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Research Paper

Effect of Cyclodextrin Complexation on the Liposome Permeability of a Model Hydrophobic Weak Acid

Vijay Joguparthi[1,2] and Bradley D. Anderson[1,3]

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Purpose. This study examines the effect of a chemically modified β-cyclodextrin on the liposome bilayer permeability of a liposomally entrapped model hydrophobic weak acid, DB-67 (7-*t*-butyldimethylsilyl-10-hydroxycamptothecin).

Materials and Methods. Permeability studies were conducted in liposomes prepared by hydration–extrusion in the presence or absence of entrapped hydroxypropyl-β-cyclodextrin (HPβCD). A gradient HPLC method with evaporative light scattering detection was developed for analysis of HPβCD. DB-67 was analyzed by HPLC with fluorescence detection.

Results. HPβCD entrapped in the aqueous compartment of liposomes was found to be membrane impermeable. Gel phase liposomes were stable in the presence of HPβCD. HPβCD complexation did not significantly alter the apparent permeability of DB67 lactone, due to its high membrane binding. However, lactone ring-opening and ionization significantly decreased the apparent permeability and improved the liposomal retention of DB-67, an effect that was amplified in the presence of 50 mM HPβCD.

Conclusions. In liposomes, cyclodextrin complexation competes with liposomal membrane binding which may temper the potential benefit of complexation in prolonging hydrophobic drug retention. Cyclodextrin complexation combined with drug ionization may nevertheless significantly enhance the retention of ionizable hydrophobic drugs in liposomes as complexation may compete more favorably with membrane binding when the drug is ionized.

KEY WORDS: camptothecins; controlled drug delivery; cyclodextrin complexation; DB-67; hydroxypropyl-β-cyclodextrin; liposomes; membrane transport.

INTRODUCTION

Liposomal encapsulation of chemotherapeutics is an attractive formulation strategy for passively targeting tumor tissue. However, the efficient use of this strategy requires that liposomes retain the entrapped drug while they are in the systemic circulation (1–3). Poor drug retention in circulating liposomes may be a major obstacle to clinical success. While several examples of commercially successful liposomal formulations of hydrophobic ionizable amine-containing drugs are available, the development of liposomal products for neutral or weakly acidic lipophilic drugs has been limited despite the availability of a several drug candidates that could benefit from the approach, possibly due to the lack of suitable loading and retention strategies for these compounds.

The lipophilic neutral camptothecins constitute a class of drugs that could benefit from liposomal encapsulation. Since the lactone form is the biologically active form (4), it is desirable to deliver this form *in vivo*. The potential of using a high intraliposomal pH to maintain the lipophilic camptothecin analog DB-67 (Fig. 1) in a membrane impermeable ionized form was recently explored in order to develop prolonged release liposomal suspensions (5,6). However, a high intravesicular pH could not be maintained under physiological conditions due to the rapid dissipation of the trans-membrane pH gradient by carbonate buffer (CO_2/H_2CO_3) (5,7). In the present study, the ability of a liposomally entrapped, chemically modified cyclodextrin complexing agent (hydroxypropyl-β-cyclodextrin, HPβCD) to decrease the fraction of membrane permeable species and thereby prolong the liposomal retention of DB-67 was explored.

Although the potential utility of complexing agents such as cyclodextrins (CDs) for improving drug loading and retention in vesicles has been recognized for more than a decade (8,9), there has been a recent resurgence in interest in this strategy (10–15). Still lacking are quantitative relationships that would enable the formulator to predict the potential benefit of a complexing agent on the liposomal loading and retention of a hydrophobic drug. Additionally, unusual release kinetics characterized by an initial burst release followed by a second phase with slower or no drug release have been observed in several studies, particularly

[1] Department of Pharmaceutical Sciences, University of Kentucky, A323A ASTeCC Bldg., Lexington, Kentucky 40536-0082, USA.
[2] *Present address:* Boehringer-Ingelheim Pharmaceuticals, 900 Ridgebury Rd, Ridgefield, Connecticut 06877, USA.
[3] To whom correspondence should be addressed. (e-mail: bande2@email.uky.edu)

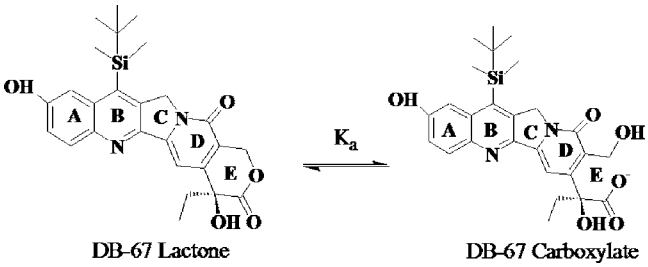

Fig. 1. Equilibrium between DB-67 lactone (*left*) and DB-67 carboxylate (*right*). Equilibrium constant K_a is the effective lactone ionization constant.

those employing liposomes comprised of liquid crystalline bilayers (8,11,14,16). It has been speculated that the initial burst phase is due to a membrane destabilizing effect of cyclodextrins (8,11,14). While interactions of some cyclodextrins with certain liposome components such as cholesterol have been reported (17,18), the reason for the observed biphasic release kinetics remains unclear. Certain cyclodextrins such as HPβCD have been found to have a negligible effect on the gel to liquid crystalline phase transition enthalpy or temperature (19,20) and to induce no significant leakage of entrapped markers (16,21,22). Therefore, such non-interacting cyclodextrins may be more useful as excipients for prolonged release liposomal suspensions.

In the present study, the effect of intravesicular pH modification combined with HPβCD complexation on the liposomal retention of DB-67 and the role of physiological levels of extravesicular carbonate at pH 7.4 were investigated. The physicochemical conditions under which cyclodextrins may serve as useful excipients in liposome formulations are also discussed.

MATERIALS AND METHODS

Materials

Hydroxypropyl-β-cyclodextrin (HPβCD, degree of substitution=2.94, MW=1,305.5) was obtained from American Maize-Products Company (Hammond, IN). DB-67 (7-*t*-butyldimethylsilyl-10-hydroxycamptothecin) was obtained from Novartis Pharmaceuticals Corporation (East Hanover, NJ, USA). Phospholipids (1, 2-distearoyl-*sn*-glycero-3-phosphatidylcholine (DSPC) and 1, 2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[polyethylene glycol 2000] [m-PEG DSPE, MW=2,806]) were purchased as powders from Avanti Polar Lipids (Alabaster, AL, USA). Sephadex® G-25M prepacked size exclusion columns were purchased from GE Healthcare Bio-sciences Corporation (Piscataway, NJ). Dialysis tubes (Float-A-Lyzer®, MWCO: 100,000) were obtained from Spectrum Laboratories (Rancho Dominguez, CA, USA). All other reagents and HPLC solvents were obtained from Fisher Scientific (Florence, KY, USA).

Preparation and Pegylation of Unilamellar Vesicles for Membrane Binding and Permeability Studies

Various types of unilamellar vesicles were prepared for membrane binding and permeability studies, including vesicles containing both drug and HPβCD, blank vesicles loaded with HPβCD but containing no drug, vesicles containing drug but no HPβCD, and blank vesicles containing no drug or HPβCD. Buffers (60 mM Na citrate [pH 3.5–6.5], 70 mM Na phosphate [pH 7–7.8], 50 mM Na borate [pH 8.5–8.8] and 60 mM Na carbonate [pH 9.5]) were employed in the preparation of all vesicles.

Preparation of Vesicles Containing HPβCD

HPβCD solutions were prepared by adding a weighed amount of HPβCD to 2 mL of buffer (pH 3.5–9.5). Solutions were prepared at varying HPβCD concentrations (10–50 mM) at low pH (3.5–4) while solutions at pH>4 contained 50 mM HPβCD. An aliquot of a stock solution of DB-67 in DMSO (1 mM) was added to the HPβCD solutions to provide DB-67 concentrations of 1–10 μM for use in preparing vesicles containing both HPβCD and DB-67. The final osmolality of each of the above solutions was adjusted to 300 mOsm with NaCl. Two milliliter aliquots of each HPβCD solution (with or without DB-67) were added to test tubes containing a 100 mg film of DSPC (prepared as described previously (6)) and unilamellar vesicle suspensions containing 50 mg DSPC/mL (diameter=200 nm) were prepared by the hydration–extrusion procedure (6) at 60°C. The final osmolalities of the extravesicular solutions in all vesicle preparations were adjusted to match the osmolality of the entrapped solution.

All vesicle suspensions containing HPβCD were pegylated following their preparation by first storing them at room temperature for 30 min, then transferring them into a dialysis tube to remove the unentrapped HPβCD by dialyzing against 1 L of buffer having a pH and osmolality matching that of the entrapped solution at 37°C for 6 h. (The removal of unentrapped HPβCD by the dialysis method was validated in separate experiments using blank vesicles spiked with HPβCD). Following removal of the unentrapped HPβCD, the vesicle suspensions were equilibrated at 60°C and pegylated by addition of a stock solution of m-PEG DSPE (100 mg/mL (prepared in corresponding buffer)) to obtain vesicles pegylated on the outer lipid monolayer (5 mol%). Following addition of m-PEG DSPE, vesicles were stored at 60°C for 1 h and for 2 h at room temperature.

Preparation of Vesicles Containing No HPβCD

Vesicles containing drug but no HPβCD and blank vesicles containing no drug or HPβCD were prepared as described earlier but in the absence of cyclodextrin. The final osmolalities of the blank buffers or drug solutions were adjusted to 300 mOsm with NaCl and 2 mL of unilamellar vesicles were prepared containing 50 mg DSPC /mL. The final osmolalities of the extravesicular solutions in all vesicle preparations containing no HPβCD were adjusted to match the osmolality of the entrapped solution.

Drug-loaded vesicles containing no HPβCD were pegylated while blank vesicles containing neither drug nor HPβCD were either pegylated or non-pegylated, depending on the application (blank vesicles used in membrane binding studies were non-pegylated). Non-pegylated vesicles were cooled at room temperature for 1 h after the extrusion process, then directly used in binding studies. Vesicle

suspensions (50 mg DSPC/mL) prepared in the absence of cyclodextrin were equilibrated at 60°C immediately after their preparation and pegylated as described above.

pH, Particle Size, and Osmolality Measurements

The pH of all the buffers and vesicles was monitored at each step of the formulation procedure and the pH of the corresponding buffers was adjusted (with 0.1 N HCl or NaOH) as required to the pH of the vesicles. The particle size of the vesicles was measured by dynamic light scattering (DLS) using a Malvern Zetasizer-3000 (Malvern Instruments Ltd, Malvern, UK) at each step of the formulation procedure. DLS was also employed as described previously (6) to validate the Sephadex® column separation of liposome entrapped *versus* free drug in the presence of HPβCD. The osmolality of drug solutions and vesicles was measured by the freezing point depression method (Model 110 Osmometer, Fiske Associates, Norwood, MA, USA). The osmolality of all the buffers used in these experiments was adjusted with NaCl to the internal osmolality of the vesicles to ensure that there were no osmolality gradients during the permeability studies.

Membrane Permeability Studies in the Presence or Absence of Entrapped HPβCD

The release of DB-67 from vesicles as a function of pH 4–8.5 was monitored by a previously (6) developed dynamic dialysis method. Liposomes were separated from the unentrapped drug and HPβCD by size exclusion chromatography on Sephadex® columns. At each pH, 0.1 mL of the liposome suspension was loaded onto a Sephadex® column (pre-conditioned with 50 mL of corresponding buffer) and eluted with 5 mL of buffer. The eluent liposome suspension (5 mL) was collected, immediately transferred to a dialysis tube, and dialyzed against 1 L of the same buffer at 37°C. At various times, 100 μL of liposome suspension was withdrawn from the dialysis tube and diluted into 900 μL of a mixture of cold (−25°C) methanol/acetonitrile (2:1; (*v*/*v*)) to quench the carboxylate/lactone interconversion reaction. The pipette tip used for sampling was washed in the same quenching solution to transfer any adsorbed drug. The quenched samples were stored at −25°C prior to their analysis for DB-67 lactone and carboxylate concentration by HPLC. Entrapped HPβCD concentration was determined after 1 mL of the original undiluted liposome suspension (pH 3.5–8.5) was dialyzed for 2 h at 37°C against 1 L of the corresponding buffer to remove any unentrapped cyclodextrin that was not completely removed during the dialysis step prior to pegylation. Following dialysis, samples were withdrawn from the dialysis tube, diluted into methanol, and stored at −25°C until HPLC analysis.

The effect of intravesicular cyclodextrin (50 mM) on drug retention under physiological conditions (pH 7.4, 296 mOsm) was investigated by studying drug release from vesicles prepared from pH 9.5 buffer in pH 7.4 carbonated phosphate buffered saline (C-PBS) as described previously (5). In these studies, the extravesicular buffer was exchanged for C-PBS buffer by size exclusion similar to the permeability studies described earlier and the vesicles were dialyzed against 1 L of C-PBS buffer while taking samples at various time intervals. The sampling and processing conditions were the same as in the pH-permeability studies described earlier.

DB-67 Membrane Binding Studies in Pegylated and Non-pegylated Liposome Suspensions

Since vesicles in the present studies were pegylated only on the outer monolayer, the effect of asymmetric lipid distribution on the membrane binding of DB-67 lactone was investigated by measuring the changes in fluorescence spectra of DB-67 with varying lipid concentration (0.05–2.5 mg lipid/mL). The fluorescence emission spectrum (FluoroMax-3, Jobin Yvon Inc, Edison, NJ, USA, λ_{ex}=380 nm) of a 2 mL aliquot of a 100 nM DB-67 lactone solution in pH 4 citrate buffer at 37°C was first recorded. Then aliquots of stock suspensions of pegylated or non-pegylated blank vesicles (50 mg DSPC/mL in pH 4 citrate buffer) were added incrementally to the corresponding drug solution and emission spectra were immediately recorded after each addition. Light scattering from vesicles was reduced by a long pass filter (400 nm cut off) in the emission pathway. Fluorescence emission spectra were recorded (in parallel) on blank vesicles and used as a control to further correct for light scattering.

HPLC Analyses

Drug (lactone or carboxylate) analyses utilized an isocratic HPLC method with fluorescence detection. Standards for DB-67 lactone and carboxylate were prepared in methanol and 10 mM carbonate buffer (pH 10.5), respectively. The solvents, column, and chromatographic system employed in the analyses and the relevant method validations have been described elsewhere (6).

HPβCD was analyzed by gradient HPLC with evaporative light scattering detection (ELSD, Sedere Inc., Lawrenceville, NJ, USA) using a Metasil® AQ (Metachem Technologies, Lake Forest, CA, USA) C-18 column (120 Å, 250×46 mm) with a linear gradient starting at 100% methanol, changing to 50% methanol: 50% acetonitrile (*v*/*v*) in 5 min, and 100% acetonitrile in 10 min. The gradient was changed back to 100% methanol in 15 min and total run time was 20 min at a flow rate of 1 mL/min. The sample injection volume was 10 μL. The sample compartment and column holder were at ambient temperature. ELSD conditions included a gain of 8, temperature of 50°C and pressure of 2.6 lb. Standards for HPβCD (100–500 μM) were prepared in methanol and all experimental samples were diluted to this concentration range in methanol for analysis. The detector response factor was calculated using a log concentration *versus* log peak area calibration curve. The retention time for HPβCD was approximately 5.5 min. The limit of quantitation was 10 μM.

Theory

Membrane Permeability Studies

In the pH region explored (pH 3.5–8.5), DB-67 exists predominantly as two different species (Fig. 1). The neutral carboxylic acid species concentration can be assumed to be negligible (23). Previous studies (5) have shown that the

transport of the neutral lactone accounts for the permeability observed at any pH. Thus, the apparent permeability is controlled by the fraction of unbound neutral species (5). In HPβCD containing vesicles, entrapped DB-67 may exist as unbound lactone or carboxylate, HPβCD complexes or as various membrane bound species. Binding of HPβCD to the lipid bilayer was assumed to be negligible. The complexation, partitioning and transport equilibria considered in the present treatment are shown in Fig. 2. In the pH region 3.5–8.5, the total concentration of drug inside the vesicle is:

$$D_i = \frac{[L^{\mathrm{w}}] + [L{-}\mathrm{CD}] + [C^{\mathrm{w}}] + [C{-}\mathrm{CD}]}{a} + \frac{[L^{\mathrm{m}}] + [C^{\mathrm{m}}]}{b} \quad (1a)$$

$$D_i = \frac{L^w}{a}\left(1 + \frac{K_a^w}{H^+}\right) + \frac{L{-}CD}{a}\left(1 + \frac{K_a^{CD}}{H^+}\right) + \frac{L^m}{b}\left(1 + \frac{K_a^m}{H^+}\right) \quad (1b)$$

where a and b are the ratios of the total intravesicular volume to that of the intravesicular aqueous volume and the inner monolayer volume, respectively; D_i is the total intravesicular concentration of DB-67; L^{w} and L^{m} are the concentrations of DB-67 in the intravesicular aqueous and membrane phases, respectively; L-CD is the aqueous concentration of lactone complex with HPβCD; C^{w} and C^{m} are the concentrations of DB-67 carboxylate in the intravesicular aqueous and membrane phase, respectively; C-CD is the concentration of carboxylate complex with HPβCD; K_a^{w}, K_a^{CD} and K_a^{m} are the effective ionization constants of DB-67 lactone in the aqueous phase, as a cyclodextrin complex, and in the membrane phase, respectively.

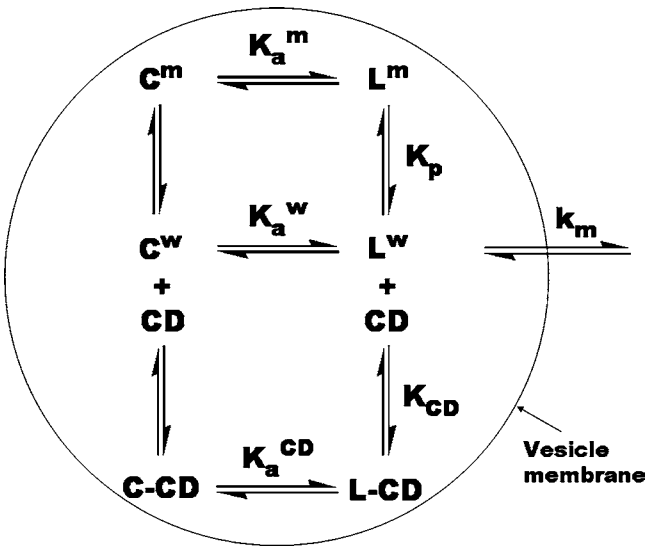

Fig. 2. The various intravesicular ionization and complexation equilibria in the presence of a cyclodextrin. K_a^{w}, K_a^{CD} and K_a^{m} are the ionization constants for free, complexed, and membrane bound lactone, respectively. K_p and K_{CD} are the membrane/water partition coefficient and cyclodextrin complexation constant of DB-67 lactone, respectively; and k_{m} is the membrane permeation rate constant of DB-67 lactone.

Equation 1b can be expressed in terms of intravesicular aqueous lactone concentration (L^{w}) as follows:

$$D_i = \frac{L^{\mathrm{w}}}{a}\left\{\left(1 + \frac{K_a^{\mathrm{w}}}{H^+}\right) + K_{\mathrm{CD}}[\mathrm{CD}]\left(1 + \frac{K_a^{\mathrm{CD}}}{H^+}\right)\right\} + \frac{K_p L^{\mathrm{w}}}{b}\left(1 + \frac{K_a^{\mathrm{m}}}{H^+}\right) \quad (1c)$$

where [CD] is the intravesicular concentration of cyclodextrin, K_{CD} is the complexation constant for DB-67 lactone with HPβCD, and K_p is the membrane/water partition coefficient of DB-67 lactone. The apparent permeability coefficient of DB-67 (P_{app}) across the vesicle is:

$$P_{\mathrm{app}} = P_{\mathrm{m}} f_{\mathrm{ub}} \quad (1d)$$

where P_{m} is the intrinsic permeability coefficient of DB-67 and f_{ub} is the fraction of unbound lactone inside the vesicle. The fraction of unbound lactone in the vesicle (from Eq. 1c) is:

$$f_{\mathrm{ub}} = \frac{bH^+}{b\{(H^+ + K_a^{\mathrm{w}}) + K_{\mathrm{CD}}[\mathrm{CD}](H^+ + K_a^{\mathrm{CD}})\} + aK_{\mathrm{p}}(H^+ + K_a^{\mathrm{m}})} \quad (1e)$$

In the absence of intravesicular CD, the fraction of unbound species is:

$$f_{\mathrm{ub}} = \frac{bH^+}{b(H^+ + K_a^{\mathrm{w}}) + aK_p(H^+ + K_a^{\mathrm{m}})} \quad (1f)$$

The concentration–time profiles obtained at each pH in the presence or absence of intravesicular HPβCD were fit to the following relations to estimate k_{app}, the apparent rate constant for release from the vesicles:

$$D_t = \frac{D_i}{x} + D_o \quad (1g)$$

$$\frac{dD_i}{dt} = -k_{\mathrm{app}}(D_i - D_o) - k_{\mathrm{d}} D_o \quad (1h)$$

where D_t is the total drug concentration in the dialysis tube, D_o is the extravesicular concentration in the dialysis tube, x is the ratio of the total intravesicular to the extravesicular volume in the dialysis tube, and k_{d} is the rate constant for transport across the dialysis tube. The apparent permeability coefficient at each pH was calculated from the apparent rate constant for release:

$$P_{\mathrm{app}} = \frac{dk_{\mathrm{app}}}{6} \quad (1i)$$

where d is the diameter of the vesicle. The ratio of lactone to carboxylate in the vesicle during release studies was used to estimate the apparent ionization constant in the intravesicular microenvironment. In the presence or absence of HPβCD, the apparent ionization constant (K_a') of DB-67 inside the vesicles can be related to the effective ionization constants of

aqueous, membrane bound and HPβCD complexed lactone; and the membrane/water partition coefficient and cyclodextrin binding constant of lactone as follows:

$$K'_a = \frac{b\{K_a^w + K_{CD}K_a^{CD}[CD]\} + aK_pK_a^m}{b\{1 + K_{CD}[CD]\} + aK_p} \quad (2a)$$

The apparent permeability coefficients of DB-67 as a function of pH and the apparent ionization constant of DB-67 lactone in the vesicle (obtained from lactone to carboxylate ratios) in the absence or presence of HPβCD were fit to Eqs. 1d–1f and 2a to obtain estimates of equilibrium constants (i.e., K_a^{CD} and K_{CD}) and the intrinsic permeability coefficient, P_m. The membrane/water partition coefficient and HPβCD complexation constant of DB-67 carboxylate were obtained from the following relations:

$$K_{CD}^C = \frac{K_a^{CD}K_{CD}}{K_a^w} \quad (2b)$$

$$K_p^C = \frac{K_a^m K_p}{K_a^w} \quad (2c)$$

where K_{CD}^C and K_p^C are the HPβCD complexation constant and the membrane/water partition coefficient of DB-67 carboxylate, respectively.

Membrane Binding Studies

Membrane binding of DB-67 lactone was estimated from the changes in fluorescence properties of DB-67 as a function of increasing lipid concentration. The half-life for liposomal transport of drug was ≥3 h. Since fluorescence measurements in the presence of the vesicles were completed within 30 min, the drug was assumed to be predominantly bound to the outer monolayer. The total fluorescence intensity of DB-67 at each wavelength is:

$$I_t = I_m f_m + I_w(1 - f_m) \quad (3a)$$

$$f_m = \frac{cK_p}{d + CK_p} \quad (3b)$$

where I_t is the total fluorescence intensity of DB-67, I_m and I_w are the intensities of the aqueous and membrane bound species, respectively, f_m is the fraction of species bound to the outer monolayer of vesicles, K_p is the membrane/water volume partition coefficient, and c and d are the ratios of the total extravesicular volume to that of the extravesicular aqueous volume and membrane outer monolayer volume, respectively. Data for the fluorescence emission intensity maximum (λ_{em}=450 nm) obtained at varying lipid concentrations were simultaneously fit to Eqs. 3a–3b to obtain the values of I_m, I_w and K_p.

All nonlinear regression analyses were performed using Scientist®, Micromath Scientific Software, St. Louis, MO, USA.

RESULTS

Validation of Experimental Methods

Drug loaded liposomes for permeability studies were prepared in the presence or absence of HPβCD. For HPβCD-containing vesicles, a dialysis step (prior to pegylation) was employed to remove the unentrapped cyclodextrin. In the absence of this step, the unentrapped drug could not be efficiently separated from the liposome loaded drug by size exclusion prior to drug release studies. This is demonstrated in Fig. 3, where the effect of extravesicular HPβCD on the separation of liposomes (monitored by DLS measurements as previously reported (6)) from free drug is shown. Panel A shows the separation of liposomes from unentrapped DB-67 when 0.1 mL of blank vesicles (50 mg DSPC/mL, pH 4) spiked to produce concentrations of 50 mM HPβCD and 100 μM DB-67 were passed through a size exclusion column. Figure 3B shows the size exclusion separation of the same vesicles after dialysis for 6 h against an isoosmolar and iso-pH buffer (1 L, 37°C). The efficiency of the dialysis method to remove the extravesicular cyclodextrin was validated by dialyzing vesicles (pH 4, [lipid]=1 mg/mL) spiked to contain DB-67 (100 nM), cyclodextrin (0.5 mM) or drug–CD complex solution (0.5 mM HPβCD, 100 nM DB-67). The apparent first-order rate constants for the loss of DB-67 from inside the dialysis tube (at 37°C) in the presence or absence of cyclodextrin were found to be 1.13±0.17 (95% CI) and 0.55±

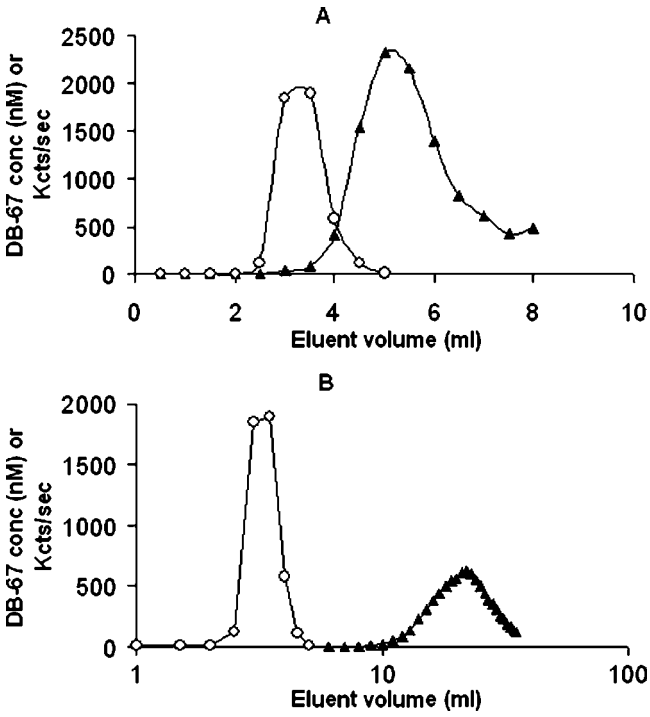

Fig. 3. Elution profiles of liposomes (*empty circles*) or DB-67 (*filled triangles*), when liposomes (0.1 mL, 50 mg/mL DSPC) spiked with drug–CD complexes (50 mM cyclodextrin, 100 μM DB-67) were passed through a Sephadex® column in the presence (**A**) or absence (**B**) of extravesicular cyclodextrin. The extravesicular cyclodextrin was removed by dialysis in **B**. DB-67 concentration (nM) was analyzed by HPLC and liposomes (Kcts/s) were analyzed by light scattering.

0.08 h^{-1}, respectively, corresponding to half-lives of 0.61±0.1 and 1.26±0.18 h. The rate constant for loss of HPβCD (in the absence of DB-67) was found to be 0.51±0.07 h^{-1} (95% CI), corresponding to a half-life of 1.35±0.18 h. Therefore, a 6 h dialysis was adequate to remove unentrapped cyclodextrin prior to pegylation.

A 50 mM HPβCD solution was used to prepare vesicles for pH permeability studies. Figure 4 shows the representative concentrations of entrapped cyclodextrin as a function of pH in the vesicles used in these studies. The intravesicular concentration of cyclodextrin was determined to be 49.1± 6.9 mM (mean±SD) and was independent of pH and drug concentration. The dashed line in Fig. 4 is the theoretical concentration assuming cyclodextrin is entrapped completely in the aqueous core. These results suggest that HPβCD is entrapped only in the intravesicular aqueous compartment and does not bind significantly to the liposome membrane. This is consistent with previous observations (16,19,20,22) that reported a lack of interaction between HPβCD and gel phase lipid bilayers.

The particle size of the vesicles used in the present study was 195±60 nm (mean±SD) and the final osmolality of all the vesicles (pH 4–8.5) used in the permeability studies was 298±9 mOsm (mean±SD). A small increase in the mean particle size (by approximately 10 nm) was observed after pegylation, likely due to the PEG chain that is not incorporated into the bilayer after pegylation, consistent with previous reports (24).

Membrane Binding

Fluorescence spectra were measured (in parallel) on blank vesicles and drug loaded vesicles (of similar lipid concentration) and the signal from blank vesicles was subtracted from the total fluorescence signal in the presence of DB-67. Figure 5 shows the effect of increasing lipid concentration on the observed changes in the emission spectra of DB-67 at 37°C. Figure 6 shows representative non-linear regression plots generated by fitting the emission intensity in the presence or absence of m-PEG DSPE to Eqs. 3a–3b. The values for K_p were found to be 2,485±261 (95% CI) and 2,040±650 (95% CI) in the presence and absence of m-PEG DSPE (on the outer monolayer), respectively. These values suggest that pegylation does not significantly alter the binding constant of lactone to the gel phase vesicles employed.

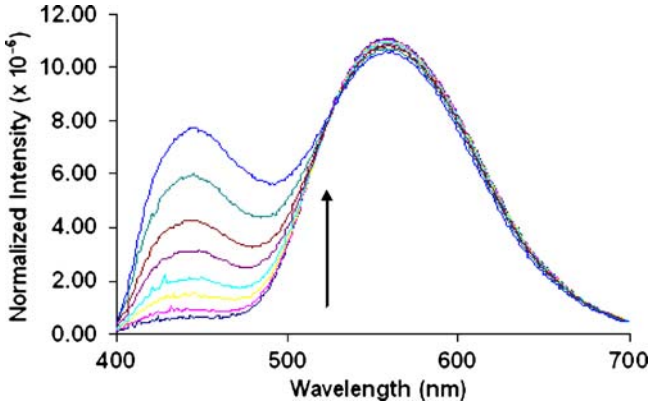

Fig. 5. Normalized fluorescence emission spectra (λ_{ex}=380 nm) of DB-67 lactone as a function of increasing suspension lipid concentration (0.05–2.5 mg lipid/mL), observed when a 100 nM DB-67 solution was titrated with pegylated unilamellar vesicles at 37°C. The arrow indicates the direction of increasing lipid.

Membrane Permeability

The liposomal transport of DB-67 at varying pH (3.5–8.5) was monitored in the presence or absence of intravesicular HPβCD (50 mM) at 1 mg/mL lipid concentration. The effect of varying HPβCD concentration (0–50 mM) was probed only at low pH (3.5–4). DB-67 has been previously found to form both 1:1 and 1:2 complexes with HPβCD [25]. Due to the low concentration (50 mM) of HPβCD employed, only 1:1 complexes were assumed to be relevant. Intra-

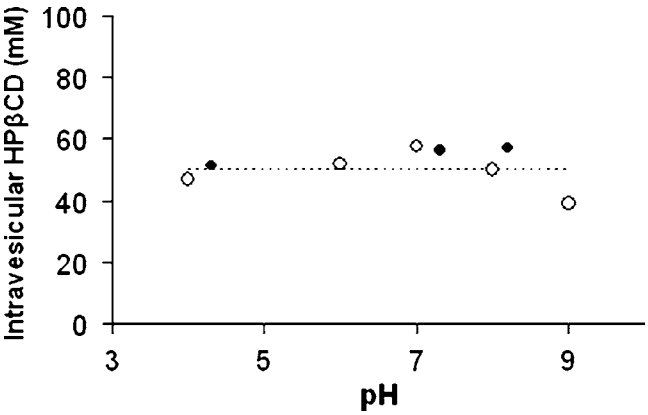

Fig. 4. Measured intravesicular concentrations of HPβCD in blank (*empty circles*) or drug-HPβCD (*filled circles*) loaded liposomes prepared at varying intravesicular pH during the pH–permeability studies. The *dashed line* represents the theoretically predicted concentration assuming aqueous entrapment. HPβCD was analyzed by HPLC with ELSD detection.

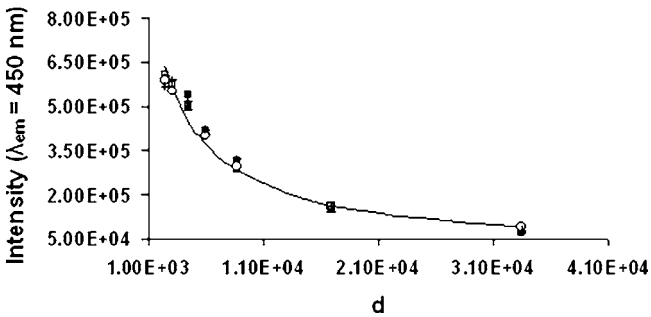

Fig. 6. Effect of decreasing suspension lipid concentration on the fluorescence emission intensity of DB-67 (λ_{em}=450 nm) in the presence of m-PEG DSPE on the outer monolayer at 37°C. Similar non-linear regression plots were generated in the absence of m-PEG DSPE on the outer monolayer (data not shown). *d* is the ratio of the total extravesicular volume to the membrane outer monolayer volume. The value of *d* was varied by increasing [lipid] in the suspensions. The *symbols* represent experimental measurements (n= 3) and the *solid lines* are fits of the experimental data to Eqs. 3a–3b.

vesicular DB-67 lactone and carboxylate can exist in three independent drug pools (i.e., free, lipid bound, or complexed) (see Fig. 2). The ionization constant of membrane bound DB-67 was previously (6, 7) shown to be approximately 1.5 pK_a units higher than that for the free drug. Cyclodextrin complexation was also expected to shift the ionization constant of DB-67. Therefore, three different ionization constants $\left(K_a^{w}, K_a^{wm} \text{ and } K_a^{wCD}\right)$ were used to represent the ionization of free, membrane bound, and complexed DB-67 lactone, respectively (see "Theory"). The concentration–time profiles obtained for the loss of DB-67 from inside the dialysis tube (at varying pH) in the presence or absence of intravesicular HPβCD were fit to Eqs. 1g–1h to estimate the apparent rate constants for release from vesicles, from which apparent permeability coefficients were determined using Eq. 1i.

Initial studies of the effect of varying intravesicular cyclodextrin (0–50 mM) at low pH (3.5–4) suggested that the apparent permeability coefficient at this pH was independent of intravesicular cyclodextrin concentration. Figure 7 shows the observed apparent permeability coefficients of DB-67 as a function of intravesicular HPβCD concentration at pH 4. At this pH, DB-67 is completely in its lactone form both in aqueous solution and in the intravesicular microenvironment. Based on the previously (25) reported complexation (1:1) constant of DB-67 with HPβCD (K_{CD}=5,800±200 M^{-1}, 25°C), the percent of DB-67 complexed inside the vesicles was predicted to vary from 14% to 64% with varying cyclodextrin concentration (0–50 mM). This was expected to alter the fraction of unbound drug inside the vesicle and consequently the apparent permeability coefficient of DB-67. However, as evident from Fig. 7, there was no significant change in the apparent permeability coefficient of DB-67 with varying intravesicular HPβCD concentration or in comparison to that obtained ($1.1\pm0.31\times10^{-10}$ cm/s [95% CI]) in the absence of cyclodextrin.

The effect of varying intravesicular pH on the apparent permeability was probed only at a single (50 mM) cyclodextrin concentration. The representative apparent release profiles for loss of DB-67 as a function of pH from inside the dialysis tube in the presence of 50 mM intravesicular cyclodextrin are shown in Fig. 8. Panel A in Fig. 9 shows the representative observed fractions of DB-67 lactone at various pH values during the release studies in the presence of 50 mM cyclodextrin. The constant fraction of lactone throughout the transport experiment at each pH (in the absence or presence of HPβCD) was consistent with previous observations [6] suggesting that aqueous, cyclodextrin and membrane bound fractions of DB-67 carboxylate and lactone exist in equilibrium inside the vesicles during the release

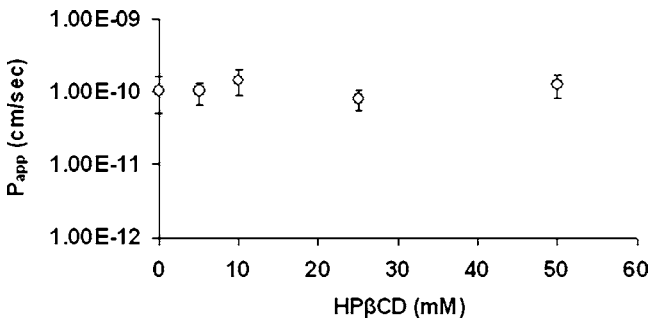

Fig. 7. Effect of varying intravesicular HPβCD concentration on the apparent (mean±SD) permeability (*empty circles*) coefficient of DB-67 at pH 4 (37°C).

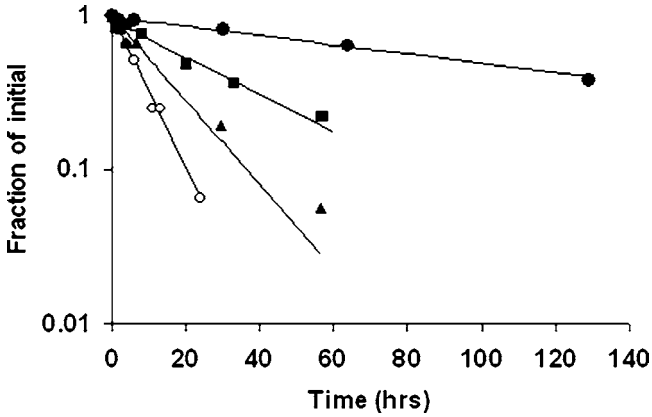

Fig. 8. Fractions of initial DB-67 remaining inside the dialysis tube at pH 3.8 (*empty circles*), 7.4 (*filled triangles*), 7.7 (*filled squares*) and 8.6 (*filled circles*) during release studies (37°C) in the presence of intravesicular HPβCD (50 mM). The *solid lines* are model fits (Eq. 1g–1h) of the experimental data assuming a first-order loss of DB-67 from inside the dialysis tube following release from vesicles.

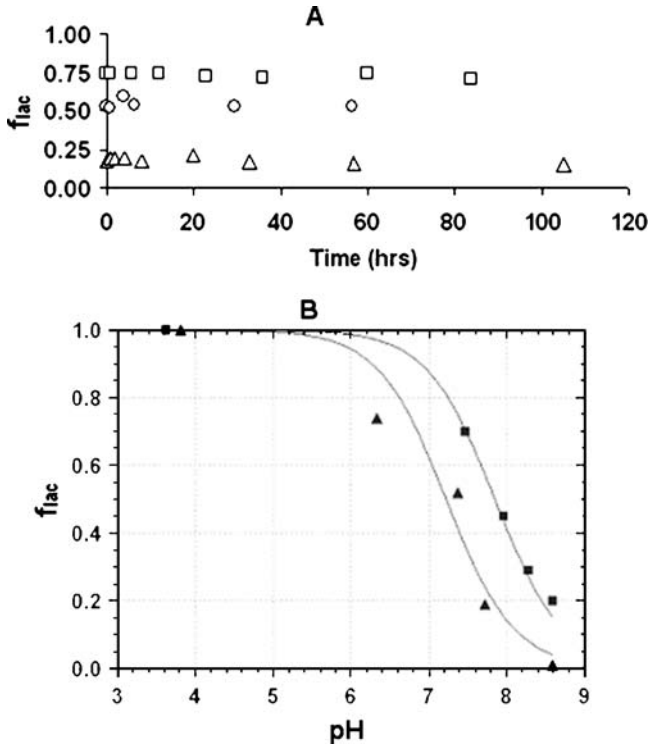

Fig. 9. A Representative fractions of DB-67 lactone inside the dialysis tube during the release studies (37°C) at pH 6.3 (*empty squares*), 7.4 (*empty circles*) and 7.7 (*empty triangles*) from vesicles prepared in the presence of 50 mM HPβCD. **B** Fractions of lactone remaining in the dialysis tube as a function of pH in the presence (*filled triangles*) or absence (*filled squares*) of intravesicular HPβCD (50 mM) during release studies (37°C). The *solid line* is a fit of the fraction of lactone to an equilibrium model ($f_{lac} = \frac{H^+}{H^+ + K_a'}$, where K_a' is the apparent ionization constant in the vesicle) assuming ionization of a monoprotic weak acid.

experiments and therefore an equilibrium permeability model was justified in the analyses of transport data. Panel B in Fig. 9 shows the fractions of lactone observed in release studies (37°C) as a function of pH. The lactone to carboxylate ratios were used to estimate the apparent ionization constant $\left(K_a'\right)$ of DB-67 in the vesicle in the absence or presence of HPβCD. These values were $1.28 \pm 0.41 \times 10^{-8}$ $\left(pK_a' = 7.9 \pm 0.3\right)$ and $6.21 \pm 0.17 \times 10^{-8}$ $\left(pK_a' = 7.2 \pm 0.3\right)$, respectively, in the absence and presence of 50 mM intravesicular HPβCD. The apparent permeability coefficients in the presence or absence of HPβCD were fit to Eqs. 1d–1f, while constraining the apparent ionization constants (obtained from the lactone/carboxylate ratios) in the vesicle to Eq. 2a, to fit for the parameters K_a^{CD}, K_{CD} and P_m. The cyclodextrin complexation constant and the membrane/water partition coefficient of DB-67 carboxylate were estimated from Eqs. 2b–2c.

Figure 10 shows the observed (symbols) and fitted (lines) apparent permeability coefficient–pH profiles in the presence or absence of HPβCD (50 mM). The intrinsic permeability coefficient of DB-67 was found to be $1.1 \pm 0.15 \times 10^{-8}$ cm/s (95% CI), approximately one-third that observed previously ($3.5 \pm 1 \times 10^{-8}$ cm/s) in vesicles pegylated on both monolayers. This suggests a change (improvement) in barrier properties in vesicles pegylated only on the outer monolayer, possibly due to improved lipid packing on the inner monolayer in the absence of m-PEG DSPE. The binding constants of DB-67 lactone and carboxylate to HPβCD were estimated to be 1,552±875 (95% CI) and 230±150 M^{-1} (95% CI), respectively. The binding constant of DB-67 lactone estimated here at 37°C is significantly lower than that generated in solubility studies of DB-67 (5,800±200 M^{-1}, 25°C). The ionization constant of HPβCD-bound DB-67 lactone was found to be $5.04 \pm 0.88 \times 10^{-8}$ $\left(pK_a^{CD} = 7.3 \pm 0.17\right)$, reflecting a 0.8 upward shift in pK_a of the complex compared to the free lactone $\left(pK_a^{w} = 6.5 \pm 0.1\right)$. The membrane/water partition coefficient of DB-67 carboxylate was estimated to be 64±23 (95% CI), lower than that obtained previously (140±24) in pre-pegylated vesicles. In the absence of intravesicular HPβCD, the ionization constant

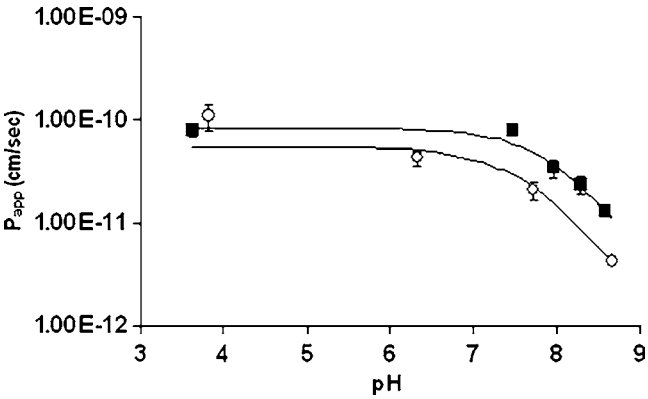

Fig. 10. Apparent (mean±SD) permeability coefficients of DB-67 as a function of pH (37°C) in the presence (*empty circles*) or absence (*filled squares*) of 50 mM HPβCD inside the vesicles. The *solid lines* were generated by fitting the observed permeability coefficients to an equilibrium permeability model (Eqs. 1d–1f), while constraining the apparent ionization constant in the vesicle to Eq. 2a.

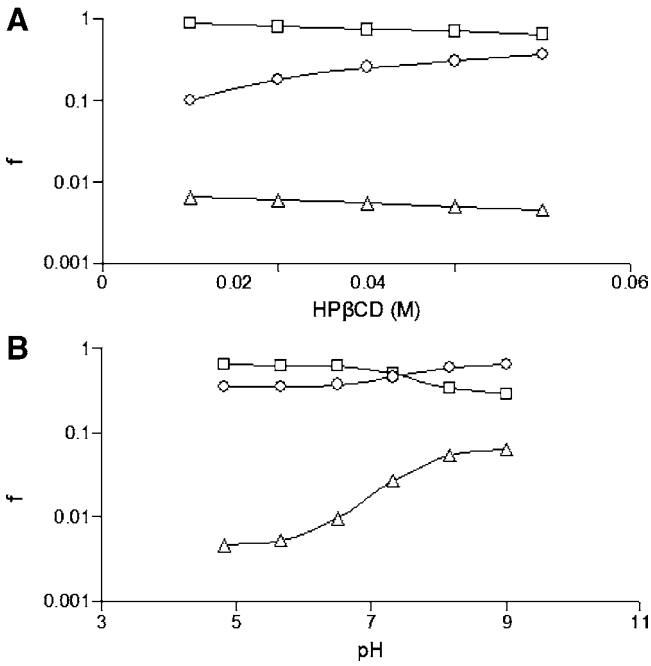

Fig. 11. Simulated fractions of free (*triangles*), cyclodextrin bound (*circles*) and membrane bound (*squares*) DB-67 in the presence of varying intravesicular HPβCD (pH 4, **A**) or pH (50 mM HPβCD, **B**).

$\left(K_a^{m}\right)$ of membrane bound DB-67 was calculated (using Eq. 2a with [CD]=0 mM) to be $1.04 \pm 0.15 \times 10^{-8}$ $\left(pK_a^{m} = 7.98 \pm 0.16\right)$, similar to that in pre-pegylated vesicles. Based on the observed membrane/water partition coefficient and HPβCD complexation constant of DB-67 lactone, panel A in Fig. 11 shows the estimated fraction of DB-67 lactone (pH 4) in various drug pools (i.e. as free, complexed, or membrane bound drug) in the presence of intravesicular HPβCD. Panel B in Fig. 11 shows the fraction of total drug in various pools as a function of varying intravesicular pH. Thus, at low intravesicular pH, DB-67 exists in its lactone form which is predominantly bound to the bilayer membrane. Upon increasing the intravesicular pH, the fraction of DB-67 bound to the bilayer membrane decreases and the fractions of free and complexed drugs increase, resulting in an increase in the fraction of ionized species inside the vesicle.

The effect of intravesicular HPβCD (50 mM) on the release of DB-67 was assessed by dialyzing vesicles (prepared in 60 mM carbonate buffer at pH 9.5) in a C-PBS buffer at pH 7.3, a buffer containing a physiological concentration of carbonate. C-PBS buffer was previously (6) shown to mimic *in vivo* conditions due to the ability of CO_2 to rapidly dissipate the trans-membrane pH gradients. Mathematical models to predict the change in intravesicular pH when dialyzing vesicles against this buffer were also developed previously (6). At an initial intravesicular pH of 9.3 and extravesicular pH of 7.3, the intravesicular pH was predicted to drop to a pH of 7.75 at equilibrium and the half-life for drug release was found to be 28.4±6.9 h. From the pH–permeability profile in the presence of HPβCD, the half-life at pH 7.75 can be estimated to be approximately 39 h. These values are similar, and significantly longer than the ~12 h half-life at the same pH in the absence of HPβCD.

DISCUSSION

Effect of HPβCD on the Liposomal Transport of DB-67, a Model Hydrophobic Weak Acid

The high membrane binding of hydrophobic weak acids results in an upward shift in pK_a leading to an increase in apparent fraction of the neutral species inside the vesicles. The dissipation in the trans-bilayer pH gradient caused by an influx of CO_2 when present at physiological concentrations at pH 7.4 (6) reduces the *in vivo* effectiveness of a strategy to retain hydrophobic weak acids in their membrane impermeable, ionized form inside liposomes. In contrast, hydrophobic weak bases can be successfully maintained in a membrane impermeable protonated form by using a low intravesicular pH (3). This inability to maintain a high intravesicular pH stimulated a search for alternative strategies to improve the retention of the model hydrophobic weak acid (DB-67). Therefore, the ability of HPβCD to complex DB-67 and decrease the fraction of membrane permeable species was explored.

Pegylated unilamellar vesicles (pH=3.5–8.5, [DSPC]= 50 mg/mL) were prepared in a two-step approach (see "MATERIALS AND METHODS") to selectively pegylate the outer monolayer of the vesicles. DSPC vesicles were equilibrated for 1 h at 60°C following addition of m-PEG DSPE to allow for incorporation of the pegylated lipid into the outer monolayer based on kinetic studies by Uster *et al.* (24). The incorporated m-PEG DSPE is not expected to cross the bilayer due to the very slow trans-bilayer flip-flop rate constant in the gel phase (26).

Previous reports (8–14) on the effect of cyclodextrins on the loading and release of drugs encapsulated in vesicles have been largely inconclusive due to the unusual release kinetics often observed, where an initial burst effect was attributed to a membrane destabilizing effect of the cyclodextrin. This has prompted several investigators (16,19–22) to probe the effect of various cyclodextrins on membrane stability. Some cyclodextrins such as methyl-β-cyclodextrin can bind to lipids and alter their thermotropic properties, while other cyclodextrins such as HPβCD and SBEβCD do not appear to interact with the bilayer. Piel *et al.* (22) and Hatzi *et al.* (16) investigated the ability of various cyclodextrins to induce release of carboxyfluorescein at cyclodextrin to lipid molar ratios as high as 20. They found gel phase (DSPC) vesicles to be very stable under these conditions in the presence of hydroxypropyl cyclodextrins. Piel *et al.* (22) reported a complexation efficiency of 0 for complexation of phosphatidylcholine to HPβCD.

An initial burst phase was also observed in preliminary release studies for the present work, which we attributed to the failure to completely remove extravesicular HPβCD and unentrapped drug (panel A, Fig. 3) and the initial rapid loss of extravesicular drug (that could not be separated by Sephadex® elution) from inside the dialysis tube. This was avoided by use of a dialysis step prior to size exclusion and release studies to separate unentrapped HPβCD after vesicle preparation. This significantly improved the separation of entrapped from unentrapped drug prior to the release studies (panel B, Fig. 3). An investigation of the amount of HPβCD entrapped in the vesicles revealed (Fig. 4) that regardless of pH, concentration (0–50 mM) of HPβCD, or the presence or absence of DB-67, loading of HPβCD is through aqueous entrapment. Further, when blank vesicles loaded with 50 mM HPβCD were dialyzed against 2 L of corresponding buffer for 40 h, the concentration of HPβCD stayed constant following loss of the unentrapped cyclodextrin (data not shown). Thus, the present HPβCD loading and release studies confirm previous observations that HPβCD may not bind to gel phase membranes or passively permeate the lipid bilayer on the time scale of these studies.

The permeability of DB-67 lactone was investigated as a function of HPβCD concentration (0–50 mM). At the highest cyclodextrin concentration (50 mM), the molar ratio of intravesicular HPβCD to lipid (125 mM) is 0.4. The intravesicular lipid concentration depends only on the size of the vesicle and therefore is relatively constant in all these studies since all the vesicles used were similar in size (195±60 nm). Panel A in Fig. 11 shows the simulated intravesicular fractions of free, complexed, and lipid bound DB-67 lactone based on its estimated membrane/water partition coefficient and HPβCD complexation constant. The fraction of unbound drug at pH 4 is relatively insensitive to the change in concentration of intravesicular HPβCD. For example, upon increasing the intravesicular concentration of HPβCD from 10 to 50 mM, the fraction of unbound lactone species decreases from 0.0064 to 0.0045. This would suggest that the apparent permeability should decrease (see "Theory") by approximately 30% upon increasing the intravesicular HPβCD from 10 mM from 50 mM. However, the apparent permeability coefficients (Fig. 7) observed at various cyclodextrin concentrations were not different from each other and were also not significantly different from that observed in the absence of HPβCD ($1.1\pm0.31\times10^{-10}$ cm/s). This is likely due to the difficulty in experimentally detecting such a small decrement in permeability given the error of permeability estimates. This relative insensitivity of lactone permeability to changing intravesicular HPβCD concentration is due to the high local concentration of lipid inside the vesicle relative to the cyclodextrin concentration. Under these conditions, DB-67 is nearly completely bound to the lipid and the fraction of unbound drug (which is the driving force for permeation) is not greatly affected by cyclodextrin complexation.

Due to the lack of an effect on the apparent permeability of DB-67 lactone at varying intravesicular cyclodextrin concentrations, further studies to probe the effect of pH were performed at 50 mM HPβCD. The HPβCD complexation (1:1) constants of DB-67 lactone and carboxylate were found to be 1,552±875 and 230±150 M^{-1}, respectively; and the membrane/water partition coefficients of DB-67 lactone and carboxylate were found to be 2,100±450 and 64±23, respectively, inside the vesicle. The higher value for the lactone complexation constant reported previously (25) in solubility studies (5,800±200 M^{-1}, 25°C) is likely due to the difference in the temperature.

The ratios of lactone to carboxylate inside the vesicle during the release studies were used to calculate the apparent ionization constant in the intravesicular microenvironment. In the presence of 50 mM HPβCD, the mean apparent pK_a in the intravesicular microenvironment was found to be 0.7 units lower than that in the absence of cyclodextrin. Based on the observed pH–permeability profiles (Fig. 10) and the lactone to carboxylate ratios in the presence or absence of HPβCD,

the apparent ionization constants for the lactone complex and membrane bound lactone were estimated to be 7.3±0.17 and 7.98±0.16, respectively. Panel B in Fig. 11 shows the fractions of free, membrane bound, and complexed drug inside vesicles as a function of varying intravesicular pH determined from the estimated binding constants, partition coefficients, and ionization constants. In the pH region 4–7 liposomal DB-67 exists predominantly as the membrane bound lactone. Upon ring opening and ionization, the fraction of drug bound to the membrane decreases while the fractions of complexed and free drug increase due to the relatively greater affinity of DB-67 carboxylate for cyclodextrin compared to the lipid. This greater binding affinity of DB-67 carboxylate for HPβCD also decreases the apparent pK_a in the intravesicular microenvironment in the presence compared to that in the absence of HPβCD. This results in a greater fraction of membrane impermeable DB-67 carboxylate species during liposomal release studies in C-PBS (pH 7.4, 296 mOSm) and therefore improved $(t_{1/2} \sim 28h)$ drug retention when HPβCD is present.

Thus, the present studies suggest that the potential benefit of drug complexation with intravesicularly entrapped cyclodextrin for the case of ionizable, hydrophobic drugs, relies on the relative drug binding to the cyclodextrin *versus* the lipid membrane. With highly hydrophobic drugs such as DB-67, cyclodextrin may not significantly enhance drug retention if the drug is highly bound to the bilayer membrane in its neutral form. In such cases, the modification of intravesicular pH in conjunction with cyclodextrin complexation may enable enhancement of retention if the ionized species has relatively greater binding to cyclodextrin than the lipid.

Utility of Cyclodextrin as an Excipient in Liposome Formulations

Given the significant improvement in the retention of DB-67 when using HPβCD in combination with a high intravesicular pH, it would be of interest to ascertain the physical and chemical conditions under which cyclodextrins would be useful excipients in liposome formulations of hydrophobic drugs. Due to concerns with membrane stability as well as limits on intravesicular osmolality (physiological osmolality=300 mOsm), the type as well as concentration of cyclodextrin that can be used as an excipient in liposome formulations is limited. The relative complexation constant of the drug to cyclodextrin *versus* lipid affects both drug loading and retention in vesicles. Figure 12 shows the simulated effect of a varying complexation constant using DB-67 as an example on the apparent permeability at two different intravesicular HPβCD concentrations (50 and 100 mM). It can be seen that achieving a permeability decrement of at least an order of magnitude for a highly membrane bound drug requires that the drug have a relatively large complexation constant with the cyclodextrin (at least 10,000). Further, due to the high effective concentration of lipid inside the vesicles and limitations on the amount of cyclodextrin that can be used (due to concerns with bilayer stability and intravesicular osmolality), a significantly higher drug binding to cyclodextrin (relative to lipid) may be required to effectively decrease the apparent permeability of the neutral species of hydrophobic drugs. The relative complexation constants of drug to cyclodextrin and lipid may serve as an initial guide in selecting a cyclodextrin (or any other complexing agent) as an excipient in liposome formulations.

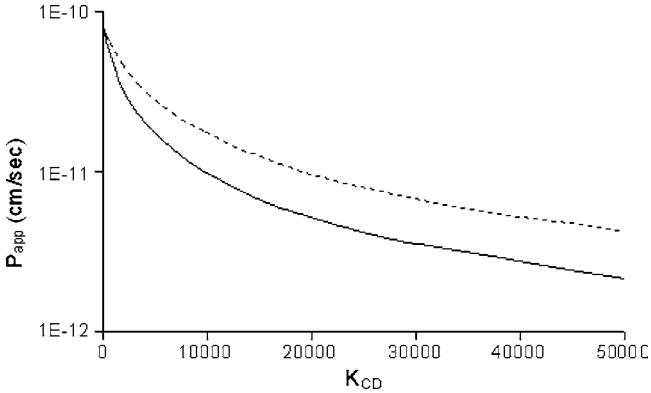

Fig. 12. Simulated effect of varying HPβCD complexation constant (K_{CD}) on the apparent permeability of DB-67 lactone in the presence of 50 (*dashed line*) and 100 mM (*solid line*) intravesicular HPβCD.

The modification of intravesicular pH in the presence of a cyclodextrin may further enhance liposomal retention of hydrophobic ionizable drugs, depending on the relative binding of the neutral *versus* the ionized species to the lipid and cyclodextrin.

CONCLUSIONS

In conclusion, the role of an entrapped complexing agent (HPβCD) on the liposomal retention of a model hydrophobic weak acid, DB-67, was systematically explored. An HPLC assay for analysis of HPβCD was developed and liposomal loading of HPβCD in the presence or absence of DB-67 was found to occur by aqueous entrapment. Within the range of HPβCD concentrations (0–50 mM) employed in these studies, the bilayer membrane was found to be stable consistent with previous studies. Due to the relatively high concentration of lipid compared to cyclodextrin in the intravesicular microenvironment, DB-67 was predominantly bound to lipid and there was no observable effect of cyclodextrin on the apparent permeability of the neutral lactone species. However, intravesicular pH modification significantly improved the retention of DB-67 in the presence compared to that in the absence of 50 mM HPβCD. With highly membrane bound drugs such as DB-67; a relatively large complexation constant is desirable if a cyclodextrin is to be used as an excipient in liposome formulations to enhance intravesicular drug retention. In the case of ionizable hydrophobic drugs, determining the relative binding constants of the neutral and ionized species to cyclodextrin *versus* lipid may facilitate the feasibility assessment for a given cyclodextrin as an excipient to prolong drug retention in liposome formulations.

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