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Encapsulation of Drugs Within Liposomes by pH-Gradient Techniques

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INTRODUCTION

It is now recognized that drug delivery systems can improve the pharmacological properties of many drugs, resulting in increased circulation lifetimes and enhanced efficacy (1). This is often due to altered pharmacokinetic and biodistribution properties of the drugs, which result from their encapsulation within a specific drug carrier system. In recent years, a variety of lipid- or polymer-based nanoparticles have been developed and characterized including liposomes (2), micelles (3), dendritic unimolecular micelles (4), and polymeric nanospheres (3,5), to name a few. Of these many systems, perhaps the best characterized, and certainly one of the first to be developed, were the liposomes, small artificial lipid bilayers with diameters in the nanometer-to-micrometer size range. Although first discovered over 35 years ago, when it was observed that lipids dispersed in water spontaneously formed large multilamellar vesicles (6), a significant amount of technological development was required before their full potential as drug delivery systems could be realized. In addition to a thorough knowledge of the physical properties

of lipids in membranes (such as the effect of lipid composition on membrane permeability), this included techniques for the rapid generation of unilamellar vesicles possessing an optimal size and narrow size distribution, and for the encapsulation of drugs and macromolecules within them. The first requirement was met by the development of extrusion technology, and the latter by the use of pH gradients as a driving force for the accumulation of weakly basic drugs in the interior of acidic vesicles.

Most of the early methods for the formation of liposomes (7) did not generate liposomes of optimal size and polydispersity, and were often technically demanding and time consuming. In contrast, the extrusion method allowed for the rapid generation of monodisperse populations of unilamellar vesicles (8–10), which in turn allowed characterization of the physical properties and in vivo characteristics of a wide variety of liposomal systems. From this work came an understanding of several key features that would be shared by all optimized liposomal drug delivery systems: (i) an appropriate rate of release of drug; (ii) a small size (on the order of 100 nm), and (iii) a long circulation lifetime (half-life $>$ five hours in mice). Drug retention is important because the drug has to stay with its carrier long enough to reach its target, at which point some drug leakage becomes acceptable and perhaps even necessary. Drug retention can be regulated by careful choice of the liposome membrane composition (see below). As far as size is concerned, most liposomal systems are based on the large unilamellar vesicle (LUV) with a diameter close to 100 nm. These systems possess sufficiently large internal volumes for the transport of encapsulated material, but are themselves small enough to circulate for a time sufficient to reach sites of disease such as tumors or sites of inflammation. Vesicles that are much larger or smaller are rapidly cleared from the circulation. However, several other factors besides size also affect circulation lifetime. The lipid composition of an LUV will affect both circulation lifetime and drug retention, both of which were found to be greatly enhanced in systems made from phosphatidylcholine (or sphingomyelin) and cholesterol (11–14). Further improvements in circulation longevity have been achieved by the inclusion of ganglioside G_{M1} in the vesicle formulation (15–17), or by grafting water-soluble polymers such as (polyethylene) glycol onto the vesicle surface, thereby generating what have come to be known as ''stealth'' liposomes (16,18–20).

A major step forward in the design of the first generation of drug transport systems came with the development of methods for achieving the encapsulation and retention of large quantities of drug within liposomal systems. Perhaps the most important insight in this area was the recognition that many chemotherapeutic drugs (and other drugs such as antifungals and antibiotics) could be accumulated within vesicles in response to transmembrane pH gradients (ΔpH) (21–23). It had long been recognized that pH gradients could influence transmembrane distributions of certain weak acids and bases (see Ref. 23 and references therein), and the fact that many chemotherapeutics were weak bases made this an obvious area of inquiry.

Subsequent studies led to considerably broader applications involving the transport and accumulation of a wide variety of drugs, biogenic amines, aminoacids, peptides, lipids, and ions in LUVs exhibiting a ΔpH (for a review, see Ref. 23). To date, the application of this technology has led to the development of several liposomal anticancer systems that exhibit improved therapeutic properties over free drug. Our initial efforts led to the development of a liposomal version of doxorubicin, the most commonly employed chemotherapeutic agent, which is active against a variety of ascitic and solid tumors, yet exhibits a variety of toxic side effects. The pH-gradient approach (11,12,24–26) was expected to provide significant improvements in overall efficacy, or reductions in side effects, due to high drug-to-lipid (D/L) ratios and excellent retention observed both in vitro and in vivo. This has been realized in liposomal doxorubicin preparations that are currently either in advanced clinical trials (27,28), or have been approved by the U.S. Food and Drug Administration (FDA) for clinical use (29). Other liposomal doxorubicin formulations (30–39) are in various Phase I or II clinical trials, often with promising results. A variety of other liposomal drugs are currently in preclinical or clinical development: these include vincristine (13,14,40–42), mitoxantrone (22,43–46), daunorubicin (22,29,47,48), ciprofloxacin (49,50), topotecan (51), and vinorelbine, to name a few. Of these, our group has been prominent in devising methods for the encapsulation of doxorubicin, vincristine, and ciprofloxacin.

Liposomal delivery systems are finally reaching a stage of development where significant advances can reasonably be expected in a short term. The first of the conventional drug carriers are reaching the market, whereas new liposomal drugs are being developed and entered into clinical trials. These advances stem from the fact that the design features required of drug delivery systems that have systemic utility are becoming better defined. Based on the studies indicated above, we now know that liposomal systems that are small (diameter ≤ 100 nm) and that exhibit long circulation lifetimes (half-life $\geq$ five hours in mice) following IV injection exhibit a remarkable property termed "disease site targeting" or "passive targeting" that results in large improvements in the amounts of drug arriving at the disease site. For example, liposomal vincristine formulations can deliver 50- to 100-fold higher amounts of drug to a tumor site relative to the free drug (11,13–15). This can result in large increases in efficacy (15). These improvements stem from the increased permeability of the vasculature at tumor sites (52,53) or sites of inflammation, which results in preferential extravasation of small, long-circulating carriers in these regions.

Over the past 20 years, our laboratory has played a major role in the development of liposomal systems optimized for the delivery of conventional drugs, almost all of which are encapsulated by pH-gradient techniques. Our initial studies led to the development of several liposomal drug delivery systems in which uptake was driven by the "citrate" method of generating pH gradients (15,21–23,27,54–58). This was followed by the development of new

approaches for the loading of drugs via generation of ΔpH (57,58). In this paper, we will describe these methods and their applications, and in the process provide a rationale for the development of new loading methods for the future.

THE FORMATION OF LARGE UNILAMELLAR VESICLES BY EXTRUSION METHODS

Many research questions in membrane science require liposomal systems that are unilamellar and which possess a specific size distribution, usually in the nanometer range. This is particularly true for questions involving the presence of pH or ion gradients, which cannot be adequately modeled using large, multilamellar systems. In our case, investigations relating ion and pH gradients to lipid asymmetry (23,59) were the driving force for the development of extrusion technology. Early methods available for the generation of unilamellar vesicles, which included dispersion of lipids from organic solvents (60), sonication (61), detergent dialysis (62), and reversed phase evaporation (63), had serious drawbacks (59). However, Papahadjopoulos and coworkers (8) had observed that sequential extrusion of multilamellar vesicles (MLVs) through a series of filters of reducing pore size under low pressure gave rise to LUV systems. Further development of this method in our laboratory led to an approach involving direct extrusion of MLVs, at relatively high pressures (200–400 psi), through polycarbonate filters with a pore size ranging from 30 to 400 nm. This allowed generation of narrow, monodisperse vesicle populations with diameters close to the chosen pore size (Fig. 1) (9,10). The method is rapid and simple, and can be performed for a wide variety of lipid compositions and temperatures. As it is necessary to extrude the lipid emulsions at temperatures 5 to 10°C above the gel-to-liquid crystalline phase transition temperature, the system is manufactured so that it may be attached to a variable-temperature circulating water bath.

We have previously (2) described the formation of LUVs from MLVs by the freeze-thaw extrusion method. Here, we describe a modification of this method, which involves extrusion in the presence of ethanol. This method allows for easier extrusion of highly saturated phospholipid preparations, and gives rise to vesicles that are more spherical and possess a slightly larger trapped volume. We will describe in detail the formation of a 20 mM solution of 100 nm LUVs composed of sphingomyelin/cholesterol, a highly ordered lipid mixture that is frequently chosen for drug delivery applications due to its good circulation lifetime and drug retention properties.

Preparation of Sphingomyelin:Cholesterol (55:45) Large Unilamellar Vesicle by Extrusion

When several experiments will be performed using the same lipid formulation, greater consistency will be achieved if each LUV sample is prepared

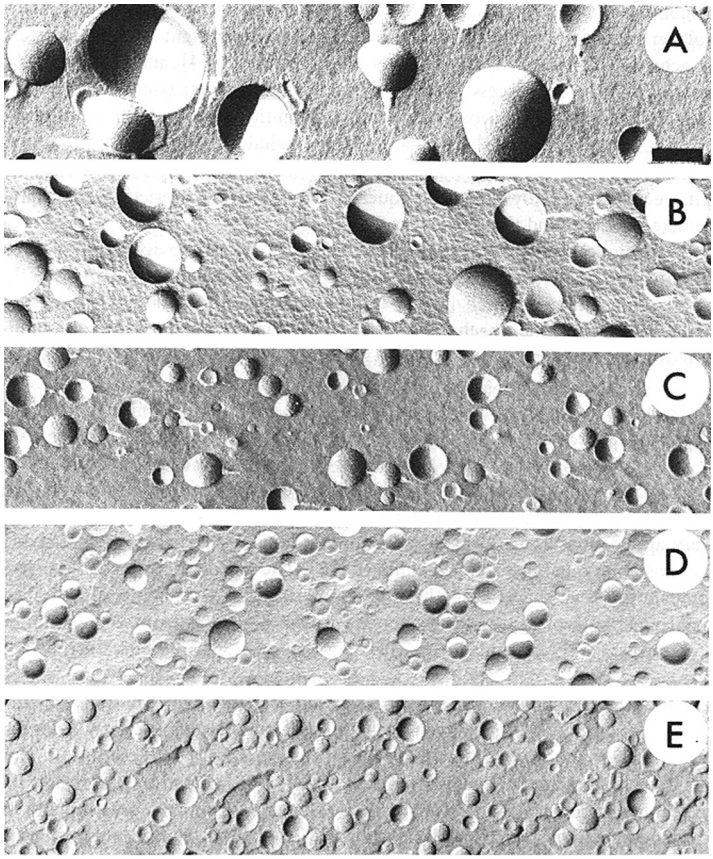

Figure 1 Freeze-fracture electron micrographs of egg phosphatidylcholine large unilamellar vesicles prepared by extrusion through polycarbonate filters with pore sizes of (**A**) 400 nm, (**B**) 200 nm, (**C**) 100 nm, (**D**) 50 nm, and (**E**) 30 nm. The bar in panel (**A**) represents 150 nm. *Source*: From Ref. 7.

from the same lipid stock solution, in this case sphingomyelin:cholesterol (55:45) in ethanol. For example, if LUV with a concentration of 20 mM is desired, the lipid stock should be in excess of 100 mM. In one case, 500 μmol of lipid [275 μmol egg sphingomyelin (Avanti Polar Lipids; Northern Lipids) and 225 μmol cholesterol (Sigma-Aldrich; Northern Lipids)] were dissolved in 3.75 mL of ethanol, giving a solution with a lipid concentration of 133 mM. If desired, an aliquot of [^{3}H]cholesterol hexadecyl ether (Perkin-Elmer Life Sciences Canada) (a nonexchangeable lipid marker) can be added to the solution. In our case, addition of approximately 0.9 μCi of [^{3}H] cholesteryl hexadecyl ether (CHE) gave rise to a lipid solution with a specific activity of 3600 dpm/μmol. This can be adjusted as needed. The specific activity can be

measured by diluting the ethanol solution and measuring both activity and lipid concentration, or by assaying the final LUV preparation. Whenever possible, lipid concentrations should be verified by an appropriate assay. Phospholipid concentrations are verified using the assay of Fiske and Subbarow for the quantitative determination of inorganic phosphate (64). The assay, which combines simplicity, accuracy, and reproducibility, is generally used for determining the concentration of phospholipid stock solutions or LUV preparations. Detailed protocols for this assay have recently been described by us (65) and will not be repeated here.

For the formation of vesicles with a final concentration of approximately 20 mM, 100 μmol of lipid (0.75 mL) is removed from the ethanol stock and placed in a scintillation vial with a tiny stir bar. While stirring rapidly, 4.25 mL of appropriate buffer (the internal buffer or hydration buffer) is added such as 350 mM citrate pH 4.0 (pH-gradient loading), 350 mM ammonium sulfate (for amine loading), or 350 mM $MgSO_4$ pH 6.5 (for ionophore loading) (these will all be discussed below). This will give internal buffer/salt concentrations of 300 mM. This lipid emulsion is then used for extrusion.

The extruder (Northern Lipids) is assembled with two polycarbonate filters (Nuclepore polycarbonate membranes; Whatman) with pore size of 0.1 μm and diameter of 25 mM, and connected to a circulating water bath equilibrated at 65°C. The lipid emulsion is extruded 10 times through the filters under a pressure of approximately 400 psi. For larger LUVs (200–400 nm), lower pressures will be adequate (100–200 psi). After each pass, the sample is cycled back to the extruder. It is important to start at a low pressure and gradually increase until each pass takes less than one minute.

Following extrusion, the LUVs are dialyzed against the hydration buffer (2×1 L) overnight to remove ethanol, followed by dialysis for a further 24 hours against the desired external buffer, usually 20 mM *N*-[2-hydroxyethyl] piperazine-*N*′-[2-ehtanesulfonic acid] (HEPES) 150 mM NaCl pH 7.5 (citrate loading), 150 mM NaCl (ammonium sulfate loading), or 20 mM HEPES 300 mM sucrose pH 6.5 (ionophore loading). Following dialysis, the lipid concentration of the final LUVs is determined by a phosphate assay (64,65) or by liquid scintillation counting, and is usually in the range of 18 to 19 mM.

GENERATION OF pH GRADIENTS VIA INTERNAL CITRATE BUFFER

Early studies in our laboratory on membrane potentials and the uptake of weak bases used for the measurement of ΔpH led to the recognition that a variety of chemotherapeutic drugs could be accumulated within LUVs exhibiting transmembrane pH gradients (59). This ''remote-loading'' technique, so named because drug is loaded into preformed vesicles, is based on the membrane permeability of the neutral form of weakly basic drugs such

as doxorubicin. When doxorubicin ($pK_a = 8.6$) is incubated at neutral pH in the presence of LUVs exhibiting a ΔpH (interior acidic), the neutral form of the drug will diffuse down its concentration gradient into the LUV interior, where it will be subsequently protonated and trapped (the charged form is membrane impermeable). As long as the internal buffer (300 mM citrate pH 4) is able to maintain the ΔpH, diffusion of neutral drug will continue until either all the drugs have been taken up, or the buffering capacity of the vesicle interior has been overwhelmed. This process is illustrated in Figure 2 for the uptake of doxorubicin into EPC/Chol and egg phosphatidylcholine (EPC) LUVs, where it is seen that uptake is dependent on time, temperature, and lipid composition (21). If conditions are chosen correctly, high D/L ratios can be achieved (D/L = 0.2 mol:mol) with high trapping efficiencies (98% and higher) and excellent drug retention. A diagrammatic illustration of this process is given in Figure 3A (also see insert). Interestingly, much higher levels of doxorubicin can be loaded than would be predicted on the basis of the magnitude of ΔpH (23,66). This would appear to result from the formation of doxorubicin precipitates within the LUV interior, which provides an additional driving force for accumulation (67,68). Doxorubicin forms fibrous precipitates that are aggregated into bundles by citrate (69) or sulfate (67,68) counteranions, and which affect the rate of doxorubicin release from LUVs (70). These precipitates can be visualized by cryoelectron microscopy, where they are seen to give the LUVs a "coffee-bean" appearance (Fig. 4). Some LUVs contain several bundles of fibers. This has been corroborated by recent observations that very high levels of uptake can be achieved in the absence of a pH gradient by the formation of doxorubicin–Mn^{2+} complexes (71–74).

The experimental procedure described below for the accumulation of doxorubicin within DSPC/Chol LUVs represents our "basic" pH-gradient method for drug loading. This basic system can be used for the uptake of a wide variety of drugs (22) and all the remote-loading methods, which follow are based on similar principles involving the generation of ΔpH, even though this may not always be immediately obvious.

Remote Loading of Doxorubicin into DSPC:Cholesterol (55:45) Large Unilamellar Vesicle

DSPC/Chol (55:45) LUVs (diameter = 100 nm) are prepared as described in section "Preparation of Sphingomyelin/Cholesterol (55:45) Large Unilamellar Vesicle by Extrusion" [(Lipid) = 20 mM, volume = 5 mL], using 350 mM citrate pH 4.0 as the hydration buffer, and 20 mM HEPES 150 mM NaCl pH 7.5 (HEPES-buffered saline) as the external buffer. In this case, the pH gradient is formed during the final dialysis step. It would also be possible to omit the final dialysis step and form the pH gradient by one of two common column methods. This could be desirable if the LUV

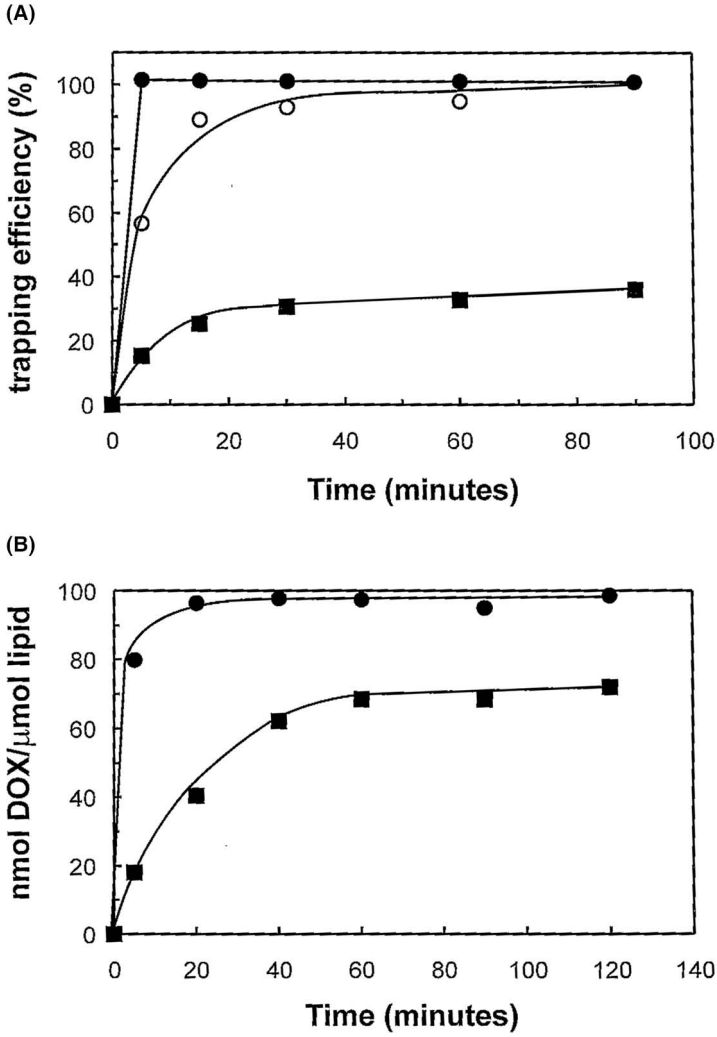

Figure 2 (**A**) Effect of incubation temperature on uptake of doxorubicin into 200 nm EPC/cholesterol (55:45 mol/mol) large unilamellar vesicles (LUVs) exhibiting a transmembrane pH gradient (pH 4 inside, 7.8 outside). Doxorubicin was added to LUVs (D/L = 0.3 wt:wt) equilibrated at 21°C, 37°C, and 60°C. (**B**) Effect of cholesterol on the uptake of doxorubicin at 20 into 100 nm LUVs exhibiting a transmembrane pH gradient (pH 4.6 inside, 7.5 outside). Lipid compositions were EPC and EPC/cholesterol (1:1 mol/mol). The initial drug-to-lipid ratio was 100 nmol/μmol. *Source*: From Refs. 12 (**A**), 21 (**B**).

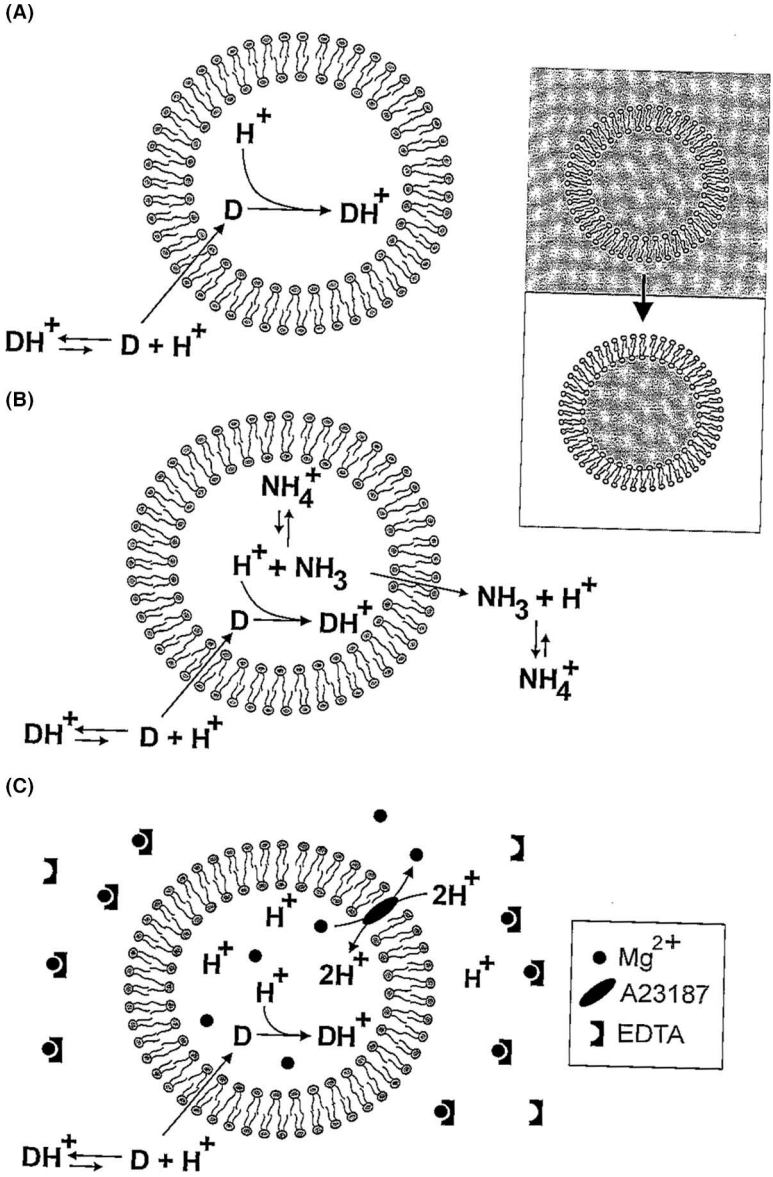

Figure 3 (*Caption on next page*)

preparation will not be used in a fairly short time, or if the lipid composition is such that pH gradients are not stable over longer periods of time. The first alternate method is passing the LUVs down a column of Sephadex G-50 (Amersham Pharmacia Biotech) equilibrated in HEPES-buffered saline

(the method described below), and the second method involves the use of spin columns. Spin columns permit the rapid separation of LUVs from their hydration buffer (or from unencapsulated drug) on hydrated gel at low centrifugation speeds. They are particularly useful for monitoring drug uptake with time (as described below). On the day prior to drug loading, a slurry of Sephadex G-50 in HEPES-buffered saline (HBS) is prepared by adding a small volume (2–3 mL) of dry G-50 powder to 200 to 300 mL HBS with frequent swirling. Small quantities of gel are added as necessary until the settled G-50 occupies about half the aqueous volume. The hydrated gel is allowed to swell overnight. To prepare the spin columns, a tiny plug of glass wool is packed into the end of a 1-mL disposable syringe (without the needle), which is then placed in a 13×100 mm glass test tube. The G-50 slurry is swirled, and the syringes are immediately filled using a Pasteur pipette. The syringes (in test tubes) are then placed in a desktop centrifuge, and the gel is packed (to 0.6 or 0.7 mL) by bringing the speed to 2000 rpm ($\times$670 g) momentarily. More G-50 slurry is added, and the centrifugation is repeated. When finished, the moist G-50 bed should be 0.9 to 1.0 mL. The spin columns are covered with parafilm to prevent drying, and are used within an eight-hour period.

If the second dialysis step against external buffer is omitted during the formation of LUV, transmembrane pH gradients can be formed by running

Figure 3 (*Figure on previous page*) Diagrammatic representations of drug uptake in response to transmembrane pH gradients. Prior to drug loading, it is necessary to establish the primary pH gradient or the primary ion gradient, which will generate a ΔpH. Lipid films or powders are initially hydrated and then extruded in the internal (or hydration) buffer, giving rise to a vesicle solution in which both the external and the internal solutions are the same, as indicated by the grey shading in the upper frame of the insert (*top right*). The vesicles are then passed down a gel exclusion column (Sephadex G-50) hydrated in the external buffer, giving rise to vesicles with a pH or ion gradient (lower frame of insert). (**A**) The standard pH-gradient method. The internal buffer is 300 mM citrate pH 4, and the external buffer is 20 mM HEPES 150 mM NaCl pH 7.5. The precipitation of certain drugs such as doxorubicin, which provides an addition driving force for uptake, is not indicated in the figure. (**B**) A second method for generating ΔpH involves the initial formation of a transmembrane gradient of ammonium sulfate, which leads to an acidified vesicle interior as neutral ammonia leaks from the vesicles. Here, the internal buffer is 300 mM ammonium sulfate and the external buffer is 150 mM NaCl. Possible drug precipitation is not indicated. (**C**) Transmembrane pH gradients can also be established by ionophores (such as A23187) in response to transmembrane ion gradients (e.g., Mg^{2+}, represented as solid circles). A23187 couples the external transport of one Mg^{2+} ion (down its concentration gradient) to the internal transport of two protons, resulting in acidification of the vesicle interior. An external chelator such as EDTA is required to bind Mg^{2+} ions as they are transported out of the vesicle. Other divalent cations such as Mn^{2+} can also be used. See text for further details. *Source*: From Ref. 2.

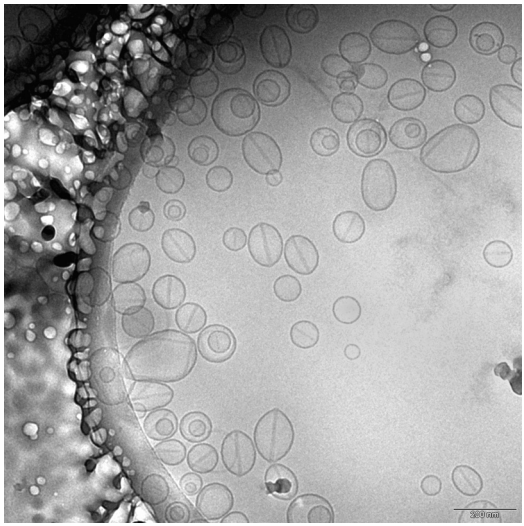

Figure 4 Cryoelectron micrograph of 100 nm DSPC/cholesterol large unilamellar vesicles (LUVs) containing doxorubicin (drug-to-lipid ratio = 0.05 wt:wt) loaded in response to a transmembrane pH gradient (inside acidic). The precipitated drug is clearly visible, and gives the LUV the appearance of a "coffee-bean." The pH gradient was generated by the ionophore A23187 in response to a transmembrane Mg^{2+} gradient. *Abbreviation*: DSPC, distearoylphosphatidylcholine. *Source*: Johnston, unpublished results.

an aliquot (400 μL) of the LUVs down a column (1.5 × 15 cm) of Sephadex G-50 eluted in HBS. The LUV fractions, which will elute at the void volume and are visible to the eye, are collected and pooled. The final volume will be approximately 2 mL and the lipid concentration will be around 5 mM. Alternatively, the pH gradient can be formed using spin columns prepared in HBS (spin 4 × 100 μL) and pooling the fractions.

Doxorubicin (Sigma-Aldrich) is often loaded at a D/L ratio of 0.2 mol:mol. A doxorubicin standard solution is prepared by dissolving 1.0 mg of drug in 0.5 mL of saline (150 mM NaCl). The concentration is verified on the spectrophotometer using the doxorubicin extinction coefficient $\varepsilon = 1.06 \times 10^4\ M^{-1}\ cm^{-1}$ (75). Aliquots of lipid (5 μmol) and doxorubicin (1 μmol, approximately 0.5 mg) are combined in a glass test tube (or plastic Ependorf tube) with HBS to give a final volume of 1 mL (5 mM lipid concentration). Drug uptake occurs during a 30-minute incubation at 65°C. This is verified at appropriate time points (0, 5, 15, and 30 minutes) by applying an aliquot (50–100 μL) to a spin column and centrifuging at 2000 rpm for two minutes. LUVs containing entrapped drug will elute off the column, while free doxorubicin will be trapped in the gel. An aliquot (50 μL) of the initial lipid–drug mixture is saved for determination of initial D/L ratio.

The initial mixture and each time point are then assayed for doxorubicin and lipid. Lipid concentrations can be quantified by the phosphate assay (see above) or by liquid scintillation counting of an appropriate radiolabel. Doxorubicin is quantified by an absorbance assay (see below). The percent uptake at any time point (e.g., $t = 30$ minutes) is determined by %-uptake $= [(D/L)_{t=30\text{minutes}}] \times 100/[(D/L)_{\text{initial}}]$. Doxorubicin can be assayed by both a fluorescence assay and an absorbance assay, but we find the latter to be more accurate. The standard curve consists of four to five cuvettes containing 0 to 150 nmol doxorubicin in a volume of 0.1 mL; samples to be assayed are of the same volume. To each tube is added 0.9 mL of 1% (v/v) Triton X-100 (in water) solution. For saturated lipid systems such as DSPC/Chol, the tubes should be heated in a boiling water bath for 10 to 15 seconds, until the detergent turns cloudy. Samples are allowed to cool, and absorbance is read at 480 nm on a UV/Visible spectrophotometer.

GENERATION OF pH GRADIENTS VIA TRANSMEMBRANE AMMONIA GRADIENTS

Despite its successful application in several drug delivery systems (22), the pH-gradient approach utilizing internal citrate buffer does not provide adequate uptake of all weakly basic drugs. A case in point is the antibiotic ciprofloxacin, a commercially successful, quinolone antibiotic widely used in the treatment of respiratory and urinary tract infections (56). Ciprofloxacin is a zwitterionic compound that is charged and soluble under acidic and alkaline conditions, but is neutral and poorly soluble in the physiological pH range, precisely the external conditions of most drug-loading techniques. This low solubility results in low levels of uptake (<20%) when the drug is loaded using the standard citrate technique. However, high levels of encapsulation can be achieved using an alternate ΔpH-loading method that is based on transmembrane gradients of ammonium sulfate (56,67,76). If a given drug is incubated with LUVs containing internal ammonium sulfate in an unbuffered external saline solution, a small quantity of neutral ammonia will diffuse out of the vesicle, creating an unbuffered acidic interior with a pH ~2.7 (56). Any neutral drug that diffuses into the vesicle interior will consume a proton and becomes charged and therefore trapped. If continued, drug uptake leads to consumption of the available protons and the diffusion of additional neutral ammonia from the vesicles will create more protons to drive drug uptake. This will continue until all the drugs have been loaded, or until the internal proton supply is depleted, leaving a final internal pH of about 5.1 (56). The technique is ideal for ciprofloxacin, because the drug is supplied as an HCl salt, and thus is acidic and soluble when dissolved in water. In addition, the amine method results in higher uptake levels for other drugs such as doxorubicin. A diagrammatic scheme of the method is given in Figure 3B.

This technique has been applied to a variety of drugs including doxorubicin (57,67,68,76), epirubicin (57), ciprofloxacin (56,57,67,77), and vincristine (57). The method is equally effective using a range of alkylammonium salts (e.g., methylammonium sulfate, propylammonium sulfate, or amylammonium sulfate) to drive uptake (57). Some drugs such as doxorubicin precipitate and form a gel in the vesicle interior (67,68,70), whereas others such as ciprofloxacin do not (67,77). Ciprofloxacin can reach intraliposomal concentrations as high as 300 mM, and while the drug does form small stacks as shown by ^{1}H-NMR, it does not form large precipitates (77), even though its solubility in buffer cannot exceed 5 mM. As a result of the lack of precipitation and rapid exchange properties, ciprofloxacin can respond rapidly to changes in electrochemical equilibria such as depletion of the pH gradient. This explains the observed rapid leakage of ciprofloxacin from LUVs in response to serum destabilization or loss of pH gradient. In contrast, doxorubicin, which is known to form insoluble precipitates within LUVs in the presence of both citrate (69,70) and ammonium sulfate (67,68), is retained within vesicles in the presence of serum. This is a clear illustration of how the physical state of encapsulated drugs will affect retention and therefore may impact efficacy.

The experimental procedure below describes the uptake of ciprofloxacin into sphingomyelin (SPM)/Chol LUVs. Drug delivery vehicles prepared from SPM/Chol often exhibit greater efficacy than those prepared from DSPC/Chol (13). Included is a description of the Bligh–Dyer extraction procedure (78), which involves partitioning the lipid and water-soluble drug into organic solvent and aqueous layers, respectively. This is necessary because lipid interferes with the ciprofloxacin assay.

Remote Loading of Ciprofloxacin into SPM/Cholesterol Large Unilamellar Vesicles

SPM/Chol (55:45) LUVs (diameter = 100 nm) are prepared as described in the section "Preparation of Sphingomyelin/Cholesterol (55:45) Large Unilamellar Vesicle by Extrusion" [(Lipid) = 20 mM, volume = 5 mL], using 350 mM ammonium sulfate ($(NH_4)_2SO_4$) as the hydration buffer, and 150 mM NaCl as the external solution. In this case, the amine and pH gradients are formed during the final dialysis step. It would also be possible to omit the final dialysis step, and form the amine and pH gradients by one of two common column methods, as described above for the citrate method. The first alternate method is passing the LUVs down a column of Sephadex G-50 (Amersham Pharmacia Biotech) equilibrated in 150 mM saline (the method described below), and the second involves the use of spin columns. Spin columns are prepared (using saline rather than HBS) for monitoring drug uptake with time.

If the second dialysis step against external buffer is omitted during the formation of LUV, the amine and pH gradients are formed by running an aliquot (200 μL) of the LUVs down a column (1.5 × 15 cm) of Sephadex

G-50 eluted in saline (150 mM NaCl), as described above. Alternatively, the gradient could be formed using spin columns (spin $2 \times 100\,\mu L$), and pooling the fractions.

Ciprofloxacin (Bayer Corporation) is often loaded at a D/L ratio of 0.3 mol:mol. After preparation of a ciprofloxacin standard solution (4 mM in water), 5 μmol of lipid and 1.5 μmol of ciprofloxacin are pipetted into a glass test tube (or plastic Ependorf tube), adding saline to give a final volume of 1 mL (5 mM lipid concentration). This solution is incubated at 65°C for 30 minutes, with aliquots (50–100 μL) withdrawn at appropriate time points (0, 5, 15, and 30 minutes) and applied to a spin column [centrifuge at 2000 rpm (×670 g) for two minutes]. An aliquot (50 μL) of the initial lipid–drug mixture is saved for determination of initial D/L.

The initial mixture and each time point are then assayed for ciprofloxacin and lipid. Lipid can be quantified using the phosphate assay (64,65) or by liquid scintillation counting. Ciprofloxacin is quantified by an absorbance assay following removal of drug from lipid by a Bligh–Dyer extraction procedure (78) (see below). The percent uptake is determined as described in the section "Remote Loading of Doxorubicin into DSPC/Cholesterol (55:45) Large Unilamellar Vesicle."

To perform the ciprofloxacin assay, a standard curve is prepared consisting of six glass test tubes containing 0, 50, 100, 150, 200, and 250 nmol ciprofloxacin (in water). The volume is made up to 1 mL with 200 mM NaOH. For the blank, 1 mL of 200 mM NaOH is used. Each LUV sample to be assayed should contain less than 250 nmol ciprofloxacin in a volume of 1 mL.

To each standard and assay sample, 2.1 mL of methanol and 1 mL of chloroform are added and vortexed gently. Only one phase should be present (if two phases form, 0.1 mL methanol is added and the solution is vortexed again). One milliliter of 200 mM NaOH and 1 mL chloroform are then added to each tube, which are then vortexed at high speed. Two phases should form, an aqueous phase containing the ciprofloxacin with a volume of 4.1 mL (top), and an organic phase containing the lipid with a volume of 2 mL (bottom). If a clean separation is not obtained, the tubes can be centrifuged at 2000 rpm for two minutes in a desktop centrifuge.

After carefully removing the aqueous phase, the absorbance at 273.5 nm is read to obtain nmol Cipro present in the original sample volume (in microliters), thereby yielding the sample drug concentration (mM).

IONOPHORE-MEDIATED GENERATION OF pH GRADIENTS VIA TRANSMEMBRANE ION GRADIENTS

The observation that improved remote loading of ciprofloxacin could be achieved using ammonium sulfate solutions rather than sodium citrate buffers highlighted the need for further investigation and development of drug-loading methodologies. In this section, we examine an approach in

which ionophores, responding to transmembrane ion gradients (involving Na^+, Mn^{2+}, or Mg^{2+}), generate a secondary pH gradient that provides the driving force for drug uptake.

Recently, we have developed a new method of remote loading that is based on the ionophore-mediated generation of a secondary pH gradient in response to transmembrane gradients of monovalent and divalent cations (58). The process is diagrammed in Figure 3C. A primary ion gradient is generated when LUVs formed by extrusion in K_2SO_4, $MnSO_4$, or $MgSO_4$ solutions are passed down a column equilibrated in a sucrose-containing buffer. The use of sulfate salts is important, because chloride ion can dissipate pH gradients by forming neutral HCl that can diffuse out of the vesicle. Likewise, sucrose is chosen as a component of the external buffer rather than saline, because chloride ion can interfere with some ionophores (79). After establishing the primary ion gradient, the drug (which to date includes doxorubicin, mitoxantrone, ciprofloxacin, vincristine, and other vinca alkaloids) is added. If the LUVs contain a potassium salt, the ionophore nigericin is added, whereas if the LUVs contain either Mn^{2+} or Mg^{2+}, the ionophore A23187 and the chelator ethylenediaminetetraacetic acid (EDTA) are used. Under the current conditions, nigericin couples the outward flow of a potassium ion (down its concentration gradient) to the inward flow of a proton. Likewise, A23187 couples the outward flow of a single divalent cation to the inward flow of a pair of protons. In both cases, ionophore-mediated ion transport is electrically neutral and results in acidification of the vesicle interior, thereby creating a pH gradient that drives drug uptake. For systems containing divalent cations, EDTA chelates manganese or magnesium as they are transported out of the vesicles, and is required to drive drug uptake. Both ionophore methods result in high levels of encapsulation for the drugs ciprofloxacin and vincristine (80–90%), and excellent in vitro retention (58). However, the A23187-loaded systems exhibit excellent in vivo circulation and drug retention properties, which are comparable to systems loaded by the citrate or amine methods, whereas the nigericin-loaded systems do not.

Ionophore-Mediated Loading of Vincristine, Vinblastine, or Vinorelbine into SPM/Chol Large Unilamellar Vesicles

LUVs (diameter = 100 nm) are prepared from SPM/Chol (55:45) as described above [(Lipid) = 20 mM]. It has been shown that liposomal vincristine prepared from SPM/Chol exhibits greater efficacy than systems prepared from DSPC/Chol (13). The hydration solution is 350 mM $MgSO_4$, which when diluted with the ethanol used in extrusion will give an internal salt concentration of 300 mM. The external buffer is 20 mM HEPES 300 mM sucrose pH 7.5 containing 15 mM Na_2EDTA. In this case, the Mg^{2+} gradient is formed during the final dialysis step. It would also be possible to omit

the final dialysis step, and form the Mg^{2+} gradient by one of two common column methods, as described above for the other methods. The first alternate method is passing the LUVs down a column of Sephadex G-50 (Amersham Pharmacia Biotech) equilibrated in HEPES-buffered sucrose containing Na_2EDTA (the method described below), and the second involves the use of spin columns.

Spin columns for monitoring drug uptake with time are hydrated in 300 mM sucrose. Assuming the final dialysis step against HEPES-buffered sucrose (20 mM HEPES 300 mM sucrose pH 7.5) containing Na_2EDTA is omitted, the Mg^{2+} gradient can be formed by running an aliquot (200 μL) of the LUVs down a column (1.5 × 15 cm) of Sephadex G-50 eluted in HEPES-buffered sucrose containing 15 mM Na_2EDTA. Alternatively, the gradient can be formed using spin columns prepared in the same buffer (spin 2 × 100 μL), and pooling the fractions.

Vincristine sulfate, vinblastine sulfate (Fine Chemicals, Australia), or vinorelbine ditartrate (OmniChem Sa, Belgium) is added to the LUVs to give a D/L ratio of 0.1 wt:wt. If desired, the drugs can be labeled with trace amounts of [^{14}C]vincristine sulfate (Chemsyn Laboratories, Lenexa, Kansas, U.S.A.), [^{3}H]vinorelbine, or [^{3}H]vinblastine (Moravek Biochemicals, Inc., Breas, California, U.S.). The sample is diluted to a final lipid concentration of 5 mM with addition of HEPES-buffered sucrose containing 15 mM Na_2EDTA. Ionophore A23187 (Sigma-Aldrich) is added to a concentration of 2 μg/mg lipid. The mixture is incubated at 60°C for two hours, saving an aliquot (50 μL) for determination of initial D/L ratio. Aliquots (100 μL) are withdrawn at appropriate time points (0, 10, 20, 30, 60, and 120 minutes) and applied to spin columns (2000 rpm for two minutes). The initial mixture and each time point are then assayed for drug and lipid to monitor drug uptake. When maximum uptake has been achieved, the remainder of the sample is dialyzed against HEPES-buffered sucrose for two hours to remove unencapsulated drug and traces of ionophore.

A representative uptake experiment is shown in Figure 5, where the effect of temperature is apparent (80). For both vinorelbine and vincristine, little uptake is observed at 25°C, far below the gel-to-liquid crystalline phase transition temperature of egg SPM (about 44°C), whereas 80% uptake is observed at 60°C where the membrane is fluid. Interestingly, the uptake of vinblastine at 25°C yields an end point nearly identical to that obtained

Figure 5 (*Figure on next page*) Effect of temperature on the uptake of (**A**) vinblastine, (**B**) vinorelbine, and (**C**) vincristine into 100 nm ESM/Chol large unilamellar vesicles (55:45) containing 300 mM $MgSO_4$. After addition of ionophore A23187 (2 μg/mg lipid), 15 mM EDTA, and drug solution to the liposome preparation (5 mM lipid; final drug-to-lipid ratio = 0.1 wt:wt), samples were incubated at 25°C (■) and 60°C (●). Aliquots were removed at different time points for determination of lipid and drug concentrations as described in the text. *Abbreviation*: ESM, egg sphingomyelin. *Source*: From Ref. 80.

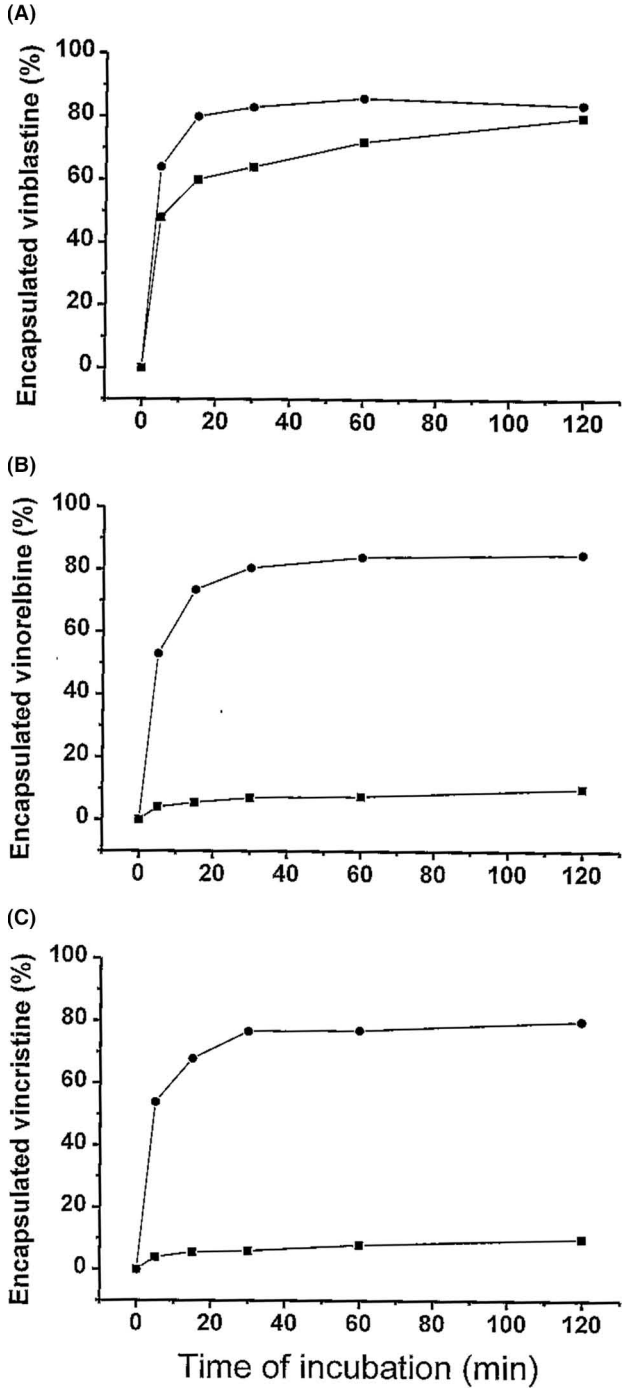

Figure 5 (*Caption on previous page*)

at higher temperature, although the rate of uptake is somewhat reduced. This was attributed to the higher hydrophobicity of this drug relative to vinorelbine and vincristine.

COMPARISON OF LOADING METHODS

We have already noted one example that demonstrates the benefits of possessing more than a single method for loading drugs via pH-gradient methods. Drugs such as ciprofloxacin that have low solubilities at near-neutral pH values will not exhibit efficient loading when the standard citrate method is used (due to the requirement for external pH values near 7, a condition that is in turn dictated by citrates optimal buffering pH near 4). In that case, the use of the ammonium sulfate method, where the external solution can be unbuffered saline, allowed for much higher D/L ratios to be obtained. Such general considerations were behind the development of the ionophore method as well, and our initial studies showed that ciprofloxacin could be as efficiently loaded by this new method as by that involving ammonium sulfate. These observations, coupled with the growing recognition that certain drugs formed insoluble precipitates within liposomes in the presence of the correct counteranion(s), and a developing appreciation of the role of drug retention in liposomal drug efficacy, have led us and others to investigate whether specific loading methods will result in optimized formulations for specific drugs. Although these studies are currently in progress and thus the final word cannot yet be made, a number of early studies point toward the superiority of certain methods over others in specific applications. We will note a few of these here, looking forward to research in this area as it unfolds in the near future.

Zhigaltsev et al. (80) have recently examined the in vitro and in vivo loading and retention properties of three closely related vinca alkaloids (vincristine, vinblastine, and vinorelbine) in liposomal systems composed of SPM/Chol. Results were obtained for both the citrate and the ionophore (using Mg^{2+}) methods of loading. Interestingly, it was noted that when loading was accomplished using an ionophore method, drug retention was significantly improved by increasing the D/L ratio (from 0.1 to 0.3 wt:wt). However, drug retention remained the same following an increase in D/L ratio when the citrate method of loading was used. It was suggested that the differences in retention observed for the two methods could be related to differences in the intravesicular forms of the drugs in the two systems.

Similar results have been described in a recent study comparing the encapsulation of topotecan within DSPC/Chol liposomes using four different loading methods (81). The four methods included the citrate method, the ammonium sulfate method, and two variations of the ionophore A23187 method (one using $MnCl_2$ and the other $MnSO_4$). Both the citrate and the A23187-$MnCl_2$ methods exhibited reduced uptake (final D/L ratios in the range of 0.1), whereas the ammonium sulfate and A23187-$MnSO_4$

methods exhibited higher final D/L ratios (in the range of 0.2; close to 100%) and much better retention. In fact, with the two superior loading systems, D/L ratios as high as 0.3 could be achieved. These two systems also exhibited improved retention as the D/L ratio was increased from 0.1 to 0.2, retaining five times as much drug at 36 hours at the higher ratio. The amount of drug retained by the ammonium sulfate and $MnSO_4$ methods was 10 times as great as that retained by the citrate and $MnCl_2$ methods as well. Cryoelectron microscopy studies revealed the presence of topotecan precipitate within the two sulfate-containing systems. Together, these results demonstrate the improvements in drug retention that can be achieved using a pH gradient in combination with the correct counteranion (in this case, sulfate).

It is clear that very different rates of drug retention are observed when encapsulation is achieved by different methods of loading, even though the driving force for uptake in all cases is a pH gradient of approximately the same magnitude. These differences may be due to interactions of the drugs with different counteranions, which can result in the formation of precipitate and have a profound effect on the rate of release. The fact that drug efficacy is highly sensitive to drug release rates, especially for drugs that only act at a certain point in the cell cycle (such as vincristine), demonstrates the importance of having a variety of methods available for regulating the rate of drug release. This highlights the need for developing new methods of drug encapsulation, and for meticulously investigating each existing technology for each drug of interest. Of particular interest at this time will be in vivo animal studies that compare drug retention rates to antitumor efficacy for different loading systems. Such studies could lead to significant improvements in the treatment of human disease.

CONCLUSIONS

The rapid development of liposomal technology over the past 15 years has led to a wide variety of delivery systems for conventional drugs, some of which are in clinical trials or have been approved for use by the U.S. FDA. At least three different methods now exist for loading weakly basic drugs into liposomes in response to transmembrane pH gradients. By appropriate selection of loading method, drug, and other parameters such as D/L ratio, delivery systems exhibiting a range of drug retention properties can be generated. The question of whether these will lead to delivery systems exhibiting optimized antitumor efficacy is an exciting current and future topic of investigation.

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