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Factors influencing uptake and retention of amino-containing drugs in large unilamellar vesicles exhibiting transmembrane pH gradients

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Abstract

The level of uptake and retention of amino-containing drugs in large unilamellar vesicles (LUVs) following uptake in response to a transmembrane pH gradient (ΔpH) can vary dramatically depending on the drug. For example, the anticancer drugs doxorubicin and epirubicin can be readily retained, whereas the anticancer drug vincristine and the antibiotic ciprofloxacin tend to leak out rapidly. In this investigation, we examine the influence of the hydrophobicity of the entrapped amines (that induce the ΔpH) and the anionic lipid content of the LUV on drug retention. It is shown that entrapment of increasingly hydrophobic monoamines (methylamine to amylamine) all lead to an induced ΔpH of 3 units and essentially complete drug uptake under the conditions employed, but do not lead to improved retention of vincristine and ciprofloxacin. However, significantly improved retention could be achieved by substitution of the anionic lipid distearoylphosphatidylglycerol (DSPG) for distearoylphosphatidylcholine (DSPC) in the LUV bilayer. Further, it is shown that if the induced ΔpH is reduced to 1.4 units (driven by entrapped diamine) nearly 100% accumulation of doxorubicin and epirubicin could be achieved, whereas only 25% loading for vincristine and ciprofloxacin was observed. Taken together these results provide methodology for improving (weak base) drug retention in liposomes and indicate that drugs that can partition into the lipid bilayer exhibit improved uptake and retention characteristics.

Keywords: Liposome; Transmembrane pH gradient; Membrane partitioning

1. Introduction

Liposomal formulations of anticancer drugs, such as doxorubicin or vincristine, and antibiotics, such as ciprofloxacin, can result in improved therapeutic activity and reduced toxic side effects [1]. However, these drugs can exhibit significantly different uptake and retention properties in the large unilamellar vesicles (LUVs) usually employed for drug delivery. For example, both doxorubicin and vincristine can be readily accumulated into LUVs exhibiting a pH gradient [2,3], but vincristine leaks out much more rapidly than doxorubicin for LUVs with the same lipid composition [3]. Alternatively, ciprofloxacin can be accumulated in response to a pH gradient induced by entrapped ammonium sulfate [4] when the exterior pH is approximately 6, but leaks from

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these LUVs relatively quickly [5]. It is clearly important to be able to control the rate of leakage of drugs from LUV delivery systems, as LUVs which do not retain their contents in vivo cannot deliver them to target sites. As a result, the efficacy of liposomal formulations is highly sensitive to drug leakage rates. In the case of vincristine, for example, improved drug retention in LUVs dramatically improves antitumor activity [3].

In this work, we investigate two methods of regulating drug release from LUVs following uptake in response to ΔpH. The first involves the use of sulfate salts of alkyl amines in place of ammonium sulfate. The increased hydrophobicity of amines with longer alkyl chains may be hypothesized to result in higher levels of encapsulated amine and increased partitioning into the inner monolayer of the LUVs, resulting in an increased positive surface charge and improved retention of the positively charged drug. The second approach is based on a model of drug uptake in response to a pH gradient which suggests that increased partitioning of drugs in the inner monolayer is correlated with high levels of entrapped drug and improved retention properties [2,6]. Membranes composed of negatively charged lipids exhibit higher membrane–water partition coefficients for positively charged drugs, which should lead to improved retention properties according to this model.

It is shown that drug retention in LUVs containing alkylammonium sulfates of increasing hydrophobicity is not improved over LUVs containing ammonium sulfate. However, improved retention is observed in LUVs containing the negatively charged lipid phosphatidyl glycerol (PG). These results support the model suggesting higher membrane–water partition coefficients lead to improved drug uptake and retention properties.

2. Materials and methods

2.1. Preparation of LUVs

Distearoyl phosphatidylcholine (DSPC), dipalmitoyl PC (DPPC), palmitoyl-oleoyl PC (POPC) and egg sphingomyelin (SM) were purchased from Avanti Polar Lipids (Alabama, USA), and were greater than 99% pure. Distearoyl PG (DSPG) was purchased from Northern Lipids (Vancouver, Canada). Cholesterol and the alkylamines were obtained from Sigma (St. Louis, MO, USA). For PC/cholesterol vesicles the acyl chain composition of the LUVs had no major influence on drug uptake. The same results were obtained for all PC/cholesterol mixtures (DSPC/Chol, DPPC/cholesterol and POPC/Chol) and for SM/cholesterol with a molar ratio of 55:45. For the experimental data shown DSPC/cholesterol (55:45 mol/mol) vesicles were used. For improved retention of vincristine and ciprofloxacin the vesicle composition was DSPC/DSPG/cholesterol (40:15:45) or DSPG/cholesterol (55:45 mol/mol). DSPC/cholesterol and SM/cholesterol lipid mixtures were prepared by lyophilization from *t*-butanol, DSPC/DSPG/cholesterol was lyophilized from benzene/methanol (95:5 v/v) and DSPG/cholesterol was dried from chloroform at a total lipid concentration of 50 mg/ml. The lipid was labeled either with [^{14}C]cholesteryl hexadecyl ether or [^{3}H]cholesteryl hexadecyl ether. [^{14}C]Cholesteryl hexadecyl ether was custom synthesized by DuPont New England Nuclear (Boston, MA) and [^{3}H]cholesteryl hexadecyl ether was also purchased from DuPont New England Nuclear.

Multilamellar vesicles (MLVs) were formed by hydration of the lipid with a 300 mM aqueous solution of the alkylammonium sulfate salts followed by five freeze–thaw cycles. These MLVs were extruded 10 times [7] through an extrusion device obtained from Lipex Biomembranes (Vancouver, BC, Canada), fitted with two stacked Nuclepore polycarbonate filters with a pore size of 100 nm. The extruder was equilibrated to 60°C. The mean diameter of the vesicles was measured with dynamic light scattering (Nicomp, Particle Sizing Systems, Santa Barbara, CA) and found to be 115 ± 20 nm.

2.2. Synthesis of amino compounds and formation of the gradient

Alkylammonium sulfate salts were synthesized by the addition of sulfuric acid to an aqueous solution of the appropriate amine under cooling and vigorous stirring until the pH of the solution was neutral. The molar ratio of amine to sulfuric acid was 2:1 except for ethylenediammonium sulfate where it was 1:1. About 95% of the water was then removed using a

Brinkmann Rotavapor-R rotary evaporator at 50–60°C. The remaining water was removed azeotropically with absolute ethanol (five to six additions) on the rotovap, giving a wet salt precipitate. The salt was washed with anhydrous ethylether and the solvent was removed by filtration through a Buchner funnel. Residual solvent was removed under high vacuum overnight. Sulfate salts were identified by ^{13}C-NMR by comparison to the alkylammonium chloride salts. The latter were purchased from Sigma. Ammonium methylsulfate was prepared according to Werner [8].

An amine gradient was generated by exchanging the extravesicular solution with 150 mM NaCl, pH 7.4 or a 2-(*N*-morpholino)ethanesulfonic acid (MES)/NaCl buffer, 20/140 mM, pH 6.0 on Sephadex G-50 spin columns [9]. To show that this procedure removed the external amine quantitatively the external solution was fluorimetrically assayed for amine after the reaction with fluorescamine [10]. Gradients formed using packed columns of Sephadex G-50 (1.5×10 cm) gave similar results. In order to match physiological conditions, the extravesicular medium was about 300 mosmol/kg. The inside and the outside of the LUVs were therefore not osmotically balanced. Reduction of the amine concentration to 150 mM for monoamines instead of 300 mM and 300 mM EDAS instead of 600 mM had no major influence on the results.

Table 1 gives the names, abbreviations and the chemical formulas of all amines used. p*K* values are given [11]. The pH and the osmolarity of the 300 mM aqueous solutions were measured at least three times and the mean values are given.

2.3. pH gradient and drug uptake

The pH gradient was determined using [^{14}C]-methylamine and was performed as previously described [12].

Drugs were added to the liposome preparation (5 mM total lipid) to give a molar drug-to-lipid ratio of 0.2 for doxorubicin, epirubicin and ciprofloxacin and 0.03 for vincristine. The samples were incubated at 60°C. Aliquots were taken for determination of drug uptake at different time points. Unentrapped drug was removed by running the aliquots over Sephadex G-50 spin columns prior to detection of the liposomally entrapped drug. Doxorubicin and epirubicin concentrations were determined by measuring the absorption at 480 nm in a 1% Triton X-100 solution. Vincristine concentration was determined either from the absorption at 297 nm in 80% ethanol or from dual label scintillation counting using [^{3}H]vincristine. Ciprofloxacin concentrations were determined by dual label scintillation counting using [^{14}C]ciprofloxacin or, in some cases, from absorption readings at 273.5 nm after a Bligh–Dyer extraction [13] into 200 mM NaOH as aqueous phase. The results from UV spectrometry and radiolabel analysis were in very good agreement. The lipid concentration was determined from scintillation counting and phosphate assays.

Doxorubicin (adriamycin RDF), epirubicin (pharmorubicin RDF) and vincristine sulfate (vincasar PFS) were obtained from Pharmacia (Mississauga, Ont., Canada) and [^{3}H]vincristine sulfate was obtained from Amersham (Oakville, Ont., Canada). Ciprofloxacin and [^{14}C]ciprofloxacin were from Bayer

Table 1
Amine compounds encapsulated in liposomes

Name	Abbreviation	Formula	p*K*	pH[a]	Osmolarity[a]
Ammonium sulfate	AS	$(NH_4)_2SO_4$	9.2	5.1	622
Methylammonium sulfate	MAS	$(CH_3NH_3)_2SO_4$	10.7	4.1	663
Ethylammonium sulfate	EAS	$(CH_3CH_2NH_3)_2SO_4$	10.8	4.2	717
Diethylammonium sulfate	DEAS	$((CH_3CH_2)_2NH_2)_2SO_4$	11.2	5.0	748
Propylammonium sulfate	PAS	$(CH_3CH_2CH_2NH_3)_2SO_4$	10.8	5.1	666
Amylammonium sulfate	AAS	$(CH_3(CH_2)_4NH_3)_2SO_4$	10.6	6.2	752
Ethylenediammonium sulfate	EDAS	$[H_3NCH_2CH_2NH_3]SO_4$	7.4, 10.2	5.1	360
Ammonium methyl sulfate	AMS	$NH_4CH_3OSO_3$	–	5.4	536

[a]pH and osmolarity are given for 300 mM aqueous solutions of the respective sulfate salt.

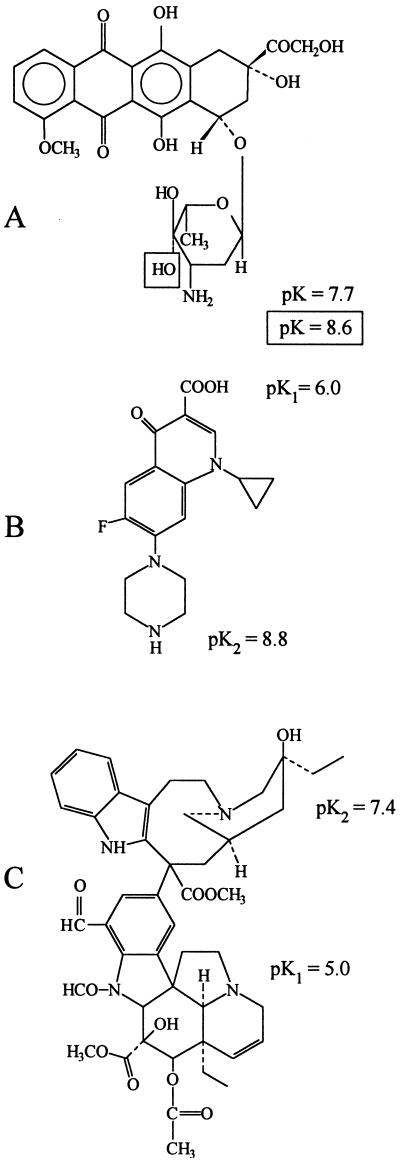

Fig. 1. Chemical structures of (A) epirubicin and doxorubicin (details circumscribed), (B) ciprofloxacin and (C) vincristine and their pK values. Doxorubicin and epirubicin have the same structure, but differ in their stereochemistry of the OH bearing C-4 of the sugar moiety. This has a strong influence on the pK value being increased for doxorubicin to pK = 8.6.

(Leverkusen, Germany). Fig. 1 shows the chemical structures of epirubicin and doxorubicin, ciprofloxacin and vincristine and their pK values. Doxorubicin differs from epirubicin only in the stereochemistry of the OH bearing C-4 on the amino sugar. The pK of doxorubicin is 8.6. Regarding the zwitterionic property of ciprofloxacin it is important to note that as the pK of the carboxyl function is rather high (pK_1 = 6.0) and the intraliposomal pH is very low the carboxyl groups are almost completely protonated. Thus the entrapped ciprofloxacin can be regarded as a weak base drug.

2.4. In vitro assay for drug retention

In vitro release experiments were carried out in test tubes by diluting samples after maximal drug uptake 1:1 (v/v) with normal mouse serum (Cedar Lane Laboratories, Hornby, Ont., Canada) followed by incubation at 37°C. Samples at various time points were taken and concentrations of entrapped drugs were determined as described for drug uptake experiments. For these experiments, the ratio of concentrations of mouse serum and total lipid is important [14]. A ratio of 1:10 (v/v) of mouse serum to sample liberated only 40% of encapsulated ciprofloxacin within 15 min compared to 90–95% with 1:1 mouse serum.

3. Results

3.1. Drug accumulation in response to monoamine sulfate gradients

It has previously been shown that when ammonium sulfate is entrapped within LUVs and exterior ammonium sulfate is removed, a transmembrane ΔpH is established that can drive the accumulation of drugs which are weak bases [4]. The ΔpH is set up due to the efflux of neutral ammonia molecules, each of which leaves a proton behind in the LUV interior, leading to a proton gradient according to the relation $[H^+]_{in}/[H^+]_{out} = [NH_4^+]_{in}/[NH_4^+]_{out}$. The mechanism of loading of drugs in response to a pH gradient is depicted in Fig. 2. In the present study, we hypothesized that sulfate salts of more hydrophobic amino compounds may lead to improved uptake and retention of weak base drugs. An increase in partitioning of the alkylamine into the inner monolayer of the LUVs may be expected to lead to higher levels of entrapped amino sulfate and the establishment of a positive surface charge at the inner monolayer interface, which may, in turn, inhibit efflux of the positively charged entrapped drug. In order to establish

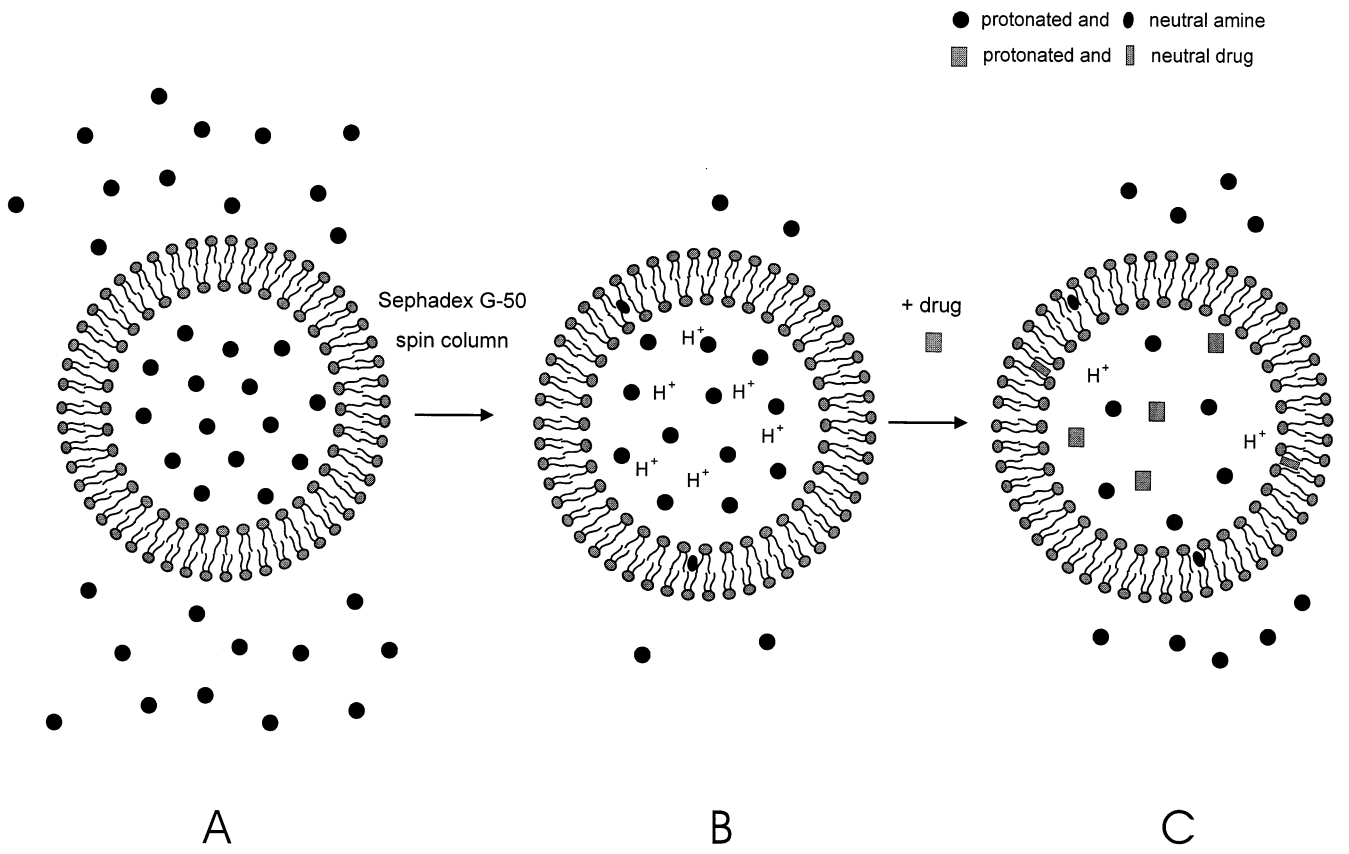

Fig. 2. Diagram of the mechanism for remote loading of drugs into LUVs comprising a ΔpH. (A) 100 nm LUVs were obtained by hydration of the dry lipid in 300 mM aqueous amine (●) solution and subsequent extrusion. (B) After exchange of the external amine with 150 mM NaCl or MES/NaCl buffer on a Sephadex G-50 spin column the amine equilibrates leading to a ΔpH (inside acidic) as the neutral form (black oval) crosses the bilayer. (C) The neutral form of the externally added drug (small gray rectangle) can cross the bilayer and is protonated (larger gray rectangle) and trapped inside the vesicles. Monobasic drugs exchange 1:1 with the amine while the ratio of amine/vincristine is 2:1.

that alkylamines with increased alkyl chain length show higher membrane–water partitioning, the fluorescence of the surface potential probe TNS was measured in the presence of amylammonium sulfate and methylammonium sulfate. 2-(*p*-Toluidino)naphthalene-6-sulfonic acid (TNS) fluorescence increased in the presence of amylammonium sulfate, but not methylammonium, consistent with a higher membrane–water partition coefficient for the more hydrophobic compound (results not shown).

A variety of alkylammonium sulfates were synthesized (see Table 1) and entrapped (300 mM) in DSPC/cholesterol (55/45 mol/mol) LUVs (100 nm diameter). After exchange of exterior alkylammonium sulfate for 150 mM NaCl pH 7.4 and incubation at 60°C for 15 min, a ΔpH of approximately 3 units was established, as shown in Fig. 3A. This ΔpH was the same for all alkylammonium sulfate salts employed, as assayed by the transbilayer distribution of trace levels of radiolabeled methylamine. This value of ΔpH corresponds well with the value expected on the basis of the transbilayer distribution of the alkylamine. In the case of entrapped methylammonium sulfate, for example, the methylamine concentration outside the LUVs after establishing the ΔpH was found to be 0.8 ± 0.05 mM and the internal methylamine concentration was 457 ± 50 mM, corresponding to a ΔpH of about 2.8 units. It should be noted that whereas other investigators [15] have found it necessary to reduce the pH of the ammonium sulfate solution to pH 2.5 in order to achieve good uptake of ciprofloxacin, no such adjustment in pH of the monoamines employed here was required.

Drug uptake also correlates well with the amount

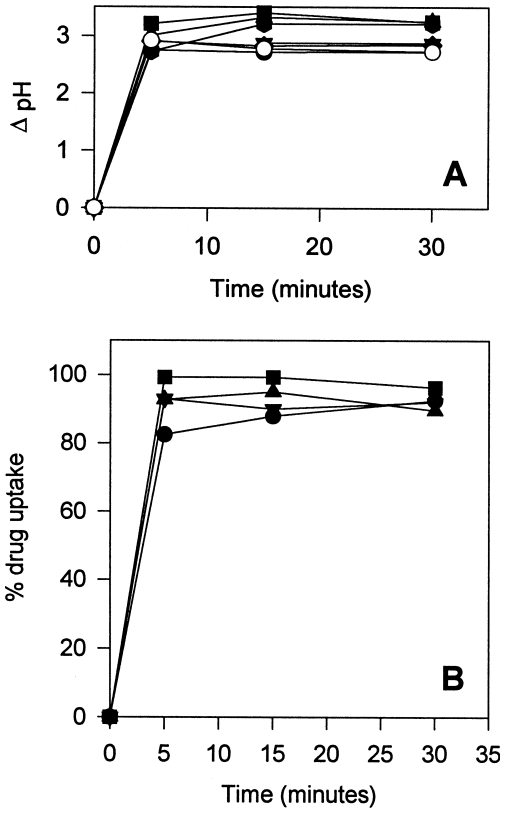

Fig. 3. (A) Formation of transmembrane pH gradient in 100 nm DSPC/cholesterol vesicles at 60°C subsequent to exchange of the external solution with 150 mM NaCl pH 7.4 and equilibration of the encapsulated amino compound, i.e. AS (●), MAS (■), EAS (△), DEAS (▲), PAS (▼), AAS (♦) and AMS (○). The equilibrium is reached when the inside and outside concentrations of the neutral form of the amino compound are equal, giving a ΔpH of 3 units as measured employing externally added [^{14}C]methylamine. (B) Comparison of the extent of drug uptake using encapsulated EAS. Experiments were conducted using 100 nm DSPC/cholesterol LUVs (55:45 mol/mol) at a total lipid concentration of 5 mM and drug-to-lipid ratios of 0.2 (mol/mol) for doxorubicin (●), epirubicin (■) and ciprofloxacin (▲) and 0.03 (mol/mol) for vincristine (▼).

of amine crossing the membrane in exchange for the drug. A previous report suggests an exchange ratio of 1:4 for doxorubicin and NH_4^+, concluding that loading was much lower than the loading capacity [4]. However, for a methylammonium sulfate gradient we found a 1:1 exchange of methylamine and drug for the monobasic drugs doxorubicin, epirubicin and ciprofloxacin while the ratio of vincristine influx to methylamine efflux was found to be about 1:2, consistent with the two basic groups of vincristine (data not shown). Thus efflux of entrapped amine is tightly coupled with drug influx.

The next set of experiments was aimed at determining the uptake and retention properties of doxorubicin, epirubicin, vincristine and ciprofloxacin for LUVs containing these alkylamines after establishing the pH gradient. As shown in Fig. 3B, all of these drugs exhibit 80% or higher loading levels after 5 min incubation (at 60°C) with DSPC/cholesterol LUVs (containing 300 mM ethylammonium sulfate) in the presence of drug at a drug to lipid ratio of 0.2 (mol/mol) for doxorubicin, epirubicin and ciprofloxacin and 0.03 (mol/mol) for vincristine. This behavior was found to be representative for all the alkylamines employed as very similar levels of drug uptake were observed for all other species.

The retention properties of doxorubicin, epirubicin, ciprofloxacin and vincristine following uptake into DSPC/cholesterol LUVs were also found to be independent of the type of alkylammonium sulfate used (results not shown). Retention was, however, highly dependent on the particular drug employed. As shown in Fig. 4, after uptake in response to entrapped methylammonium sulfate and gradient formation with 150 mM NaCl pH 7.4, on incubation at 37°C in the presence of 50% mouse serum, essentially all accumulated ciprofloxacin is released within 5 min

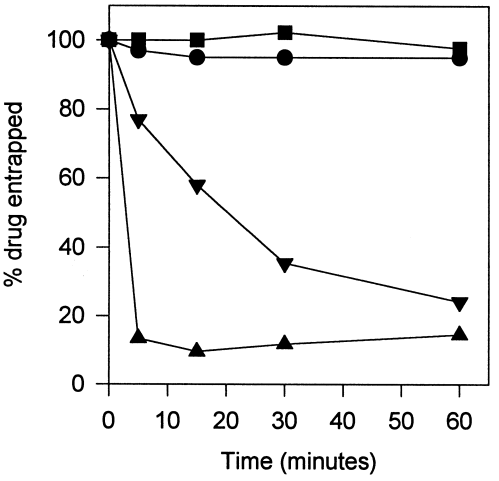

Fig. 4. Release of doxorubicin (●), epirubicin (■), ciprofloxacin (▲) and vincristine (▼) from 100 nm DSPC/cholesterol (55:45 mol/mol) vesicles (5 mM total lipid concentration) following loading under the conditions indicated in the legend to Fig. 3. Release was measured in the presence of 50% normal mouse serum at an incubation temperature of 37°C.

and only 25% vincristine remains encapsulated after 1 h. This behavior contrasts strongly with the retention properties of doxorubicin and epirubicin, which exhibit little or no leakage over the 1-h incubation time.

3.2. Drug accumulation in response to diamine sulfate gradients

It has been shown previously that more doxorubicin is accumulated into LUVs exhibiting a ΔpH than would be expected on the basis of the pH gradient alone [2]. This has been attributed to partitioning of accumulated doxorubicin into the inner monolayer, which would give rise to doxorubicin concentration gradients which are larger than the proton concentration gradients according to the equation:

$$\frac{[\text{Doxorubicin}]_i}{[\text{Doxorubicin}]_o} = (1 + K_p \frac{V_m}{V_i}) \frac{[H^+]_i}{[H^+]_o} \quad (1)$$

where K_p is the membrane–water partition coefficient of the drug, V_m is the volume of the LUV membrane and V_i is the volume of the aqueous interior. For a 100-nm-diameter LUV, $V_m/V_i \sim 0.33$ and thus for a membrane–water partition coefficient of ~ 70, as has been determined for doxorubicin [2], the concentration gradient of doxorubicin across the LUV membrane is approximately 20 times higher than would be predicted from the proton gradient. It is of interest to determine whether similar effects are observed for poorly retained drugs such as vincristine and ciprofloxacin so that their reduced membrane–water partition coefficients are related to the shorter retention times.

Accumulation in response to the entrapped monoamines, which give rise to an initial ΔpH of 3 units, results in nearly complete encapsulation of all the drugs employed under the conditions used here. At some smaller ΔpH it would be expected from Eq. 1, that drugs exhibiting large membrane–water partition coefficients should show improved uptake over those with smaller partition coefficients. In this regard, assuming the neutral form is membrane permeable, it is straightforward to show that the proton gradient resulting from an entrapped diamine will obey the relationship $[H^+]_{in}/[H^+]_{out} = ([\text{diamine}]_{in}/[\text{diamine}]_{out})^{1/2}$, and thus the ΔpH induced should be half that observed for the monoamines. As shown in Fig. 5A, the ΔpH induced in response to entrapped ethylenediammonium sulfate is 1.4 units, in good agreement with this prediction.

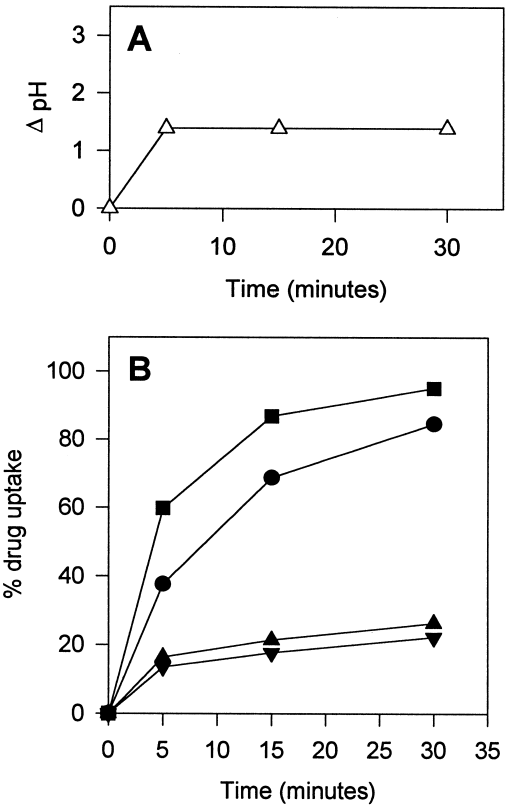

Fig. 5. (A) Formation of a transmembrane pH gradient in 100 nm DSPC/cholesterol (55:45 mol/mol) vesicles following entrapment of the diamine, EDAS. (B) Comparative uptake of doxorubicin (●), epirubicin (■), ciprofloxacin (▲) and vincristine (▼) into 100 nm DSPC/cholesterol (55:45 mol/mol) LUVs using 300 mM EDAS at 60°C for 30 min. More than 80% doxorubicin and epirubicin are accumulated in response to a pH gradient of 1.4 units, while less than 25% ciprofloxacin and vincristine is taken up.

The accumulation of doxorubicin, epirubicin, ciprofloxacin and vincristine in response to this reduced ΔpH is illustrated in Fig. 5B. It may be observed that whereas more than 80% of available doxorubicin and epirubicin are accumulated over the 30 min incubation period, less than 25% of the ciprofloxacin and vincristine is sequestered. This behavior is consistent with reduced membrane–water partition coefficients for ciprofloxacin and vincristine as compared to doxorubicin and epirubicin.

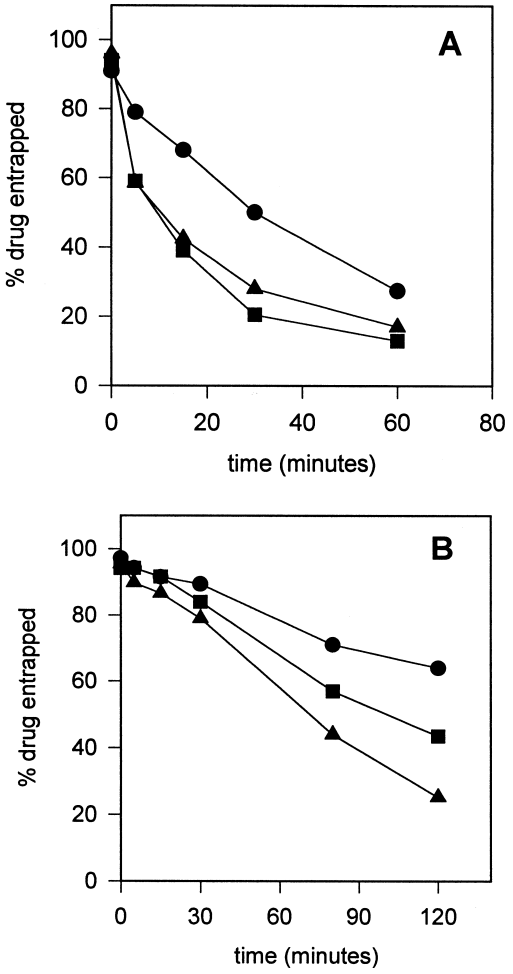

Fig. 6. Release of (A) ciprofloxacin and (B) vincristine from 100 nm DSPG/cholesterol (55:45 mol/mol) (●), DSPC/DSPG/cholesterol (40:15:45 mol/mol) (■) and DSPC/Cholesterol (55:45 mol/mol) (▲) vesicles (5 mM total lipid concentration). After exchange of the external MAS (300 mM) for MES/NaCl (20/150 mM, pH 6.0) a ΔpH of 3 units was driving uptake of ciprofloxacin (drug/lipid = 0.2 mol/mol) or vincristine (drug/lipid = 0.03 mol/mol). After 15 min of incubation at 60°C, uptake was essentially complete with close to 100% of the drug loaded. Release of ciprofloxacin and vincristine was measured in the presence of 50% normal mouse serum at 37°C following loading. Retention is significantly improved with PG-containing vesicles compared to PC-containing vesicles. Final pH_o after 2 h was 8.2.

3.3. Drug accumulation and retention in LUVs containing anionic lipids

It is well known that the membrane–water partition coefficients of charged molecules are highly sensitive to the charge on the membrane [16]. It would be expected, for example, that the use of negatively charged lipids in the LUVs experiencing a ΔpH would increase the membrane–water partition coefficients of the positively charged drugs employed here. In turn, according to Eq. 1, it would be expected that these drugs should exhibit improved retention properties in LUVs containing anionic lipids. In order to test this possibility, the retention properties of ciprofloxacin and vincristine were examined in LUVs containing varying amounts of the negatively charged phospholipid, distearoylphosphatidylglycerol (DSPG). Using a methylammonium sulfate gradient (external methylammonium sulfate was exchanged with MES/NaCl buffer at pH 6.0), essentially complete uptake of all drugs was reached after 15 min of incubation at 60°C.

As shown in Fig. 6A, the half-times for retention of ciprofloxacin in the presence of serum are increased from less than 10 min for DSPC/cholesterol (55:45 mol/mol) LUVs to approximately 30 min for DSPG/cholesterol (55:45 mol/mol) LUVs. Alternatively, for vincristine (Fig. 6B), the inclusion of as little as 15 mol% DSPG in the DSPC/cholesterol LUVs increases the retention half-times from 1 h to approximately 2 h, and considerably better retention is observed for the DSPG/cholesterol LUVs. The pH of the external serum solution rose to 8.2 during the course of this experiment. It should be noted that the improved drug retention for vincristine and ciprofloxacin with PG-containing vesicles compared to PC-containing vesicles was more pronounced at this higher external pH. Although a significantly higher amount of amine is associated with PG vesicles, about 30% more than with PC vesicles, control experiments showed that this does not influence retention. Uptake and retention of doxorubicin and epirubicin in PG containing vesicles was similar to that observed for neutral PC vesicles. Both drugs were readily taken up and did not leak out of vesicles diluted with mouse serum (1:1) during a 1-h incubation (data not shown).

4. Discussion

This study was performed to identify parameters which lead to enhanced retention of amino containing drugs in LUV systems exhibiting a transmembrane pH gradient (interior acidic). The results pre-

sented indicate that factors that lead to enhanced drug partitioning into the LUV lipid bilayer improve retention properties. The observations that support this conclusion include the lack of improvement in retention for uptake in response to entrapped hydrophobic alkylamines, the much higher uptake of doxorubicin and epirubicin versus ciprofloxacin and vincristine for LUVs containing diammonium sulfate and the improved retention of ciprofloxacin and vincristine in LUVs comprised, at least in part, of the negatively charged phospholipid, phosphatidylglycerol. Finally, the results presented here provide potential methods to improve drug retention and potency in vivo. We discuss these aspects in turn.

The fact that increased hydrophobicity of entrapped amino compounds does not affect the uptake and retention of the drugs employed here suggests that a positive surface charge on the inner monolayer–water interface does not influence the uptake and release of entrapped positively charged drug. The largest effects would be expected for entrapped amylammonium sulfate, where the hydrophobic pentyl chain should lead to the largest partitioning effects and the largest inner monolayer surface charge; however, retention of ciprofloxacin and vincristine on incubation with 50% mouse serum was not improved.

The uptake properties of doxorubicin and epirubicin versus vincristine and ciprofloxacin in response to the reduced ΔpH of 1.5 units achieved in response to entrapped diammonium sulfate are of particular interest. As indicated by Eq. 1, the ability of a drug to partition into the inner monolayer after uptake is expected to lead to equilibrium transmembrane concentrations of drug which exceed the transmembrane concentration of protons by the ratio $1+K_p V_m/V_i$, where K_p is the membrane–water partition coefficient. For drugs such as doxorubicin, for which $K_p \sim 70$ [2], this has the effect of causing much higher drug accumulation into LUVs than would be expected on the basis of the ΔpH. For example, for a drug with $K_p = 0$, the equilibrium transbilayer drug concentration gradient is 32 for a ΔpH of 1.5 units, whereas if $K_p = 70$ the equilibrium concentration gradient is about 760. Under the conditions employed for the results shown in Fig. 5 the expected encapsulation efficiency resulting from a ΔpH of 1.5 units would be 85% for $K_p = 70$ and only 19% for $K_p = 0$. As a result, the much larger accumulation of doxorubicin and epirubicin as compared to vincristine and ciprofloxacin for ΔpH = 1.5 (Fig. 4B) could be directly related to differences in the membrane–water partition coefficients of these drugs.

The improvement in retention for vincristine and ciprofloxacin entrapped in LUVs containing phosphatidylglycerol is also clearly consistent with the partitioning model. It should be noted that in the absence of membrane partitioning effects the presence of PG would be expected to increase, rather than decrease, membrane permeability. This is because negatively charged lipids generally give rise to more permeable bilayers than phosphatidylcholine bilayers with the same acyl chain composition [17] and also give rise to significantly higher binding of serum proteins [18], which strongly promote leakage.

It could be argued that the drugs with higher pK values should exhibit superior retention. Higher pK values will lead to lower concentrations of the deprotonated, membrane permeable, form of the drug. However, the rates of leakage do not correlate with the pK values of the drugs employed here. The pK values of the amino functions are 7.4 for vincristine, 7.7 for epirubicin, 8.6 for doxorubicin and 8.8 for ciprofloxacin (Fig. 1). Epirubicin and vincristine on the one hand and doxorubicin and ciprofloxacin on the other hand have very similar pK values. Their retention properties are, however, very different. While epirubicin and doxorubicin are readily retained, vincristine and ciprofloxacin leak out rapidly. This indicates that the different retention properties of the drugs are not related to their pK values.

The results presented here are also relevant to the difficulty in discerning between membrane partitioning and precipitation of accumulated drug in the LUV interior as the cause of drug encapsulation levels which exceed values expected on the basis of transmembrane pH gradients. As pointed out elsewhere, if all of the accumulated drug is in the aqueous interior the concentration of drug can exceed the solubility of the drug by orders of magnitude, potentially leading to drug precipitation in the vesicle interior [2,4,6]. This would have similar effects as drug partitioning into the membrane, as more drug would be present in the vesicle interior than would be expected on the basis of the ΔpH. The observation that improved retention is observed for the PG-contain-

ing LUVs is consistent with the partitioning model, and offers an interesting direction to pursue to resolve this area of contention.

A final point is that the results presented here demonstrating improved drug retention in vitro for vesicles containing negatively charged lipids, such as PG, offer a potential method for improving drug retention in vivo, which, for drugs such as vincristine, can result in significant improvements in efficacy. For example, methods resulting in improved retention of vincristine in liposomes in vitro resulted in improved retention in vivo [3] and dramatically enhanced efficacy in a murine P388 lymphocytic leukemia model [19]. Future studies will address the possibility that inclusion of negatively charged lipids in liposomes containing vincristine can offer similar benefits.

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