prepare PEG modified phospholipids results in the generation of an anionic lipid from a zwitterionic lipid. The presence of this charged lipid will influence drug release properties, as noted by others [66], and this is presumably due to enhanced partitioning of the encapsulated drug. Drug partitioning behavior will be dependent on a number of processes which are dictated, in part, by the equilibrium between protonated and unprotonated drug forms as they transfer from the precipitated complex trapped in the core of the liposome (see Fig. 6) through the bilayer interface and the bilayer itself. Although we have not

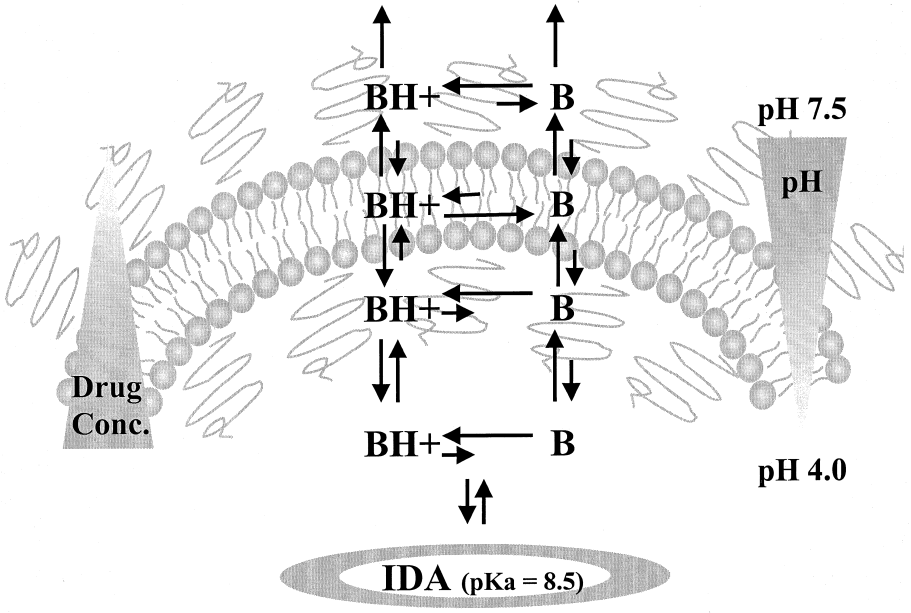

Fig. 8. Membrane partitioning of drugs encapsulated in liposomes through use of pH gradients and formation of a crystalline precipitate. IDA, protonated (BH+) and unprotonated (B) forms, are present in a dynamic equilibrium as they transfer from the precipitated complex trapped in the aqueous core of the liposome, through the bilayer interface and the bilayer itself. The release of IDA is dependent on the partitioning behavior of the drug, which is dependent on pH, membrane surface charge, and the chemical attributes of the lipid acyl chains. It is assumed that the rate limiting step in drug release is governed by permeation through the membrane rather than dissolution of the drug precipitate.

investigated the degree of partitioning of IDA within cholesterol-free and cholesterol-containing liposomes, we believe that membrane interactions are the most critical determinant of drug release.

In conclusion, we have demonstrated that cholesterol-free liposomes with surface grafted PEG may have unforeseen advantages over cholesterol-containing formulations. Inclusion of surface stabilizing components such as PEG eliminates the requirement of cholesterol within a membrane that exhibits very different drug release properties. In our case, we achieved enhanced retention of idarubicin, a hydrophobic agent readily released from conventional formulations. Future studies will focus on investigating drug release kinetics by altering loading parameters and PEG chemistry. In addition we will assess whether altering circulation longevity of IDA will impact the pharmacological properties of the drug in preclinical tumor models.

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