

## Influence of Drug-to-Lipid Ratio on Drug Release Properties and Liposome Integrity in Liposomal Doxorubicin Formulations

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*Recent studies have shown that the release properties of vincristine encapsulated in large unilamellar vesicles (LUV) can be regulated by varying the drug-to-lipid (D/L) ratio. In this work it is shown that the drug-to-lipid ratio technique for regulating drug release also applies to doxorubicin encapsulated in LUV. In particular it is shown that the half-times ( $T_{1/2}$ ) for doxorubicin release from distearoylphosphatidylcholine (DSPC)/cholesterol LUV in vitro can be increased more than six-fold by increasing the D/L ratio from 0.05 (wt/wt) to 0.39 (wt/wt). This behavior is consistent with the behavior expected for drugs that precipitate following accumulation into liposomes. It is shown that the release properties of ciprofloxacin—a drug that does not precipitate following accumulation into LUV—are not affected by the D/L ratio. It is also shown that the crystalline intravesicular doxorubicin precipitates observed as the D/L ratio is raised from 0.05 to 0.46 adopt increasingly unusual morphologies. Linear crystals are observed at lower D/L values, however triangular and rectangular variations are observed as the D/L ratio is increased, and induce considerable distortion in vesicle morphology. It is noted that trapping efficiency following uptake of external doxorubicin into LUV is reduced from nearly 100% at a D/L ratio of 0.05 (wt/wt) to less than 70% at an (initial) D/L ratio of 0.8 (wt/wt). It is suggested that this arises, at least in part, from membrane-disrupting effects of internal drug crystals as they increase in size.*

**Keywords** liposomes, drug precipitation, liposomal doxorubicin, drug loading

### Introduction

The utility of liposomal carrier systems in the treatment of malignancies has been clearly demonstrated in numerous preclinical and clinical studies (Balazsovits et al., 1989; Grunau et al., 1998; Hofheinz et al., 2005; Lim et al., 2000). The enhanced efficacy observed with liposomal systems containing antineoplastic agents can be attributed to a

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number of factors including reduced toxic side effects (Forssen and Tokes, 1981; Herman et al., 1983), accumulation at tumor sites (Gabizon, 1992), protection of encapsulated drug from degradation (Abraham et al., 2004a), and the ability to regulate drug release (Boman et al., 1998; Webb et al., 1995). Regulation of drug release is most critical for those agents that function in a cell-cycle-specific manner, such as the vinca alkaloids (Bruce et al., 1969; Horton et al., 1988). Many approaches that demonstrate differing drug release rates have been employed in the formulation of liposomal vincristine systems, including the use of different phospholipids, altering fatty acyl chain length, and saturation of the lipids (Boman et al., 1993), the inclusion of cholesterol (Mayer et al., 1989), and using temperature- or pH-sensitive lipids (Tomita et al., 1989; Ning et al., 1994; Zellmer and Cevc, 1996). Recently, studies have shown that inducing vincristine precipitation by loading liposomes at high drug-lipid ratios is also an effective means of regulating liposomal drug release, leading to optimized rates of drug release in human tumor models (Johnston et al., 2006). In vivo half-lives of release could be regulated in ESM/Chol liposomal formulations from hours (6.2h) to days (117h) by varying the drug-to-lipid ratio between 0.05 (wt/wt) and 0.6 (wt/wt). This phenomenon has been attributed to precipitation of the encapsulated vincristine at increasing drug-to-lipid ratios (Johnston et al., 2006).

The precipitation of certain drugs within liposomal formulations has been observed previously. Doxorubicin (Li et al., 1998), mitoxantrone (Almgren et al., 2000), and topotecan (Abraham et al., 2004a) form clearly defined linear bundle-like structures. Li and coworkers have extensively investigated the nature of doxorubicin precipitates within liposomes and have proposed that the drug forms fibers composed of stacked doxorubicin molecules (Li et al., 1998). These fibers are grouped into hexagonally arranged bundles aligned along the line of sight at rotation multiples of 60°, giving the appearance of repeating striations on the fiber bundle (Li et al., 1998). The vinca alkaloids (vincristine and vinorelbine) tend to form amorphous precipitates (Johnston et al., 2006; Semple et al., 2005; Zhigaltsev et al., 2005).

It has been noted for vincristine (Johnston et al., 2006) and topotecan (Abraham et al., 2004a), that increasing the drug-to-lipid ratio leads to increasing drug precipitation, which correlates with decreasing release rates of encapsulated drug. However, it was also observed that increasing initial drug-to-lipid ratios also leads to decreased loading efficiencies. Increasing the initial drug-to-lipid ratio from 0.3 (wt/wt) to 0.4 (wt/wt) decreased the loading efficiency by 30% when using an ionophore (MnSO<sub>4</sub>/A23187) loading method (Abraham et al., 2004a). For liposomal vincristine systems, a 45% decrease in loading efficiency was observed as the initial drug-to-lipid ratio was increased from 0.05 (wt/wt) to 1.6 (wt/wt) (Johnston et al., 2006).

In this present study we investigate the potential of modulating drug-to-lipid ratio as a method of regulating encapsulated drug release for doxorubicin, and further examine potential causes of the reduced loading efficiency observed when loading drugs at high drug-to-lipid ratios.

## Materials and Methods

### Materials

Distearoylphosphatidylcholine (DSPC) and cholesterol (Chol) were obtained from Sigma (St. Louis, MO) and egg sphingomyelin (ESM) was purchased from Northern Lipids (Vancouver, BC, Canada); all were used without further purification. [<sup>3</sup>H]-cholesterylhexadecyl ether (CHE) was obtained from Perkin Elmer Life Sciences

(Boston, MA). Vincristine sulfate was obtained from Fine Chemicals (Cape Town, South Africa) and ciprofloxacin hydrochloride was obtained from Bayer (Leverkusen, Germany). [ $^{14}\text{C}$ ]-vincristine sulfate was obtained from Chemsyn Laboratories (Lenexa, KS) and [ $^{14}\text{C}$ ]-ciprofloxacin hydrochloride was a gift from Inex Pharmaceuticals Corporation (Burnaby, BC, Canada). [ $^{14}\text{C}$ ]-sucrose was obtained from Amersham Biosciences (Arlington Heights, IL). Doxorubicin hydrochloride and all other chemicals were obtained from Sigma (St. Louis, MO).

### ***Liposome Preparation***

Lipids (ESM, DSPC or Chol) and trace amounts of [ $^3\text{H}$ ]-CHE were codissolved at appropriate molar ratios in ethanol. Multilamellar vesicle (MLV) suspensions were generated after the addition of a 300 mM aqueous solution of magnesium sulfate, yielding a final ethanol concentration of 10% (v/v). Large unilamellar vesicles (LUVs) were generated by extrusion of MLVs through two stacked Nuclepore polycarbonate filters with a pore size of 100 nm (10 passes) using an extrusion device obtained from Northern Lipids (Vancouver, BC, Canada) (Hope et al., 1985; Mayer et al., 1986). A transmembrane ion gradient was established through the removal of the external 300 mM magnesium sulfate, and remaining ethanol, by dialysis against SEH loading buffer (300 mM sucrose, 3 mM EDTA, 20 mM HEPES, pH 7.4). The mean diameters of the LUVs were determined by dynamic light scattering using a NICOMP 370 particle sizer (Nicom Particle Sizing Inc., Santa Barbara, CA) and found to be  $110 \pm 25$  nm. Phospholipid concentrations were determined using established techniques (Fiske and Subbarow, 1925) and the specific activity of the liposomes was determined using a Beckman LS3801 scintillation counter (Fullerton, CA).

### ***Drug Encapsulation in Liposomes***

Doxorubicin, vincristine, or ciprofloxacin were encapsulated using an ionophore-mediated drug loading procedure, as described previously (Fenske et al., 1998). Briefly, vincristine sulfate, doxorubicin hydrochloride, or ciprofloxacin hydrochloride were added to LUVs (2.5 mM final lipid concentration) at appropriate drug-to-lipid ratios (wt/wt), and subsequently pre-incubated at 65°C prior to the addition of the calcium ionophore A23187. Trace amounts of [ $^{14}\text{C}$ ]-vincristine or [ $^{14}\text{C}$ ]-ciprofloxacin were included where appropriate. The LUV/drug/ionophore mixture was then incubated at 65°C for up to 90 min. Non-encapsulated drug was removed using dialysis against SEH loading buffer (Fenske et al., 1998) for samples that were used for drug leakage assays, or 1 ml sephadex G50 chromatography spin columns for samples that were used to measure loading only. Quantification of liposome-entrapped drug was achieved via dual-label scintillation counting on a Beckman LS 3801 scintillation counter for vincristine- and ciprofloxacin-loaded liposomes. Quantification of doxorubicin-loaded liposomes was via [ $^3\text{H}$ ]-liquid scintillation counting and a doxorubicin absorbance assay. Briefly, this spectrophotometric assay involved the addition of Triton<sup>®</sup> 100 to liposomal doxorubicin to produce a 1% (v/v) final concentration of detergent. The liposomal drug/detergent was heated to 90°C for 30 s prior to reading at 480 nm on a UV160U Shimadzu spectrophotometer (Kyoto, Japan) and compared to a doxorubicin standard curve.

### ***In Vitro Drug Release***

In vitro drug-release assays were performed to make quantitative comparisons of drug leakage between formulations of varying drug-to-lipid ratios, and were conducted using

ammonium chloride to enhance the drug release rate (Maurer et al., 1998). Drug-loaded vesicles were diluted with release buffer (2 mM ammonium chloride, 300 mM sucrose, 20 mM HEPES, 3 mM EDTA, pH 7.4) to a lipid concentration of 1.25 mM. The diluted liposomal drug was then placed into dialysis tubing (12–14K MW cut off) and dialyzed against release buffer at 50°C. This temperature was chosen to provide an optimal and convenient in vitro drug leakage rate, generally 20–30% after 60 min for a drug-to-lipid ratio of 0.05 (wt/wt). Leakage of doxorubicin or ciprofloxacin from the loaded LUVs was assayed by the removal of aliquots for spin column analysis, with lipid quantified using liquid scintillation counting and doxorubicin with the spectrophotometric assay previously described (Fenske et al., 1998; Mayer et al., 1990). Lipid and remaining encapsulated ciprofloxacin was quantified via dual-label scintillation counting. The half-time for drug release ( $T_{1/2}$ ) was calculated from the release profiles as the time at which the internal drug concentration was half the initial concentration at time zero.

### ***Cryo-Transmission Electron Microscopy***

Cryogenic transmission electron microscopy (cryo-TEM) was performed on empty and drug-loaded SM/Chol liposomes using a Zeiss EM 902A transmission electron microscope (LEO Electron Microscopy, Oberkochen, Germany) operated at 80kV in the zero-loss bright-field mode. Digital images were recorded under low-dose conditions with a BioVision Pro-SM Slow Scan CCD camera (Proscan GmbH, Scheuring, Germany) and analySIS software (Soft Imaging System, GmbH, Münster, Germany). In order to visualize maximum detail, an underfocus of 1–2  $\mu\text{m}$  was used to enhance the image contrast. Sample preparation was performed at 25°C and approximately 99% relative humidity within a climate chamber. A small drop (~2  $\mu\text{l}$ ) of sample was deposited on a copper grid covered with a perforated polymer film coated with carbon on both sides. Excess liquid was removed by blotting with filter paper, leaving a thin film of the solution on the grid. Immediately after blotting, the sample was vitrified by plunging the grid into liquid ethane held at  $-182^\circ\text{C}$ . Samples were maintained below  $-165^\circ\text{C}$  and protected against atmospheric conditions during both transfer to the TEM and examination.

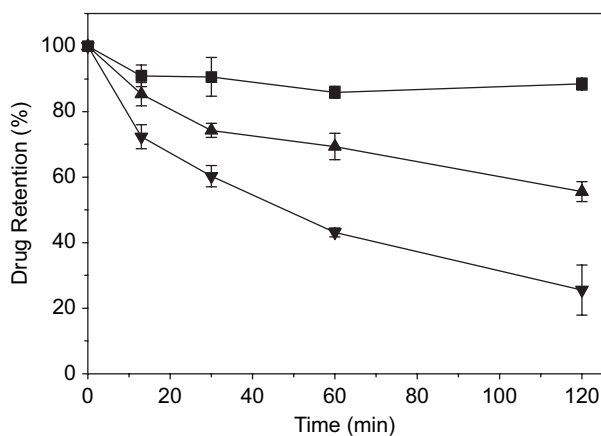
### ***Assessing Membrane Integrity Following Drug Accumulation***

To assess the potential of doxorubicin precipitation and fiber bundle formation to rupture liposomes and reduce loading efficiency, DSPC/Chol (55/45 mol%) liposomes were produced, as described previously, containing a trace amount of [ $^{14}\text{C}$ ]-sucrose (3.38  $\mu\text{M}$ ). Doxorubicin was loaded using the ionophore method at a total lipid concentration at 2.5 mM. Unloaded doxorubicin and any released [ $^{14}\text{C}$ ]-sucrose were removed employing spin column chromatography. Quantification of remaining encapsulated [ $^{14}\text{C}$ ]-sucrose and [ $^3\text{H}$ ]-labeled CHE was performed using dual-label scintillation counting on a Beckman LS 3801 scintillation counter.

## **Results**

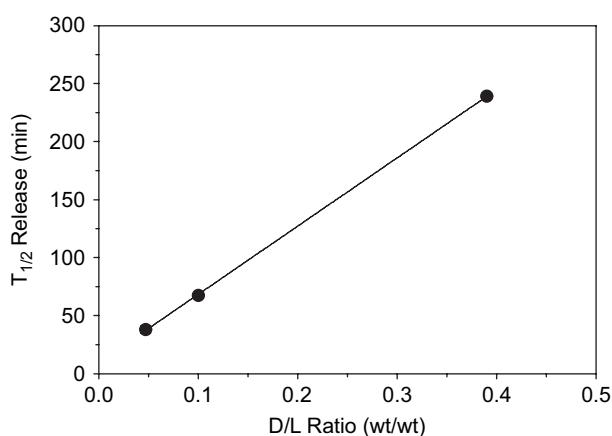
### ***The Half-Time of Doxorubicin Release from LUVs is Linearly Dependent on the Drug-to-Lipid Ratio***

The retention properties of doxorubicin in DSPC/Chol (55/45 mol%) liposomes at various D/L ratios were assessed using an in vitro release assay and are shown in Fig. 1.



**Figure 1.** In vitro drug retention for liposomal formulations of doxorubicin (DSPC/Chol-55/45 mol%) in 2 mM ammonium chloride at drug-to-lipid ratios (wt/wt) of 0.047 ( $\blacktriangledown$ ), 0.1 ( $\blacktriangle$ ), and 0.39 ( $\blacksquare$ ). Data points represent mean drug retention ( $\pm$  standard deviations) calculated from 3 samples.

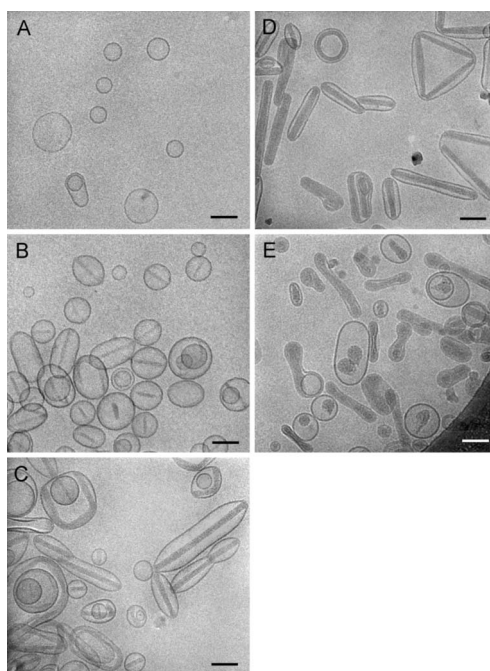
A dramatic enhancement of drug retention was observed as D/L ratios were increased from 0.047 (wt/wt) to approximately 0.39 (wt/wt) for liposomal doxorubicin. Over this range, the release half-life increased from approximately 38 min to 239 min, representing a more than 6-fold increase in drug retention. In an effort to confirm the mechanisms previously put forward to explain this phenomenon, the  $T_{1/2}$  values calculated from the release data were plotted as a function of initial D/L ratio. As shown in Fig. 2, a linear relationship is observed between the D/L ratio and the  $T_{1/2}$  values ( $R^2 = 0.999$ ), which is consistent with a large proportion of the encapsulated drug being in a precipitated (nonsoluble) form (Johnston et al., 2006).



**Figure 2.** Correlation of drug-to-lipid ratio and doxorubicin release half-life in ammonium chloride ( $R^2 = 0.999$ ). Half-lives were interpolated as described in Materials and Methods, and were 38, 67, and 239 min for D/L ratios of 0.047, 0.1, and 0.39, respectively, in vitro. Error bars represent the 95% confidence interval for the linear regression.

### ***Liposomes Containing High Levels of Doxorubicin Show Internal Morphology Consistent with Drug Precipitation***

As stated in the Introduction, previous cryo-transmission electron microscopy (cryo-TEM) studies of liposomal doxorubicin formulations have demonstrated the existence of linear precipitates of encapsulated drug resulting in a “coffee bean” shaped liposomal morphology. Therefore it is no surprise that when samples here were examined with cryo-TEM, increasingly linear structures and deformed liposomal morphology were observed with increasing D/L ratio. Representative images ( $n = 20$  per D/L ratio) from the cryo-TEM studies are shown in Fig. 3. What was unexpected however was the appearance of triangular and occasionally rectangular structures as the D/L ratio was increased (Fig. 3, panel D). Further, at the highest D/L ratio examined (D/L = 0.46 wt/wt) the defined linear structures were less apparent. At these D/L ratios the internal precipitate is similar in appearance to liposomal vincristine formulations at high drug-to-lipid ratios (Johnston et al., 2006), with the precipitated doxorubicin appearing to coalesce into an amorphous precipitate with no clearly defined structural organization (Fig. 3, panel E). However, a greater degree of liposomal deformation is observed with liposomal doxorubicin as compared to liposomal vincristine.



**Figure 3.** Cryo-transmission electron microscopy of DSPC/Chol (55/45 mol%) liposomes containing doxorubicin at different drug-to-lipid ratios. Liposomes containing 300 mM internal magnesium were loaded with vincristine using the ionophore method as described in Materials and Methods. Panels represent empty liposomes (A) and D/L ratios (wt/wt) of 0.05 (B), 0.18 (C), 0.37 (D), and 0.46 (E). The bar in panels A to E represents 200 nm in all micrographs and all micrographs (A–E) are shown at the same magnification. Each panel is a representative image from at least 20 images per D/L ratio.

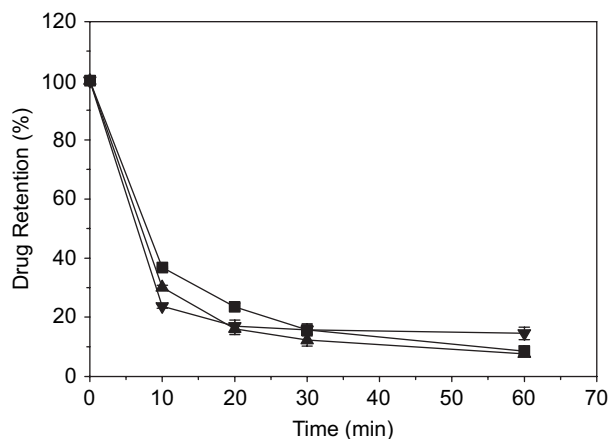
### ***Drug Precipitation is Necessary for Release Half-Life to be Dependent on Drug-to-Lipid Ratio***

Certain drugs, such as ciprofloxacin, do not precipitate inside liposomes even when loaded to extremely high D/L ratios (Maurer et al., 1998). For such drugs, increases in the D/L ratio would not be expected to result in reduced rates of drug leakage. In order to show that this is the case, the release properties of ciprofloxacin were investigated over a D/L range from 0.08 to 0.27 (wt/wt). As shown in Fig. 4 increasing the drug-to-lipid ratio over this range did not affect the halftime for drug release from ESM/Chol (55/45 mol%) LUV. The *in vitro* assay relying on the presence of 2 mM ammonium chloride (as described in Materials and Methods) to achieve release rates over an experimentally convenient timeframe was used in these experiments.

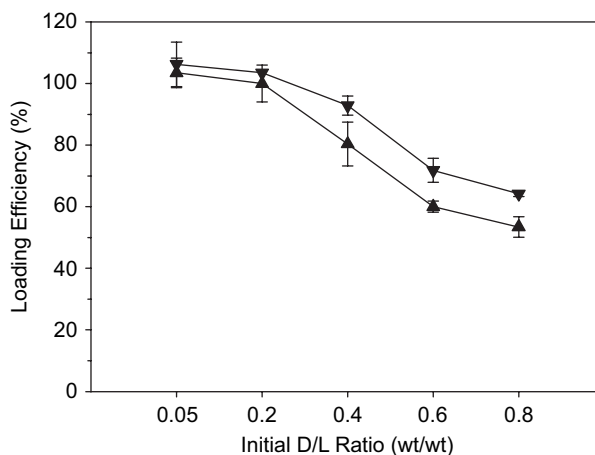
### ***Formulations with High Doxorubicin-to-Lipid Ratios Exhibit Reduced Trapping Efficiencies and Leakage of Internal Buffer During the Loading Process***

The morphological changes observed for doxorubicin-loaded liposomes as the D/L ratio is increased (Fig. 3) lead to questions regarding the influence of internal precipitated drug on the integrity of the surrounding liposome membrane. In particular, it appears possible that the drug crystals could disrupt the liposome bilayer at the higher D/L values. If this were the case the trapping efficiencies (expressed as the percentage of the initial external drug that is loaded into the liposomes) would be expected to decrease at the higher D/L values due to leakage of drug or  $Mg^{2+}$  arising from membrane disruption. As shown in Fig. 5 for doxorubicin, loading efficiencies are reduced from nearly 100% at an initial external drug-to-lipid ratio of 0.05 (wt/wt) to less than 70% at an initial external D/L ratio of 0.8 (wt/wt).

Reduced trapping efficiencies at high D/L ratios might be explained by a number of facts other than membrane disruption, including the possibility that the ion gradient giving rise to the pH gradient driving the uptake process is exhausted by the influx of high levels



**Figure 4.** *In vitro* drug retention for liposomal formulations of liposomal ciprofloxacin (ESM/Chol-55/45 mol%) in 2 mM ammonium chloride at drug-to-lipid ratios (wt/wt) of 0.08 (▼), 0.15 (▲), and 0.27 (■). Data points represent mean drug retention ( $\pm$  standard deviations) calculated from 3 samples.



**Figure 5.** Loading efficiencies for liposomal formulations of doxorubicin at increasing initial drug-to-lipid ratios for 15 minutes (▲) and 60 minutes (▼) using the ionophore method into liposomes containing 300 mM magnesium (DSPC/Chol 55/45 mol%). Data points represent mean loading efficiencies ( $\pm$  standard deviations) calculated from 3 samples.

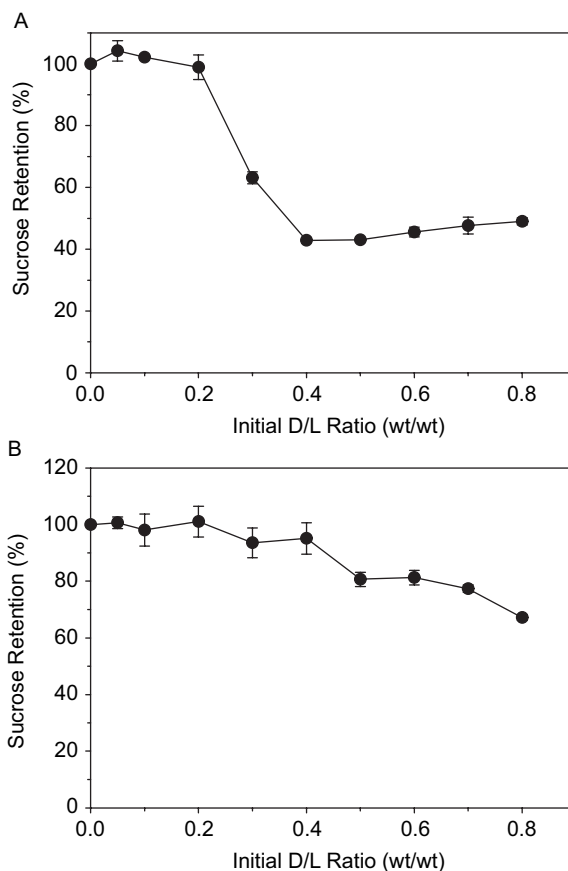
of (neutral) doxorubicin molecules, each of which is protonated on arrival in the liposome interior. In order to ascertain whether membrane disruption could account, at least in part, for the reduced trapping efficiencies observed at high D/L ratios, the effect of doxorubicin loading on release of a liposomally entrapped solute was investigated. Specifically, DSPC/Chol (55/45 mol%) liposomes were formulated containing trace amounts of [ $^{14}$ C]-labeled sucrose and trace amounts of labeled CHE as described in Materials and Methods. Doxorubicin was loaded into these liposomes employing the ionophore technique and exterior drug (and released sucrose) was removed following loading by spin-column chromatography. Measures of the labeled CHE to [ $^{14}$ C]-labeled sucrose ratio then allowed the percent release of entrapped solutes, as assayed by the release of sucrose, to be calculated. As shown in Fig. 6A, as the initial D/L ratio is increased from 0.2 to 0.4 more than 50% of the liposomally encapsulated sucrose was released during the loading process.

It was of interest to extend this approach to vincristine loading, a drug that can be loaded to achieve D/L ratios as high as one, but which does not give rise to the linear internal crystalline precipitates that are observed for doxorubicin (Johnston et al., 2006). Rather, an amorphous internal precipitate is observed, with some punctate precipitates at the highest D/L values. As shown in Fig. 6B, leakage of sucrose was also observed as vincristine was loaded at increasing initial drug-to-lipid ratios. However, the sucrose leakage was more gradual and less extensive than for doxorubicin, with only 33% sucrose release at the highest initial vincristine D/L ratio of 0.8.

## Discussion

This study demonstrates that varying the drug-to-lipid ratio, as a method for regulating drug release and ultimately obtaining therapeutically optimized liposomal drug formulations, is also applicable to liposomal drugs other than vincristine. There are three main points of interest. The first concerns the utility of varying the drug-to-lipid ratio to regulate doxorubicin release from DSPC/Chol (55/45 mol%) liposomes. The second involves the





**Figure 6.** Release of encapsulated sucrose from DSPC/Chol (55/45 mol %) liposomes when doxorubicin (A) and vincristine (B) are loaded at increasing initial D/L ratios (wt/wt). Samples were loaded at a total lipid concentration of 2.5 mM at 65°C with aliquots removed at appropriate time points and subjected to spin column chromatography and dual-label liquid scintillation counting.

mechanism by which variations in the drug-to-lipid ratio affects drug release. A final point concerns the reasons why reduced trapping efficiencies are seen when drugs are encapsulated at high drug-to-lipid ratios.

Our results demonstrate that varying the drug-to-lipid ratio provides an effective means for regulating drug release from liposomal doxorubicin formulations. As noted previously for vincristine (Johnston et al., 2006), the relative release rates determined employing the  $\text{NH}_4\text{Cl}$ -based *in vitro* assay accurately reflect the relative release rates *in vivo*. It is therefore likely that the ability to increase the half-times for drug release *in vitro* by a factor greater than 6 by changing the D/L ratio from 0.047 (wt/wt) to 0.38 (wt/wt) will result in a corresponding increase in the half-times for release *in vivo*. Studies conducted by Parr and coworkers indicated that a DSPC/Chol liposomal formulation of doxorubicin at a D/L ratio of 0.18 (wt/wt) possessed a  $T_{1/2}$  release for the encapsulated drug of approximately 5.9 days in Lewis Lung Carcinoma-bearing BDF1 mice (Parr et al., 1997). Based on data presented here, doubling the D/L ratio to 0.36 should increase the half-life of drug release to nearly 12 days. However, it should be noted that liposomal

doxorubicin formulations that demonstrate increased drug retention may not result in increased anti-tumor activity (as was observed for vincristine (Johnston et al., 2006)) due to the cell cycle independent activity of doxorubicin (Minotti et al., 2004). In this regard previous studies demonstrate that increasing the half-life of release from approximately 7h to over 10 days results in only a 50% increase in antitumor efficacy (Charrois and Allen, 2004).

The mechanism by which the drug-to-lipid ratio influences the release rate is of particular interest. If the efflux of drug from the liposome is governed by Fick's law and the traditional view of drug release, the efflux should be proportional to the drug encapsulated within the liposome and drug release, as a function of time, should be independent of the internal drug concentration. As shown here, this is the behavior exhibited by liposomal formulations of ciprofloxacin, a drug that does not precipitate in the liposome interior (Maurer et al., 1998). However, as shown previously for vincristine (Johnston et al., 2006; Zhigaltsev et al., 2005) and topotecan (Abraham et al., 2004a) and shown here for doxorubicin, the halftime for drug release is highly dependent on the D/L ratio. As indicated elsewhere (Johnston et al., 2006; Zhigaltsev et al., 2005), this behavior is consistent with the presence of drug precipitates in the liposome interior (Johnston et al., 2006; Zhigaltsev et al., 2005), a feature that has been observed for all three drugs. Assuming that soluble and precipitated forms of the drug are in equilibrium, the efflux rate will then be proportional to the concentration of the soluble form of the drug, which will remain constant until the precipitated form is dissolved. Thus the time taken for drug release will be proportional to the amount of drug in the precipitated form, as observed experimentally.

As seen here and in previous studies (Abraham et al., 2004a; Johnston et al., 2006) a decrease in trapping efficiency is observed as the drug is loaded to increasing drug-to-lipid ratios. A possible explanation for this is the physical disruption of the liposome bilayer by the growing drug precipitate, resulting in either the leakage of encapsulated drug and/or degradation of the Mg<sup>2+</sup> ion gradient giving rise to the pH gradient driving drug loading. Here we demonstrate that entrapped sucrose is released as doxorubicin is loaded at increasing drug-to-lipid ratios, indicating that the growing crystals lead to significant membrane disruption at higher D/L ratios above 0.2 (wt/wt). Leakage of sucrose was also observed when liposomal vincristine was loaded at increasing drug-to-lipid ratios but the extent of leakage was much lower. This may arise due to differences in the structures of precipitated doxorubicin and vincristine. In particular, the linear precipitates of doxorubicin seen at all drug-to-lipid ratios (Fig. 3) result in a very small radius of membrane curvature when they contact the liposome membrane, possibly resulting in reduced stability for that region of the bilayer. This could have at least two effects. First, at some point the surface tension experienced by the lipid bilayer could exceed some critical value, leading to membrane failure and contents leakage. Alternatively, regions of high membrane curvature have been associated with regions that are susceptible to membrane fusion events. Lipid membranes that display high membrane curvature have been shown to fuse more readily (Nir et al., 1982; Wilschut et al., 1981). Structures that could correspond to an intra-liposomal fusion event are present in the cryo-TEM micrographs presented here (see Fig. 3, Panel D), and may explain why some larger vesicles are seen at higher doxorubicin-to-lipid ratios. Vincristine on the other hand forms amorphous or gel-like precipitates with little liposomal deformation, even at drug-to-lipid ratios of 1 (wt/wt). This lack of membrane deformation may explain why vincristine loaded at increasing drug-to-lipid ratios does not induce membrane leakage to the same extent as doxorubicin.

In summary, the results presented here show that doxorubicin can be loaded into liposomes to achieve D/L values as high as 0.46 (wt/wt), that by varying the D/L values the rates of drug release from the liposomes can be varied over a wide range, and that this behavior is consistent with drug precipitation in the LUV interior. It is also shown that drug precipitation results in remarkable internal structures that lead to deformation and disruption of the liposomal membrane at D/L ratios above 0.2 (wt/wt).

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