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The cationic lipid stearylamine reduces the permeability of the cationic drugs verapamil and prochlorperazine to lipid bilayers: implications for drug delivery

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Abstract

The therapeutic activity of a wide variety of drugs is significantly improved when their longevity in the circulation is extended by encapsulation in liposomes. To improve the retention of cationic drugs in liposomes, we have investigated the effect of the cationic lipid stearylamine on the permeability of the calcium channel blocker verapamil and the antipsychotic drug prochlorperazine, both of which are also multidrug resistance modulators. Both drugs were efficiently incorporated into liposomes composed of DSPC/cholesterol that possessed a transmembrane pH gradient (inside acidic). However, the efflux of the loaded drugs was relatively rapid (i.e., 50% of the encapsulated verapamil was released after 4 h at 37°C), despite the presence of a 3 unit pH gradient ($pH_i = 4.0$, $pH_o = 7.5$). Drug retention within the liposomes was improved by increasing the magnitude of the transmembrane pH gradient to approx. 5 units ($pH_i = 2.0$, $pH_o = 7.5$). Further improvements in drug retention were achieved by the addition of 10 mol% of the cationic lipid stearylamine in the DSPC/cholesterol liposomes. The combination of the 5 unit pH gradient and stearylamine resulted in increases of the retention of verapamil and prochlorperazine by approx. 20- and 5-fold, respectively. Calculation of the permeability coefficients for the charged (cationic) and neutral forms of the drugs indicated that the neutral forms of both drugs were approx. 10^4-fold more permeable than were the cationic forms of the drugs. Further, the presence of stearylamine reduced the permeability coefficient for the cationic species of the drugs by approximately an order of magnitude, but had no effect on the neutral species of the drugs. The efflux curves observed for both verapamil and prochlorperazine could be mathematically modeled by assuming that the primary influence of stearylamine was on the development of a positive surface charge density on the inner monolayer of the liposome. Taken in sum, these results indicate that stearylamine is effective at decreasing the leakage of cationic drugs from liposomes, and may prove to be a valuable component of liposomal drug formulations.

Keywords: Liposome; Drug delivery; Permeability; Cancer; Stearylamine; Multidrug resistance

1. Introduction

The clinical usefulness of a number of drugs is frequently limited by the occurrence of deleterious side effects at concentrations similar to, or lower than, those required for maximum therapeutic activity. The utility of such compounds may be significantly enhanced, and their toxic activities significantly reduced, by encapsulation within delivery systems such as liposomes. For example, encapsulation of the anticancer agents doxorubicin and vincristine within liposomes reduces the toxicities of these compounds and improves their antitumor efficacy [1–3]. It is likely that the therapeutic activity of liposome-encapsulated agents is due, in part, to accumulation of the carrier and encapsulated drug at the disease site [4]. Therefore, effective delivery of therapeutic drugs to selected tissues requires that the delivery vehicle does not allow significant leakage of the drug prior to its accumulation at the target site. Consequently, our optimization studies have focused on the development of liposomes possessing enhanced drug retention characteristics. Specifically, we have attempted to improve drug retention by determining some of the factors that influence the permeability of lipid bilayers to amphipathic amines.

Abbreviations: DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine.

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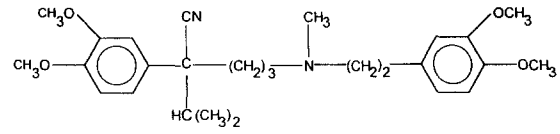
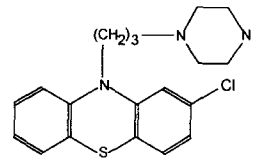
An example of a drug successfully encapsulated into well-characterized liposomal systems is the anticancer agent doxorubicin [5]. After uptake of doxorubicin into liposomes in response to a transmembrane pH gradient, the leakage of intraliposomal doxorubicin is very slow [6]. It is possible that the slow leakage of encapsulated doxorubicin is a consequence of both the partitioning of entrapped doxorubicin into the inner monolayer of the liposomal membrane [5] and the formation of intraliposomal aggregates [7]. However, other therapeutic compounds, such as vincristine, may not partition into the membrane or readily precipitate in the intraliposomal space and, therefore, are less likely to be as effectively retained within comparable liposomal systems. Indeed, a survey of a variety of drugs loaded by the transmembrane pH gradient method indicated that many compounds (including vincristine, vinblastine, quinidine, diphenhydramine, quinine and chloroquine) leak from liposomes during extended periods [8]. Therefore, it is likely that the low liposomal leakage observed for doxorubicin is an exception, and that rapid leakage of encapsulated drugs will pose a significant technical obstacle to the development of many liposomal drug formulations.

Many of the compounds possessing therapeutic activity are lipophilic or amphipathic amines. This class of compounds are weak bases and, therefore, can be trapped inside unilamellar liposomes by imposing a transmembrane pH gradient (inside acidic) such that the agents redistribute across the bilayer until the internal/external concentration gradient approximates the proton gradient. Previous studies have demonstrated that a decrease of the intraliposomal pH below 4.0 can increase the retention of these drugs [9]. We speculated that the leakage of encapsulated cationic drugs could be further reduced or precluded if the inner monolayer of the liposomes possessed a positive surface charge that would reduce cationic drug partitioning into the bilayer via electrostatic repulsion. It has been previously established that stearylamine quantitatively redistributes to the inner monolayer of a liposome in response to a transmembrane pH gradient, inside-acidic [10,11]. Therefore, in the present study we have utilized stearylamine to generate liposomes possessing a positive surface charge located on the inner monolayer.

This paper describes experiments examining the permeability of large unilamellar liposomes composed of distearoylphosphatidylcholine (DSPC) and cholesterol to the cationic drugs verapamil and prochlorperazine, and evaluates the relative contributions of the intraliposomal pH and surface charge on drug retention. Verapamil is an effective calcium channel blocker while prochlorperazine is used as an antipsychotic and neuroleptic agent. Both verapamil and prochlorperazine possess titratable tertiary amine functions (Fig. 1) and were expected to be readily encapsulated into liposomes possessing a transmembrane pH gradient. Verapamil and prochlorperazine were also of interest due to their ability to inhibit the activity of plasma membrane



Verapamil



Prochlorperazine

Fig. 1. Chemical structures of verapamil and prochlorperazine.

P-glycoprotein which is responsible for acquired and inherent resistance of some tumors to anticancer drugs such as doxorubicin and vincristine [12]. However, verapamil achieves up to 100% inhibition, in vitro, of P-glycoprotein at concentrations in the range of 2–10 μM but has serious cardiovascular side-effects, in vivo, at approx. 6 μM [12]. Therefore, the development of liposomal formulations of verapamil and prochlorperazine is expected to have significant therapeutic benefit in the treatment of tumors that are resistant to chemotherapy.

2. Materials and methods

2.1. Materials

Distearoylphosphatidylcholine (DSPC) was obtained from Avanti Polar Lipids. Verapamil (hydrochloride), prochlorperazine (edisylate salt) and all other chemicals were obtained from Sigma. *N*-[*methyl*-^{3}H]Verapamil, di[1-^{14}C]palmitoylphosphatidylcholine, [4-^{14}C]cholesteryl hexadecyl ether and [4-^{3}H]cholesteryl hexadecyl ether were obtained from New England Nuclear. [^{14}C]Methylamine was obtained from Amersham.

2.2. Liposome preparation

Dry lipids were weighed, dissolved in $CHCl_3$, and mixed in the molar ratios of DSPC/cholesterol (55:45) or DSPC/cholesterol/stearylamine (50:40:10). Typical preparations consisted of 100 mg of lipid and 0.5–1.0 μCi of either [^{14}C]DPPC, [^{3}H]cholesteryl hexadecyl ether or [^{14}C]cholesteryl hexadecyl ether. Excess solvent was removed under a stream of N_2 gas, then trace solvent removed by holding the lipid film under high vacuum for 3–16 h.

Lipids were dispersed by the addition of 1.0 ml of 0.3 M citrate buffer at either pH 2.0 or 4.0 and were then heated to 60°C, vortexed extensively, and subjected to five

freeze/thaw cycles between −196° and 60°C to facilitate homogeneous solute and buffer distribution between the intra- and extra-liposomal compartments [13]. After the final freeze/thaw cycle, the multilamellar vesicles were converted to large unilamellar vesicles by repeated extrusion through two 0.1 μm filters employing a Thermobarrel Extruder at 65°C (Lipex Biomembranes, Vancouver, BC). Vesicle size distribution was determined by quasielastic light scattering (QELS) using a Nicomp 370 Particle Sizer; all liposome preparations had mean diameters in the range between 125 and 135 nm.

2.3. Measurement of transmembrane pH gradient

Large unilamellar liposomes of either DSPC/cholesterol or DSPC/cholesterol/stearylamine containing 1.0 μCi of [^{3}H]cholesteryl hexadecyl ether were prepared at pH 4.0 or 2.0 as described above. Aliquots of 10 mg of lipid were alkalinized by the addition of 0.5 M Na_2HPO_4 then the lipid concentration was brought to 2.0 mg/ml by the addition of 150 mM NaCl, 20 mM Hepes (pH 7.5, HBS). Liposomes were equilibrated at 60°C for 10 min then 1 μCi of [^{14}C]methylamine was added (in 1 μl ethanol) and the probe was allowed to redistribute for an additional 10 min at 60°C. After cooling to room temperature, the liposomes and encapsulated [^{14}C]methylamine were recovered by centrifugation of 1 ml aliquots of the suspension on micropartition concentrators (Amicon Centrifree or Amicon Microcon-30) for 10 min at 2000 × g, 4°C. Aliquots of the suspensions taken before and after centrifugation were assayed by LSC to calculate the inside/outside ratio of [^{14}C]methylamine and, consequently, the transmembrane pH gradient.

2.4. Drug uptake and leakage

Verapamil and prochlorperazine were loaded into liposomes in response to a transmembrane pH gradient. For liposomes in 0.3 M citrate buffer at pH 4.0, the external medium was alkalinized by the addition of a small volume of 0.5 M Na_2CO_3 (65 μl/100 μl liposomes). Similarly, liposomes in 0.3 M citrate buffer at pH 2.0 were alkalinized by the addition of 85 μl of 0.5 M Na_2CO_3 per 100 μl of liposomes. The final pH outside of the liposomes using this method was 7.3–7.6.

Uptake of either verapamil (containing 0.1 μCi [^{3}H]verapamil/mg verapamil) or prochlorperazine was initiated by the addition of 2 mg of drug in 200 μl of 150 mM NaCl or HBS, respectively, to 10 mg of liposomes at 12.5 mg lipid/ml. The drug/lipid ratio of 0.2:1 (w/w) for verapamil (hydrochloride salt) and prochlorperazine (edisylate salt) represents free drug/lipid (mol/mol) ratios of 0.185 and 0.133, respectively. Uptake was allowed to proceed for 10 min at temperatures of 21°C, 37°C or 60°C and the encapsulation of verapamil and prochlorperazine was determined after removal of unentrapped drug by column chromatography. Aliquots of 0.1 ml of liposomes were centrifuged on a 1 ml mini-column of Sephadex G-50 that was pre-equilibrated in HBS. Lipid and verapamil content were measured by liquid scintillation counting (LSC) and prochlorperazine content was measured by dissolving an aliquot of the liposomes in ethanol and measuring absorbance at 314 nm.

After drug uptake, the leakage of the entrapped drug was determined by dialysis. Specifically, liposomes with uptake efficiencies > 90% were transferred directly to dialysis tubing. Preparations with entrapment efficiencies < 90% were subjected to column chromatography to remove the non-encapsulated drug prior to dialysis. All samples were dialyzed against 1000 volumes of HBS at 37°C. At various times, aliquots were removed and assayed for lipid and for the amount of drug remaining encapsulated as described above. The data presented are from side-by-side comparisons. The relationship between the different samples was very consistent between different experiments, even though the absolute flux rates were somewhat more variable.

2.5. Calculation of permeability coefficients

Permeability coefficients (P, cm/s) were calculated as described previously [14] using:

$$P = J/(A \cdot \Delta C)$$

where J is the flux (μmol s^{-1}), ΔC is the concentration gradient of the drug (μmol cm^{-3}) and A is the total surface area of the liposomes (cm^2). The flux was obtained from the initial slope of the drug efflux curves (Fig. 2A and Fig. 3A) over which the variation of drug content vs. time was linear ($r^2 > 0.98$). The concentration gradient was calculated from the known average vesicle diameter obtained from the QELS data and from the initial drug/lipid ratio. Membrane surface area (A) was calculated from the lipid concentration and assuming an average lipid molecular area of 0.5 nm^2.

For the purposes of calculating the permeability coefficients for the neutral forms of the drugs, the starting assumption that the drug efflux observed at $pH_i = 4.0$ was due exclusively to the neutral form of the drug used. The intraliposomal concentration of the neutral species was obtained from the pK_a values of 6.8 and 8.1 for verapamil and prochlorperazine, respectively (data not shown) and the initial drug/lipid ratio of the liposomes after drug uptake. Therefore, at $pH_i = 4.0$, the ratio of neutral/protonated forms of the total intraliposomal verapamil and prochlorperazine were $< 5 \cdot 10^{-3}$:1 and $5 \cdot 10^{-4}$:1, respectively. To obtain the permeability coefficient for the charged species of the drugs, the permeability coefficients calculated for the neutral forms of the drugs (i.e., the P value calculated at $pH_i = 4.0$) were used to estimate the proportion of the total flux observed at $pH_i = 2.0$ that was due to the neutral form of the drug. This flux was sub-

tracted from the total flux that was observed at $pH_i = 2.0$, and the flux remaining at $pH_i = 2.0$ was taken to be due to the charged forms of the drugs. The permeability coefficients for the charged drugs were then calculated as described above using the corrected concentration gradients for the charged forms of verapamil and prochlorperazine. The presented results are those obtained using this calculation procedure, however, it should be noted that very similar permeability coefficients were obtained for the neutral and charged forms of the drugs when the calculation was initiated with the assumption that all of the flux observed at $pH_i = 2.0$ was due exclusively to the charged species. First-order rate constants, k, for drug leakage were calculated from the slope ($-k$) of the ln (% drug remaining) vs. time plots and the half-lives ($T_{1/2}$) obtained using $T_{1/2} = 0.693/k$.

3. Results

3.1. pH gradient-mediated drug uptake

The uptake of verapamil and prochlorperazine into large unilamellar vesicles composed of DSPC/cholesterol or DSPC/cholesterol/stearylamine is summarized in Table 1. Drug uptake reached their maximum values within one minute of drug addition and were stable during uptake periods as long as 30 min (data not shown). The efficiency of entrapment of verapamil in DSPC/cholesterol liposomes with a $pH_i = 4.0$ was 60–65% at 21°C, 37°C and 60°C. Encapsulation efficiency improved to 85% to 89% when the pH_i was 2.0. The presence of 10 mol% stearyl-

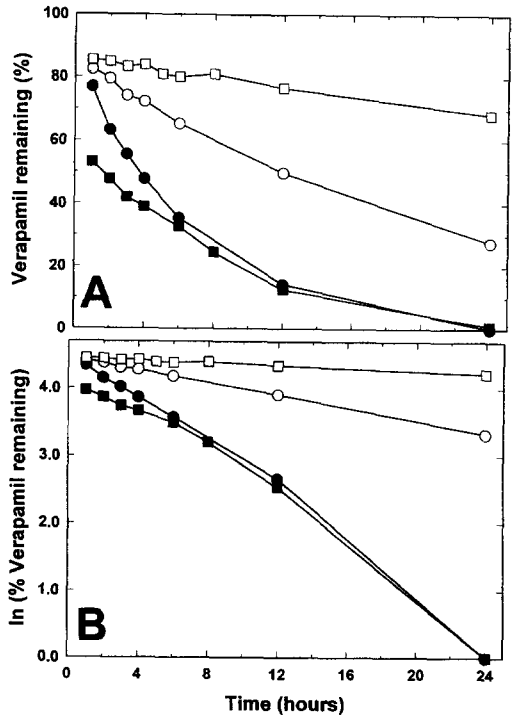


Fig. 2. Release of verapamil from large unilamellar liposomes composed of DSPC/cholesterol (55:45, mol/mol) and DSPC/cholesterol/stearylamine (50:40:10, mol/mol) during dialysis at 37°C. Curves represent data for DSPC/cholesterol (○,●) and DSPC/cholesterol/stearylamine (□,■) with intraliposomal pH of 4.0 (●,■) or 2.0 (○,□). Curves represent the % of initial verapamil (A) and the ln of the % of verapamil remaining (B). The standard deviations for these samples were typically < 4%. Linear regressions of the lines presented in (B) had r^2 values at least 0.98 and the slopes were used for calculation of $T_{1/2}$ values.

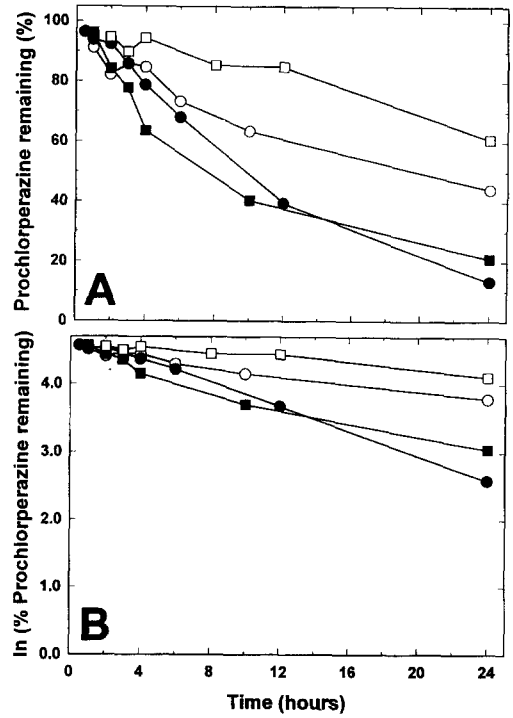


Fig. 3. Release of prochlorperazine from large unilamellar liposomes composed of DSPC/cholesterol (55:45, mol/mol) and DSPC/cholesterol/stearylamine (50:40:10, mol/mol) during dialysis at 37°C. Curves represent data for DSPC/cholesterol (○,●) and DSPC/cholesterol/stearylamine (□,■) with intraliposomal pH of 4.0 (●,■) or 2.0 (○,□). Curves represent the % of initial prochlorperazine (A) and the ln of the % of prochlorperazine remaining (B). The standard deviations for these samples were typically < 4%. Linear regressions of the lines presented in (B) had r^2 values at least 0.94 and the slopes were used for calculation of $T_{1/2}$ values.

Table 1
Summary of the uptake efficiency of verapamil and prochlorperazine into liposomes of either DSPC/cholesterol (DSPC/Chol) or DSPC/cholesterol/stearylamine (DSPC/Chol/SA) with a pH_i of either 2.0 or 4.0

Lipid	pH_i	Drug uptake (%)				
		verapamil			prochlorperazine	
		21°C	37°C	60°C	21°C	60°C
DSPC/Chol	4.0	59.5	64.5	67.8	96.5	92.0
DSPC/Chol	2.0	84.8	82.4	88.7	100	–
DSPC/Chol/SA	4.0	–	–	63.8	100	–
DSPC/Chol/SA	2.0	–	–	80.8	100	–

All liposomes had an average diameter of approx. 130 nm and $pH_0 = 7.3–7.6$. Uptake of 100% represents drug/lipid (mol/mol) ratios of 0.185 and 0.133 for verapamil and prochlorperazine, respectively.

amine in the vesicles did not improve the encapsulation efficiency (Table 1). In contrast to verapamil, the encapsulation of prochlorperazine into both DSPC/cholesterol and DSPC/cholesterol/stearylamine liposomes occurred at efficiencies of 95–100% under milder conditions than for verapamil. That is, encapsulation efficiencies for prochlorperazine were approx. 100% at 21°C and $pH_i = 4.0$ (Table 1). The loading of both verapamil and prochlorperazine into liposomes was dependent on the presence of a pH gradient; i.e., no uptake was observed in liposomes with $pH_i = pH_0 = 4.0$ (data not shown).

The magnitude of the transmembrane pH gradients has been evaluated using [^{14}C]methylamine. Prior to drug uptake, transmembrane pH gradients of 3.1 to 3.4 pH units were measured for both DSPC/cholesterol and DSPC/cholesterol/stearylamine liposomes dispersed at pH 4.0, corresponding to calculated intraliposomal pH values of 4.0 to 4.4. In those liposomes dispersed in citrate buffer at pH 2.0, the magnitude of the pH gradient (approx. 5 units) was greater than that which can be reliably determined using methylamine (approx. 3.8 units [15]). For these systems in buffer at pH 2.0, we typically measured transmembrane pH gradients of approx. 3.8 pH units, consistent with the presence of a pH gradient of approx. 5 units.

3.2. Drug leakage

The leakage of verapamil from DSPC/cholesterol and DSPC/cholesterol/stearylamine liposomes during dialysis at 37°C as a function of intraliposomal pH is shown in Fig. 2A. Verapamil leakage followed first order kinetics under all experimental conditions used here as shown by the linear relationship between the ln of intraliposomal verapamil vs. time (Fig. 2B). At a $pH_i = 4.0$, verapamil efflux from DSPC/cholesterol liposomes resulted in the loss of 50% of the entrapped drug ($T_{1/2}$) after 3.7 h. The presence of 10 mol% stearylamine in DSPC/cholesterol liposomes with $pH_i = 4.0$ had no influence on the leakage kinetics of verapamil; the $T_{1/2}$ for release was 4.0 h. The retention of verapamil in DSPC/cholesterol liposomes with a larger pH gradient ($pH_i = 2.0$) improved the $T_{1/2}$ for leakage from 3.7 h at $pH_i = 4.0$ to 14.5 h with $pH_i = 2.0$. The retention of verapamil in DSPC/cholesterol liposomes with $pH_i = 2.0$ was significantly improved ($T_{1/2} = 73.4$ h) by the presence of 10 mol% of stearylamine.

The leakage of prochlorperazine from liposomes composed of DSPC/cholesterol or DSPC/cholesterol/stearylamine is shown in Fig. 3A. As observed for the leakage of verapamil, first order efflux kinetics were observed for the release of prochlorperazine from these liposomes (Fig. 3B). Prochlorperazine was slightly less permeable than verapamil. Release of prochlorperazine from large unilamellar liposomes composed of DSPC/cholesterol with $pH_i = 4.0$ occurred with a $T_{1/2}$ of 8.1 h, compared to 3.7 h for verapamil leakage from identical liposomes. The presence of 10 mol% stearylamine in the DSPC/cholesterol liposomes with a $pH_i = 4.0$ did not substantially improve the retention of prochlorperazine ($T_{1/2} = 10.8$ h). Increasing the magnitude of the transmembrane pH gradient, effected by a decrease of the intraliposomal pH to 2.0, significantly improved the retention of prochlorperazine in DSPC/cholesterol liposomes ($T_{1/2} = 22.2$ h). The efflux of prochlorperazine from DSPC/cholesterol liposomes with $pH_i = 2.0$ was further reduced by the presence of 10 mol% stearylamine ($T_{1/2}$ increased to 36.0 h), representing a 4.5-fold improvement over the results obtained at $pH_i = 4.0$.

Table 2
Permeability coefficients (P) for the leakage of verapamil and prochlorperazine from liposomes of DSPC/cholesterol (DSPC/Chol) or DSPC/cholesterol/stearylamine (DSPC/Chol/SA)

Lipid	P (cm/s)			
	verapamil		prochlorperazine	
	neutral	cationic	neutral	cationic
DSPC/Chol	$1.2 \cdot 10^{-8}$	$5.5 \cdot 10^{-12}$	$8.3 \cdot 10^{-8}$	$1.2 \cdot 10^{-12}$
DSPC/Chol/SA	$6.2 \cdot 10^{-9}$	$3.1 \cdot 10^{-13}$	$7.8 \cdot 10^{-8}$	$5.7 \cdot 10^{-13}$

Permeability coefficients for the neutral and cationic forms of verapamil and prochlorperazine were calculated as described in the Materials and methods.

Previous studies have suggested that drug encapsulation and release properties could be explained entirely on the basis of the permeation of the neutral form of the drug [5]. If this were also the case with verapamil and prochlorperazine, then the presence of a cationic lipid in the membrane would not be expected to affect drug release, since there should be negligible effect of a surface charge on neutral drugs. However, the data presented in Figs. 2 and 3 clearly indicate a pH-dependent effect of the cationic lipid on drug permeability. To further investigate the effect of intraliposomal pH and the presence of stearylamine on drug release, the permeability coefficients of the neutral and cationic forms of verapamil and prochlorperazine (Table 2) have been calculated from the drug efflux curves (Fig. 2A and Fig. 3A). For the neutral form of verapamil, the permeability coefficient in DSPC/cholesterol liposomes ($1.2 \cdot 10^{-8}$ cm/s) was similar to that in DSPC/cholesterol/stearylamine liposomes ($0.62 \cdot 10^{-8}$ cm/s; Table 2). In contrast, the permeability coefficient for the charged form of verapamil was decreased by more than an order of magnitude in the presence of stearylamine ($5.5 \cdot 10^{-12}$ vs. $3.1 \cdot 10^{-13}$ cm/s; Table 2). In liposomes composed of either DSPC/cholesterol or of DSPC/cholesterol/stearylamine, the permeability coefficients for the neutral form of verapamil were 10^3- to 10^4-fold greater than those for the cationic form of verapamil. Results similar to those observed for verapamil were also obtained for prochlorperazine. The permeability coefficient for the neutral form of prochlorperazine in DSPC/cholesterol liposomes ($8.3 \cdot 10^{-8}$ cm/s) was very similar to that obtained in DSPC/cholesterol/stearyla-

mine liposomes ($7.8 \cdot 10^{-8}$ cm/s, Table 2). However, the permeability coefficient for the charged species of prochlorperazine was decreased by the presence of stearylamine (Table 2). In both liposome types, the calculated permeability coefficients for the neutral form of prochlorperazine were approx. 10^5-fold higher than for the cationic form of the drug. Overall, the presence of 10 mol% stearylamine in DSPC/cholesterol liposomes had no effect on the calculated permeability coefficients for the neutral forms of either verapamil or prochlorperazine, but significantly reduced the permeability coefficients for the charged forms of both drugs. For both verapamil and prochlorperazine, the neutral drug was 10^3 to 10^5 orders of magnitude more permeable than was the cationic form of the drug.

To account for the possibility that the pH gradients were partially collapsed during drug uptake, we have also calculated the permeability coefficients for the charged and neutral species of verapamil and prochlorperazine under conditions where the transmembrane pH gradient had collapsed by 2–3 pH units. Specifically, it was assumed that the pH 2.0 systems had collapsed to an intraliposomal pH of 5.0 and that the pH 4.0 systems had collapsed to an intraliposomal pH of 6.0. Using these assumptions, the calculated permeability coefficients for the charged forms of verapamil and prochlorperazine were in the range of 10^{-12} to 10^{-14} cm/s and were decreased by approximately one order of magnitude by the presence of stearylamine whereas the permeability coefficients calculated for the neutral forms were not affected by the presence of stearylamine (data not shown). Consequently, significant collapse of the transmembrane pH gradient did not affect the qualitative relationship between the neutral and charged species reported in Table 2.

3.3. A mathematical model for drug release kinetics

In order to achieve a better understanding of the factors that contribute to the permeability of neutral and cationic forms of verapamil and prochlorperazine, we have developed a mathematical model for the kinetics of drug release from liposomes. This model described the passive diffusion of both the charged and neutral forms of the drug from the liposome and incorporates the effects of intraliposomal pH, surface charge of the inner monolayer of the liposomes, charge on the permeating drug and the effect of intraliposomal citrate buffer on both surface charge and drug leakage.

The change in the number of molecules of the neutral form of the drug on the interior of the liposome, A_i, over time can be denoted as dNA_i/dt where N = number and dt = the change in time, t. According to Fick's Law:

$$\frac{dNA_i}{dt} = k([A]_0 - [A]_i)$$

where k = the rate constant, $[A]_0$ = the concentration of the neutral form on the outside of the liposome and $[A]_i$ = the concentration of the neutral form of the drug on the inside of the liposome. Similarly:

$$\frac{dNA_0}{dt} = k([A]_i - [A]_0)$$

$$\frac{dNA_i^+}{dt} = k([A^+]_0 - [A^+]_i)$$

$$\frac{dNA_0^+}{dt} = k([A^+]_i - [A^+]_0)$$

where $[A^+]_i$ and $[A^+]_0$ represent the concentration of the charged form on the inside and outside of the liposome, respectively. If $[A]_0$ and $[A^+]_0$ are kept effectively at zero by removing free drug from the outside of the liposome, then the flux equations are further simplified:

$$\frac{dNA_i}{dt} = \left(\frac{d[A]_i}{dt}\right) = -k[A]_i \quad (1)$$

$$\frac{dNA_i^+}{dt} = \left(\frac{d[A^+]_i}{dt}\right) = -k[A^+]_i \quad (2)$$

The concentrations of A and A^+ at the intraliposomal membrane surface ($[A]_i(0)$ and $[A^+]_i(0)$, respectively) are;

$$\frac{d[A]_i(0)}{dt} = -k[A]_i \quad (3)$$

$$\frac{d[A^+]_i(0)}{dt} = -k[A^+]_i \quad (4)$$

However, since the surface charge should have no effect on the neutral form of the drug, Eqs. (1) and (3) are identical.

A positively charged membrane exerts a repulsive electrostatic force on positively charged solutes such that the concentration of these molecules is lowest at the membrane surface and increases exponentially with increasing distance from the membrane surface according to [16];

$$[A^+](0) = [A^+]\exp\left(\frac{-e\psi_0}{KT}\right) \quad (5)$$

where $[A^+]$ = the bulk concentration of the charged form of the drug, e = the charge of an electron ($1.602 \cdot 10^{-19}$ Coulombs), K = the Boltzmann constant ($1.380 \cdot 10^{-23}$ J K^{-1}), T = temperature in Kelvin and ψ_0 = the membrane surface potential in V.

The membrane surface potential is related to the surface charge density, σ, by the equation [16]:

$$\sigma = \frac{1}{272}\sqrt{\Sigma C_i \exp\left(\frac{-z_i e\psi_o}{KT}\right) - 1} \quad (6)$$

where C_i = the concentration of the ith species of molecule in solution and z_i = the valence of the ith charged species of molecule in solution. The surface charge density is the percentage of the charged lipid in the membrane divided by the surface area it occupies. A lipid carrying one positive charge at a concentration of 10 mol% and occupy-

ing an average lipid surface area of 60 Å^2 would give a surface charge density of $0.1/60 = 0.00167$ charges Å^{-2}. Therefore, any ions in solution will alter the membrane surface potential when the surface charge density is held constant (Eq. (6)). When C_i in Eq. (6) is substituted by any ionic forms of the citrate buffer, the charged form of the drug and ionic forms of salt present in solution, then Eq. (6) becomes:

$$\sigma = \frac{1}{272}\left\{[B^-]\left(\exp\left(\frac{e\psi_0}{KT}\right) - 1\right) + [B^{2-}]\left(\exp\left(\frac{2e\psi_0}{KT}\right) - 1\right) + [B^{3-}]\left(\exp\left(\frac{3e\psi_0}{KT}\right) - 1\right) + [A^+]\left(\exp\left(\frac{-e\psi_0}{KT}\right) - 1\right) + [C^+]\left(\exp\left(\frac{-e\psi_0}{KT}\right) - 1\right) + [C^-]\left(\exp\left(\frac{e\psi_0}{KT}\right) - 1\right)\right\}^{1/2} \quad (7)$$

where

$$[B^-] = \frac{k_1}{[H^+]}\frac{[B]^{tot}}{f(H^+)}$$

$$[B^{2-}] = \frac{k_1 k_2}{[H^+]^2}\frac{[B]^{tot}}{f(H^+)}$$

$$[B^{3-}] = \frac{k_1 k_2 k_3}{[H^+]^3}\frac{[B]^{tot}}{f(H^+)}$$

and where

$$f(H^+) = \left(1 + \frac{k_1}{[H^+]} + \frac{k_1 k_2}{[H^+]^2} + \frac{k_1 k_2 k_3}{[H^+]^3}\right)$$

Here, $[B^-],[B^{2-}],[B^{3-}]$ = the charged species of the buffer, k_1, k_2, k_3 = the dissociation constants of the buffer, $[B]^{tot}$ = the total buffer concentration in solution, $[C^+]$ = the concentration of singly positively charged ions in solution necessary for charge balance, $[C^-]$ = the concentration of singly negatively charged ions in solution necessary for charge balance.

The membrane surface potential was determined from Eq. (6) by iteration at each time increment, dt. To calculate the intraliposomal concentrations of neutral and charged forms of the drug, Eqs. (3), (4), (5) and (7) were solved at each time increment dt. In order to facilitate this calculation, we have used an intraliposomal buffer concentration of 0.3 M and three pK_a values of 3.1, 4.8 and 6.4 ($= k_1, k_2, k_3$). We have modeled the release of a cationic drug with a single charge and a pK_a = 9.0, whose total intraliposomal concentration after loading was 180 mM and whose concentration outside the liposome was negligible. The release of this drug was modeled using the assumption that the intraliposomal pH values (2.0 and 4.0) were not altered by drug loading. However, we have also calculated the extent of collapse of the transmembrane pH gradient that is expected to occur due to drug uptake and have also modeled the drug release kinetics using the resultant intraliposomal pH values. Under these conditions, the rates of drug release had the same qualitative relationships between liposomes as a function of intraliposomal pH and surface charge, but the absolute rates were modestly different from those obtained if it was assumed that the intraliposomal pH was not altered by drug loading.

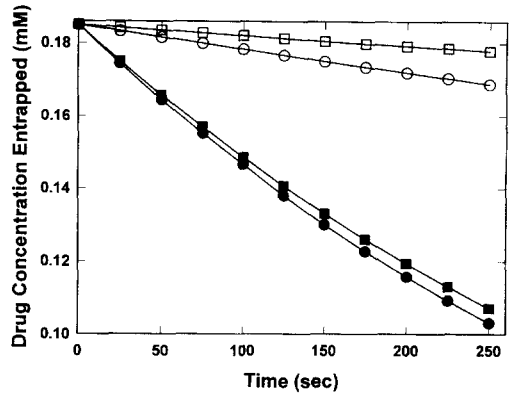


Fig. 4. Mathematical model of the effect intraliposomal pH and the effect of a 10% surface charge density on the efflux of a drug with pK_a = 9 from large unilamellar liposomes during dialysis. Curves represent drug leakage from liposomes with intraliposomal pH of 4.0 (●,■) or 2.0 (○,□) in the absence (○,●) and presence (□,■) of a 10% positive surface charge density.

The results of the mathematical modeling of drug efflux kinetics, as affected by intraliposomal pH and the presence of a surface charge density, are shown in Fig. 4. With an intraliposomal pH of 4.0, the presence of 10 mol% stearylamine did not affect the retention of the drug within the liposomes. Since citrate has a pK at 3.1, at pH 4.0 a large amount of the citrate buffer is in the monovalent anion form and effectively neutralizes the positive membrane surface potential. Consequently, there is very little effect of a surface charge on the rate of efflux at pH 4.0; calculated half-lives for drug leakage at pH 4.0 were within 7% of each other in the presence and absence of 10 mol% stearylamine (Fig. 4). In contrast, at pH 2.0, most of the citrate is uncharged and does not screen the positive membrane surface charges. Therefore, a significant membrane surface potential will exist in the presence of a positive surface charge density. It is obvious that when the membrane surface potential is high, $[A^+](0)$ is lower than when no surface potential is present. Therefore, in the presence of a membrane surface potential the effective transmembrane concentration gradient of the drug A^+ is lower and the rate of efflux is expected to be slower. In this model, there was a 6.3-fold increase in the half-life of drug leakage when the intraliposomal pH was decreased

from 4.0 to 2.0 in the absence of stearylamine (Fig. 4). With the further addition of 10 mol% stearylamine at an intraliposomal pH of 2.0, the calculated half-life for drug leakage increased by an additional 2.3-fold (Fig. 4). Overall, the modeled drug retention was improved by 14.7-fold when the liposomes were changed from uncharged liposomes at pH 4.0 to liposomes possessing a 10 mol% surface charge at pH 2.0. This compares favorably to the observed increases in drug retention of 19.8-fold for verapamil and 4.5-fold for prochlorperazine in the comparable liposomal systems.

4. Discussion

The therapeutic activity of many liposomal formulations of membrane-permeant drugs will likely depend on the success of efforts to design liposomes having enhanced drug retention characteristics. Previous studies with liposomal vincristine have indicated that antitumor activity can be significantly improved by using liposomes with enhanced drug retention characteristics [17]. The rationale for the experiments described here was that the permeability of cationic drugs could be significantly reduced if the inner monolayer of the liposome possessed a positive surface potential. In these experiments, the surface charge on the inside monolayer was created by the presence of the cationic lipid stearylamine since it is known to redistribute to the inner monolayer of liposomes in the presence of a transmembrane pH gradient with the inside acidic (inside acidic) [10,11].

The results presented in this paper confirm the prediction that drug permeability could be significantly reduced if the inner monolayer of the liposome possessed a positive surface charge. In the presence of 10 mol% stearylamine at $pH_i = 2.0$, the leakage of both verapamil (Fig. 2A) and prochlorperazine (Fig. 3A) was significantly reduced. This was observed in the empirical results as increased $T_{1/2}$ values for drug release (Fig. 2A and Fig. 3A) and as decreased permeability coefficients in the presence of 10 mol% stearylamine at $pH_i = 2.0$ (Table 2) calculated from the efflux curves. It is likely that the reduced drug leakage observed in the presence of stearylamine at $pH_i = 2.0$ was primarily a consequence of the 10-fold reduction of the permeability coefficients for the charged forms of both verapamil and prochlorperazine (Table 2). If the neutral form of the drug is the only form that will cross the membrane, then the presence of a membrane surface charge should have negligible effect on drug efflux. However, if a positive membrane surface potential affects the rate of efflux, then it must be assumed that the protonated form of the drug contributes significantly to drug permeation, since neutral molecules are unaffected by membrane surface charge.

While the surface charge density on the inner monolayer of the liposomes has not been explicitly measured, it should be noted that the observed efflux of both verapamil and prochlorperazine could be accurately modeled by assuming that a surface potential developed as a consequence of stearylamine redistribution to the inner monolayer of the liposome. In this model, the leakage of drug is directly proportional to the concentration of the drug at the membrane surface. Therefore, charge repulsion due to the presence of stearylamine effected a reduction of the local concentration of verapamil and prochlorperazine adjacent to the membrane surface, effectively reducing their concentration gradients across the membrane. It should be added that the absence of an effect of stearylamine on drug leakage at $pH_i = 4.0$ is also accounted for by this analysis. Since citric acid has a pK at 3.1, at $pH_i = 4.0$ there is a significant concentration of anionic citrate within the liposome which screens the positive surface charges due to stearylamine and, consequently, reduces the surface charge density and membrane potential to negligible levels. In contrast, at $pH_i = 2.0$ the anionic citrate concentration is lower than that necessary to neutralize the positive surface potential due to stearylamine. The positive surface potential effectively prevents the close approach of the charged drugs to the membrane surface via long-range electrostatic repulsion. This results in both a reduction of the effective drug concentration gradient across the membrane and a reduction of drug partitioning into the bilayer. As an alternative explanation for the absence of an effect of stearylamine on drug leakage at $pH_i = 4.0$, it is possible that the predominant species permeating the bilayer was the neutral forms of verapamil and prochlorperazine, as was assumed for the calculation of the permeability coefficient. The neutral forms of these drugs would not be affected by the presence of stearylamine, as was observed (Figs. 2 and 3).

The results presented here indicate that both the charged and the neutral species of the drugs verapamil and prochlorperazine are membrane permeable. However, the uncharged forms of these drugs are approximately four orders of magnitude more permeable than are the charged forms (Table 2). A consequence of this difference in permeability coefficients is that the total flux of drug at $pH_i = 4.0$ is dominated by flux due to the neutral species of the drug, despite the fact that the protonated drug is the predominant form at this pH. Similarly, at $pH_i = 2.0$ the neutral form of verapamil represented only 0.0005% of the intraliposomal drug but accounted for 1.1% of the flux of the drug from DSPC/cholesterol liposomes and 9.2% of the flux from DSPC/cholesterol/stearylamine liposomes. At $pH_i = 2.0$ the neutral form of prochlorperazine represented 0.00005% of the total drug, but accounted for 3.4% of the flux from DSPC/cholesterol liposomes and 6.4% of the flux from DSPC/cholesterol/stearylamine liposomes.

While it may be considered a surprise that the charged forms of verapamil and prochlorperazine are membrane permeable, it is notable that the permeability coefficients for the protonated forms of these drugs, approx. 10^{-12}

cm/s in DSPC/cholesterol liposomes, are comparable to those measured previously at 10^{-12} to 10^{-14} cm/s for cations such as Na^+ and K^+ [18]. The difference in molecular size of verapamil and prochlorperazine, compared to Na^+ and K^+, suggests that the charge on the compound is an important determinant of the permeation rate, a conclusion that is consistent with the effect of stearylamine on the permeability coefficients. In addition, the uptake of a positively charged analogue of dibucaine (*N*-methyldibucaine) in response to a membrane potential [19] supports our conclusion that cationic drugs are membrane-permeable.

Our experience with both DSPC/cholesterol and DSPC/cholesterol/stearylamine liposomes indicates that they are present as a highly ordered liquid-crystalline phase. This is consistent with published results by other groups showing that similar DSPC/cholesterol mixtures have no detectable phase transition between 35°C and 70°C [20]. Further, the presence of 10 mol% stearylamine in egg PC liposomes had no effect on either the magnitude of the transmembrane pH gradient or liposome size [10]. Both characteristics are reliable indicators of the structural integrity of the liposomes. To evaluate the effect of drug encapsulation on bilayer structure, we have used the published octanol/water partition coefficient for prochlorperazine hydrochloride [21] to estimate the amount of prochlorperazine that would be present in the lipid bilayer after drug loading. At maximum levels of encapsulation, the intraliposomal concentration of prochlorperazine was approx. 180 mM. In 120 nm diameter liposomes, the amount of neutral prochlorperazine partitioning into the lipid bilayer would represent approx. 3.2 mol% of the total lipid. However, at intraliposomal pH values of 4.0 and 2.0, the drug is predominantly in the charged form. Therefore, intraliposomal concentrations of neutral prochlorperazine would be < 0.01 mM and the proportion of neutral prochlorperazine in the lipid bilayer would be < 0.01 mol%. Further, due to the charge present on the intraliposomal prochlorperazine after drug loading, and because the partition coefficient for the charged from is expected to be significantly lower than that for the neutral form, it is likely that the partitioning of intraliposomal prochlorperazine (predominantly the charged form) into the bilayer is very low. Although a partition coefficient is not available for verapamil, its similarity with the structure and solubility of prochlorperazine suggests that it would not be significantly different from prochlorperazine in its partitioning into the hydrophobic phase of the bilayer. We conclude, therefore, that the bilayer partitioning of these drugs is negligible and that they have no significant impact on the physical state of the bilayers.

In summary, we have demonstrated that the retention of cationic drugs within liposomes can be significantly improved when the liposomes contain the cationic lipid stearylamine and a transmembrane pH gradient utilizing a low entrapped pH. In principle, this approach is applicable to a wide variety of cationic drugs and may significantly improve the therapeutic activity of liposomal drug formulations.

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