

[1] Entrapment of Small Molecules and Nucleic Acid-Based Drugs in Liposomes

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Abstract

In the past two decades there have been major advances in the development of liposomal drug delivery systems suitable for applications ranging from cancer chemotherapy to gene therapy. In general, an optimized system consists of liposomes with a diameter of ~ 100 nm that possess a long circulation lifetime (half-life >5 h). Such liposomes will circulate sufficiently long to take advantage of a phenomenon known as disease site targeting, wherein liposomes accumulate at sites of disease, such as tumors, as a result of the leaky vasculature and reduced blood flow exhibited by the diseased tissue. The extended circulation lifetime is achieved by the use of saturated lipids and cholesterol or by the presence of PEG-containing lipids. This chapter will focus on the methodology required for the generation of two very different classes of liposomal carrier systems: those containing conventional small molecular weight (usually anticancer) drugs and those containing larger genetic (oligonucleotide and plasmid DNA) drugs. Initially, we will examine the encapsulation of small, weakly basic drugs within liposomes in response to transmembrane pH and ion gradients. Procedures will be described for the formation of large unilamellar vesicles (LUVs) by extrusion methods and for loading anticancer drugs into LUVs in response to transmembrane pH gradients. Three methods for generating transmembrane pH gradients will be discussed: (1) the use of intravesicular citrate buffer, (2) the use of transmembrane ammonia gradients, and (3) ionophore-mediated generation of pH gradients via transmembrane ion gradients. We will also discuss the loading of doxorubicin into LUVs by formation of drug-metal ion complexes. Different approaches are required for encapsulating macromolecules within LUVs. Plasmid DNA can be encapsulated by a detergent-dialysis approach, giving rise to stabilized plasmid-lipid particles, vectors with potential for systemic gene delivery. Antisense oligonucleotides can be spontaneously entrapped upon electrostatic interaction with ethanol-destabilized cationic liposomes, giving rise to small multilamellar systems known as stabilized antisense-lipid particles (SALP). These vectors have the potential to regulate gene expression.

Introduction

It has now been over 35 years since it was discovered that vigorous dispersal of purified phospholipids in water resulted in the formation of microscopic closed membrane spheres (Bangham, 1968). These artificial membranes, referred to as liposomes, were found to consist of one or more lipid bilayers arranged concentrically about a central aqueous core. Studies on the membrane permeability of small molecules demonstrated that polar and charged molecules could be retained within liposomes, an observation that immediately suggested their potential as systems for the systemic delivery of drugs (Sessa and Weissmann, 1968). Unfortunately, a significant amount of technological development was required before this potential could be realized. In addition to a better understanding of the physical properties of membranes and their lipid components, techniques were required for the generation of unilamellar vesicles and encapsulation of drugs and macromolecules within them. Although a wide variety of methods were developed for the formation of liposomes (Hope *et al.*, 1986; Lichtenberg and Barenholz, 1988), many of them did not generate liposomes of optimal size and polydispersity and often were technically demanding and time consuming. Furthermore, the drug-loading technology at the time was based on passive entrapment methods, which resulted in low encapsulation levels (<30%) and poor retention of drugs (Mayer *et al.*, 1990a). Nevertheless, early animal studies using liposomal drug carriers were encouraging enough to warrant further development (see Mayer *et al.*, 1990a and references therein).

The development of extrusion technology for the rapid generation of monodisperse populations of unilamellar vesicles (Hope *et al.*, 1985; Mayer *et al.*, 1986b; Olson *et al.*, 1979) allowed characterization of the physical properties and *in vivo* characteristics of a wide variety of liposomal systems. This information revealed that optimized drug delivery systems would possess two key parameters: a small size (on the order of 100 nm) and long circulation lifetimes (half-life >5 h in mice). The basic structural framework on which most delivery systems are based is the large unilamellar vesicle (LUV) with a diameter close to 100 nm. These systems possess internal volumes large enough to carry adequate quantities of encapsulated material but are 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, circulation lifetime is determined by factors other than size. Both circulation lifetimes and drug retention are dependent on lipid composition and were found to be greatly enhanced in systems made from phosphatidylcholine (or sphingomyelin) and cholesterol (Mayer *et al.*,

1989, 1993; Webb *et al.*, 1995, 1998a). Further improvements in circulation longevity were achieved by the inclusion of ganglioside G_{M1} in the vesicle formulation (Boman *et al.*, 1994; Gabizon and Papahadjopoulos, 1988; Woodle *et al.*, 1994) or by grafting water-soluble polymers such as poly (ethylene glycol) (PEG) onto the vesicle surface, thereby generating what have come to be known as “stealth” liposomes (Allen, 1994, 1998; Allen *et al.*, 1991; Woodle *et al.*, 1994).

A major advance 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 could be accumulated within vesicles in response to transmembrane pH gradients (ΔpH) (Cullis *et al.*, 1997; Madden *et al.*, 1990; Mayer *et al.*, 1986a). The ability of ΔpH to influence transmembrane distributions of certain weak acids and bases had long been recognized (see Cullis *et al.*, 1997 and references therein). The fact that many chemotherapeutics were weak bases led us to investigate the transport of these substances into liposomes in response to membrane potentials and ΔpH . Subsequent studies led to considerably broader applications involving the transport and accumulation of a wide variety of drugs, biogenic amines, amino acids, peptides, lipids, and ions in LUVs exhibiting a ΔpH (for a review, see Cullis *et al.*, 1997). Application of this technology has led to the development of several liposomal anticancer systems that exhibit improved therapeutic properties over free drug. Early studies (see Mayer *et al.*, 1990a and references therein) had shown that reduced side effects with equal or enhanced efficacy could be obtained in liposomal systems, despite low encapsulation levels and poor drug retention. This led to our initial efforts to develop 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 (Mayer *et al.*, 1989, 1990a–c, 1993) was expected to provide significant improvements in overall efficacy due to high drug-to-lipid 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 (Cheung *et al.*, 1999; Chonn and Cullis, 1995) or have been approved by the U.S. FDA for clinical use (Muggia, 2001). Other liposomal doxorubicin formulations (Burstein *et al.*, 1999; Campos *et al.*, 2001; Coukell and Spencer, 1997; Gokhale *et al.*, 1996; Gordon *et al.*, 2000; Grunaug *et al.*, 1998; Israel *et al.*, 2000; Judson *et al.*, 2001; Northfelt *et al.*, 1998; Shields *et al.*, 2001) 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 (Gelmon *et al.*, 1999; Millar *et al.*, 1998; Tokudome *et al.*, 1996; Webb *et al.*, 1995, 1998a), mitoxantrone (Adlakha-Hutcheon *et al.*, 1999; Chang *et al.*, 1997; Lim *et al.*, 1997, 2000; Madden *et al.*, 1990), daunorubicin (Gill *et al.*, 1996; Madden *et al.*, 1990; Muggia, 2001; Pratt *et al.*, 1998), ciprofloxacin (Bakker-Woudenberg *et al.*, 2001; Webb *et al.*, 1998b), topotecan (Tardi *et al.*, 2000), 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 the short term. The first of the conventional drug carriers are reaching the market while 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 ≥ 5 h in mice) following intravenous (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 (Boman *et al.*, 1994; Mayer *et al.*, 1993; Webb *et al.*, 1995, 1998a). This can result in large increases in efficacy (Boman *et al.*, 1994). These improvements stem from the increased permeability of the vasculature at tumor sites (Brown and Giaccia, 1998; Dvorak *et al.*, 1988) or sites of inflammation, which results in preferential extravasation of small, long-circulating carriers in these regions.

The insights gleaned from conventional drug carriers have implications for the design of liposomal systems for the delivery of larger macromolecules. There is currently much interest in developing systemic vectors for the delivery of the therapeutic genetic drugs such as antisense oligonucleotides or plasmid DNA. To obtain appreciable amounts of a vector containing the antisense oligonucleotides or therapeutic gene to the site of disease, the vector must be stable, small, and long-circulating. Of course, the vector must also be accumulated by target cells, escape the endocytotic pathway, and be delivered to the nucleus.

Over the past 20 years, our laboratory has played a major role in the development of liposomal systems optimized for the delivery of both conventional drugs and, more recently, genetic drugs. Our early studies on the production of LUVs by extrusion led to the characterization of several liposomal drug delivery systems (Bally *et al.*, 1988; Boman *et al.*, 1993, 1994; Chonn and Cullis, 1995; Cullis *et al.*, 1997; Fenske *et al.*, 1998;

Hope and Wong, 1995; Madden *et al.*, 1990; Maurer-Spurej *et al.*, 1999; Mayer *et al.*, 1986a), the development of new approaches for the loading of drugs via generation of ΔpH (Fenske *et al.*, 1998; Maurer-Spurej *et al.*, 1999) or other ion gradients (Cheung *et al.*, 1998), and finally new methods for the encapsulation of antisense oligonucleotides (Maurer *et al.*, 2001; Semple *et al.*, 2000, 2001) and plasmid DNA (Fenske *et al.*, 2002; Maurer *et al.*, 2001; Mok *et al.*, 1999; Tam *et al.*, 2000; Wheeler *et al.*, 1999) within liposomes. In this chapter we will provide an overview of these methods, along with detailed descriptions of procedures for the encapsulation of both conventional and genetic drugs within liposomes.

Encapsulation of Small, Weakly Basic Drugs within LUVs in Response to Transmembrane pH and Ion Gradients

The Formation of LUVs by Extrusion Methods

Many research questions in membrane science, specifically those involving the dynamic properties of lipid bilayers, can be addressed using very basic model membrane systems, such as the multilamellar vesicle (MLV) formed spontaneously upon vigorous agitation of lipid–water mixtures. These large (1–10 μm) multilamellar liposomes are ideal for biophysical investigations of lipid dynamics and order using techniques such as fluorescence, electron spin resonance (ESR), or broadband (^2H and ^{31}P) nuclear magnetic resonance (NMR). However, many properties of biological membranes, such as the presence of pH or ion gradients, cannot be adequately modeled using large, multilamellar systems. These kinds of studies require the use of unilamellar vesicles in the nanometer size range. In our case, investigations relating ion and pH gradients to lipid asymmetry (Cullis *et al.*, 1997, 2000) were the driving force for the development of extrusion technology. While it was clear that MLVs were not appropriate for such topics, it was also apparent that the methods available for the generation of unilamellar vesicles, which included dispersion of lipids from organic solvents (Batzri and Korn, 1973), sonication (Huang, 1969), detergent dialysis (Mimms *et al.*, 1981), and reverse-phase evaporation (Szoka and Papahadjopoulos, 1978), had serious drawbacks (Cullis, 2000). However, Papahadjopoulos, Szoka, and co-workers (Olson *et al.*, 1979) had observed that sequential extrusion of 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 a narrow size distribution and diameters close to the chosen pore size (Fig. 1) (Hope *et al.*, 1985; Mayer *et al.*, 1986). 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 5–10° 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.

Initially, we will describe in some detail the formation of a 30 mM solution of 100 nm LUVs composed of distearoylphosphatidylcholine (DSPC)/cholesterol (Chol), a highly ordered lipid mixture that is frequently chosen for drug delivery applications due to its good circulation lifetime and drug retention properties.

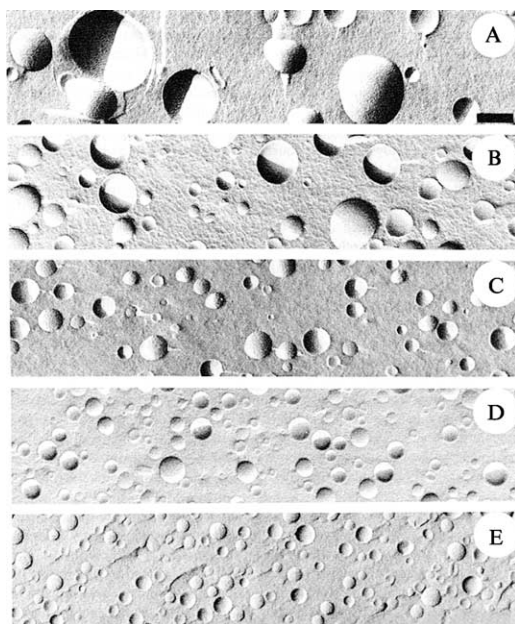


FIG. 1. Freeze-fracture electron micrographs of egg phosphatidylcholine LUVs prepared by extrusion through polycarbonate filters with pore sizes of (A) 400 nm, (B) 200 nm, (C) 100 nm, (D) 50 nm, and (E) 30 nm. The bar in (A) represents 150 nm. [Reprinted from Hope, M. J., Bally, M. B., Mayer, L. D., Janoff, A. S., and Cullis, P. R. (1986). *Chem. Phys. Lipids* **40**, 89–107, with permission.]

*Experimental Procedure: Preparation of DSPC/Chol (55:45)
LUVs by Extrusion*

Stock solutions of DSPC and Chol in CHCl_3 should be prepared with concentrations ranging from 100 to 200 mM. Whenever possible, lipid concentrations should be verified by an appropriate assay. Phospholipid concentrations are verified using the assay of Fiske and Subbarow (1925) for the quantitative determination of inorganic phosphate, which is still in use today. The assay, which combines simplicity, accuracy, and reproducibility, is generally used for determining the concentration of phospholipid stock solutions or of LUV preparations. The assay is based on the ability of perchloric acid to liberate and oxidize phosphorus, forming phosphate ions, which form a colored complex that is quantified by absorbance spectrophotometry. The entire assay takes about 2 h, including preparation of standard curve and clean up. Detailed protocols for this assay have been described recently by Fenske *et al.* (2003) and will not be repeated here.

To prepare an LUV preparation with a final lipid concentration of 30 mM and a DSPC/Chol ratio of 55:45, DSPC (33 μmol) (Avanti Polar Lipids, Alabaster, AL; Northern Lipids, Vancouver, BC, Canada) and cholesterol (27 μmol) (Sigma-Aldrich, St. Louis, MO; Northern Lipids) are codissolved in a 13×100 -mm test tube. If required, a trace of a radioactive tracer such as [^3H]cholesterol hexadecyl ether ([^3H]CHE) (PerkinElmer Life Sciences, Woodbridget, Ontario, Canada) is added to give a specific activity of 10–30 dpm/nmol lipid. The CHCl_3 is then mostly removed under a gentle stream of nitrogen gas while immersing the tube in hot water (50–60°), following which the lipid film is placed under high vacuum for a minimum of 1 h. The dry lipid film can be used immediately or stored in the freezer for later use.

For the formation of vesicles, the lipid film is hydrated with 2 ml of appropriate buffer (internal buffer or hydration buffer), such as 300 mM citrate pH 4.0 (pH gradient loading), 300 mM ammonium sulfate (for amine loading), or 300 mM MgSO_4 , pH 6.5 (for ionophore loading) (these will all be discussed below). The lipid–water mixture is vortexed at 65° until a lipid emulsion is obtained and the lipid film can no longer be seen on the glass tube. This step must always be performed at a temperature approximately 10° higher than the gel-to-liquid crystalline phase transition temperature (T_m) of the phospholipid being used.

The lipid emulsion is then transferred to cryovial tubes for five cycles of freeze-thawing. The cryovials are immersed in liquid nitrogen for 3–5 min, then transferred to lukewarm water (for 1 min), and then to a water bath at 65°. After thawing completely, the emulsions are vortexed vigorously, and the freeze–thaw cycle is repeated four more times.

The extruder (Northern Lipids) is assembled with two polycarbonate filters (Nuclepore polycarbonate membranes; Whatman, Clifton, NJ) with a pore size of $0.1 \mu\text{m}$ and a diameter of 25 mm, and connected to a circulating waterbath equilibrated at 65° . 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 1 min.

The lipid concentration of the final LUVs is determined by a phosphate assay (Fenske *et al.*, 2003; Fiske and Subbarow, 1925) or by liquid scintillation counting.

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 (Cullis, 2000). 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 ($\text{p}K_{\text{a}} = 8.6$) 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 drug has been taken up or the buffering capacity of the vesicle interior has been overwhelmed. This process is illustrated in Fig. 2 for the uptake of doxorubicin into egg phosphatidylcholine (EPC)/Chol and EPC LUVs, where it is seen that uptake is dependent on time, temperature, and lipid composition (Mayer *et al.*, 1986a). If conditions are chosen correctly, high drug-to-lipid ratios can be achieved ($\text{D/L} = 0.2 \text{ mol:mol}$) with high trapping efficiencies (98% and higher) and excellent drug retention. A diagrammatic illustration of this process is given in Fig. 3A (also see inset). Interestingly, much higher levels of doxorubicin can be loaded than would be predicted on the basis of the magnitude of ΔpH (Cullis *et al.*, 1997; Harrigan *et al.*, 1993). This would appear to result from the formation of doxorubicin precipitates within the LUV interior, which provides an additional driving force for accumulation (Lasic *et al.*, 1992, 1995). Doxorubicin forms fibrous precipitates that are aggregated into bundles by citrate (Li *et al.*, 1998) or sulfate (Lasic *et al.*,

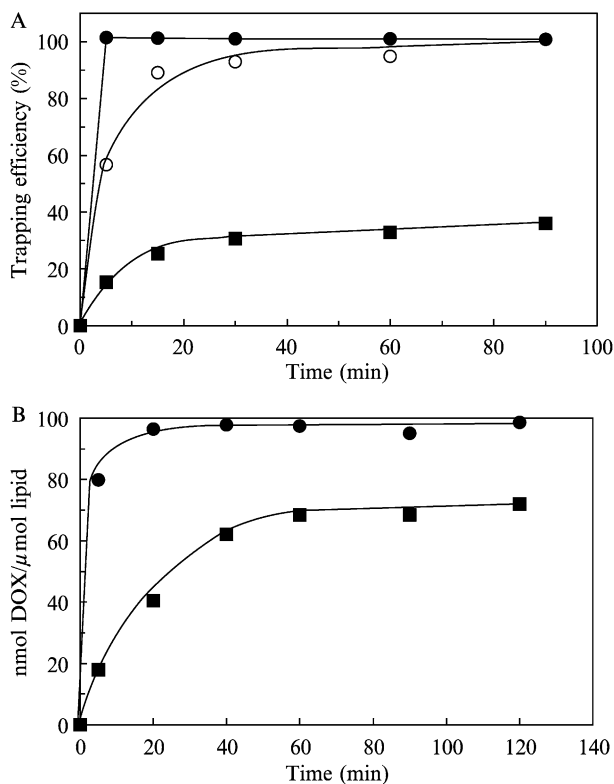


FIG. 2. (A) Effect of incubation temperature on uptake of doxorubicin into 200 nm EPC/cholesterol (55:45 mol/mol) LUVs exhibiting a transmembrane pH gradient (pH 4 inside, 7.8 outside). Doxorubicin was added to LUVs (D/L = 0.3 wt:wt) equilibrated at 21(■), 37(○), and 60°. [Reprinted from Mayer, L. D., Tai, L. C., Ko, D. S., Masin, D., Ginsberg, R. S., Cullis, P. R., and Bally, M. B. (1989). *Cancer Res.* **49**, 5922–5930, with permission.] (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 D/L ratio was 100 nmol/μmol. [Reprinted from Mayer, L. D., Bally, M. B., and Cullis, P. R. (1986a). *Biochim. Biophys. Acta* **857**, 123–126, with permission.]

1992, 1995) counteranions, which affect the rate of doxorubicin release from LUVs (Li *et al.*, 2000). These precipitates can be visualized by cryoelectron microscopy, where they are seen to give the LUVs a “coffee-bean” appearance (Fig. 4). 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–Mn²⁺ complexes (Cheung *et al.*, 1998), as will be described below.

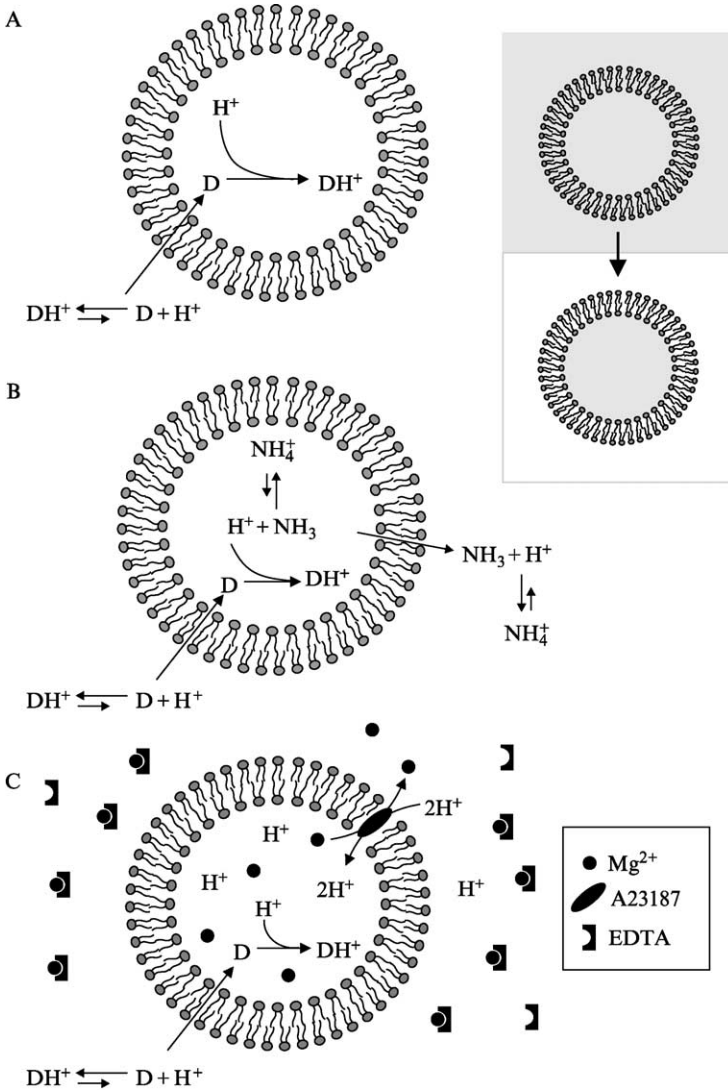


FIG. 3. Diagrammatic representations of drug uptake in response to transmembrane pH gradients. Prior to drug loading, it is necessary to establish the primary pH gradient or the primary ion gradient that will generate a ΔpH . Lipid films or powders are initially hydrated and then extruded in the internal (or hydration) buffer, giving rise to a vesicle solution in which both the external and internal solutions are the same, as indicated by the gray 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



FIG. 4. Cryoelectron micrograph of 100 nm EPC/cholesterol LUVs containing doxorubicin ($D/L = 0.3$ mol/mol) loaded in response to a transmembrane pH gradient (inside acidic). The arrow indicates the presence of precipitated doxorubicin fibers within the LUV, which gives the vesicle the appearance of a “coffee-bean.” [Reprinted from Fenske, D. B., Maurer, N., and Cullis, P. R. (2003). *In* “Liposomes: A Practical Approach” (V. P. Torchilin and V. Weissig, eds.). Oxford University Press, Oxford, with permission.]

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 additional 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.

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 (Madden *et al.*, 1990), and all the remote-loading methods that follow are based on similar principles involving the generation of ΔpH , even though this may not always be immediately obvious.

Experimental Procedure: Remote Loading of Doxorubicin into DSPC/Chol (55:45) LUVs

DSPC/Chol (55:45) LUVs (diameter = 100 nm) are prepared as described previously ([Lipid] = 30 mM, volume = 2 ml), using 300 mM citrate, pH 4.0, as the hydration buffer. There are two common methods for formation of the pH gradient, the first of which is passing the LUVs down a column of Sephadex G-50 (Amersham Pharmacia Biotech) equilibrated in HEPES-buffered saline (HBS) (20 mM HEPES, 150 mM NaCl, pH 7.5) (the method described below), and the second of which involves the use of spin columns.

Spin columns permit the rapid separation of LUVs from their hydration buffer (or from unencapsulated drug) on a 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 HBS is prepared by adding a small volume (2–3 ml) of dry G-50 powder to 200–300 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 (670g) momentarily. More G-50 slurry is added, and the centrifugation is repeated. When finished, the moist G-50 bed should be 0.9–1.0 ml. The spin columns are covered with parafilm to prevent drying and are used within an 8-h period.

Transmembrane 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 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 ~2 ml, and the lipid concentration will be around 15 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 drug-to-lipid (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 $\varepsilon = 1.06 \times 10^4 M^{-1} \text{ cm}^{-1}$ (Rottenberg, 1979). Aliquots of lipid (5 μmol) and doxorubicin (1 μmol , approximately 0.5 mg) are combined in a glass test tube (or plastic Eppendorf tube) with HBS to give a final volume of 1 ml (5 mM lipid concentration). Drug uptake occurs during a 30-min incubation at 65°. This is verified at appropriate time points (0, 5, 15, 30 min) by applying an aliquot (50–100 μl) to a spin column and centrifuging at 2000 rpm for 2 min. 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 the 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), by liquid scintillation counting of an appropriate radiolabel, or by measuring the fluorescence of LUVs containing 0.5–1 mol% of rhodamine-PE (see below). Doxorubicin is quantified by an absorbance assay (see below). The percent uptake at any time point (e.g., $t = 30 \text{ min}$) is determined by $\% \text{ uptake} = [(D/L)_{t = 30 \text{ min}}] \times 100 / [(D/L)_{\text{initial}}]$. 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 or five cuvettes containing 0–150 nmol doxorubicin in a volume of 0.1 ml; samples to be assayed are the same volume. To each tube is added 0.9 ml of 1% (v/v in water) Triton X-100 solution. For saturated lipid systems such as DSPC/Chol, the tubes should be heated in a boiling waterbath for 10–15 s, until the detergent turns cloudy. The samples are allowed to cool, and the 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 (Madden *et al.*, 1990), 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 (Hope and Wong, 1995). 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 Δ pH-loading method that is based on transmembrane gradients of ammonium sulfate (Haran *et al.*, 1993; Hope and Wong, 1995; Lasic *et al.*, 1995). 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 \sim 2.7 (Hope and Wong, 1995). Any neutral drug that diffuses into the vesicle interior will consume a proton and become charged and therefore trapped. If continued drug uptake leads to consumption of the available protons, the diffusion of additional neutral ammonia from the vesicles will create more protons to drive drug uptake. This will continue until all the drug has been loaded or until the internal proton supply is depleted, leaving a final internal pH of about 5.1 (Hope and Wong, 1995). The technique is ideal for ciprofloxacin as 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 Fig. 3B.

This technique has been applied to a variety of drugs including doxorubicin (Haran *et al.*, 1993; Lasic *et al.*, 1992, 1995; Maurer-Spurej *et al.*, 1999), epirubicin (Maurer-Spurej *et al.*, 1999), ciprofloxacin (Hope and Wong, 1995; Lasic *et al.*, 1995; Maurer *et al.*, 1998; Maurer-Spurej *et al.*, 1999), and vincristine (Maurer-Spurej *et al.*, 1999). The method is equally effective using a range of alkylammonium salts (e.g., methylammonium sulfate, propylammonium sulfate, or amylammonium sulfate) to drive uptake (Maurer-Spurej *et al.*, 1999). Some drugs, such as doxorubicin, precipitate and form a gel in the vesicle interior (Lasic *et al.*, 1992, 1995; Li *et al.*, 2000), while others, such as ciprofloxacin, do not (Lasic *et al.*, 1995; Maurer *et al.*, 1998). Ciprofloxacin can reach intraliposomal concentrations as high as 300 mM, and while the drug does form small stacks as shown by $^1\text{H-NMR}$, it does not form large precipitates (Maurer *et al.*, 1998), 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 (Li *et al.*, 1998, 2000) and ammonium sulfate (Lasic *et al.*, 1992, 1995), 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 (Webb *et al.*, 1995). Included is a description of the Bligh–Dyer extraction procedure (Bligh and Dyer, 1959), which involves partitioning the lipid and water-soluble drug into organic solvent and aqueous layers, respectively. This is necessary as lipid interferes with the ciprofloxacin assay.

Experimental Procedure: Remote Loading of Ciprofloxacin into SPM/Chol LUVs

When preparing radiolabeled lipid stock solutions, it is often convenient to prepare a large batch for determination of specific activity and then divide that batch into smaller aliquots for later use. For the preparation of 10 lipid samples of 50 μmol each, 275 μmol egg sphingomyelin (193 mg) (Avanti Polar Lipids; Northern Lipids; Sigma Aldrich) is codissolved with 225 μmol Chol (87 mg) in 7 ml *t*-butanol. If desired, 12 μCi of [^3H]cholesteryl hexadecyl ether (CHE) or [^{14}C]CHE is added. The specific activity (in dpm/ μmol lipid) of the lipid mixture is easily obtained by measuring the activity of an aliquot by liquid scintillation counting, and measuring SPM concentration via the phosphate assay (Fenske *et al.*, 2003; Fiske and Subbarow, 1925), removing *t*-BuOH by lyophilization prior to assay (freeze tubes in liquid nitrogen and place under high vacuum for 30 min). The lipid mixture is aliquoted into 10 glass Pyrex tubes (50 μmol lipid per tube), frozen in liquid nitrogen, and lyophilized for at least 4 h. SPM/Chol (55:45) LUVs (diameter = 100 nm) are prepared using 50 μmol lipid as described above ([Lipid] = 25 mM, volume = 2 ml). The hydration solution is 300 mM ammonium sulfate [(NH₄)₂SO₄]. Spin columns are prepared (using saline rather than HBS) for monitoring drug uptake with time.

The amine gradient is formed by running an aliquot (200 μl) of the LUVs down a column (1.5 \times 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 μl) and pooling the fractions.

Ciprofloxacin (Bayer Corporation) is often loaded at a drug-to-lipid (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 Eppendorf tube), adding saline to give a final volume of 1 ml (5 mM lipid concentration). This solution is incubated at 65° for 30 min, with aliquots (50–100 μl) withdrawn at appropriate time points (0, 5, 15, 30 min) and applied to a spin column [centrifuge at 2000 rpm (670g) for 2 min]. 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 (Fenske *et al.*, 2003; Fiske and Subbarow, 1925) or by liquid scintillation counting. Ciprofloxacin is quantified by an absorbance assay following removal of drug from lipid by a Bligh–Dyer extraction procedure (Bligh and Dyer, 1959) (see below). The percent uptake is determined as previously described.

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 <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 is 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 2 min in a desktop centrifuge.

After carefully removing the aqueous phase, the absorbance at 273.5 nm is read to obtain nmol ciprofloxacin present in the original sample volume (in μl), 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 and the next we examine two approaches that utilize transmembrane ion gradients to generate the driving force for uptake. In the first method, uptake is driven by a secondary pH gradient that is generated through the action of ionophores responding to a primary ion gradient (involving Na^+ , Mn^{2+} , or Mg^{2+}). In the second method, drug uptake is driven by the formation of intravesicular drug–ion complexes and does not involve a ΔpH at all. These will be discussed in turn below.

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

(Fenske *et al.*, 1998). The process is diagrammed in Fig. 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, as 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, as chloride ion can interfere with some ionophores (Wheeler *et al.*, 1994). After establishing the primary ion gradient, the drug (which to date includes doxorubicin, mitoxantrone, ciprofloxacin, and vincristine) 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 and 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 (Fenske *et al.*, 1998). However, the A23187-loaded systems exhibit excellent *in vivo* circulation and drug retention properties that are comparable to systems loaded by the citrate or amine methods, whereas the nigericin-loaded systems do not.

Experimental Procedure: Ionophore-Mediated Loading of Vincristine into SPM/Chol LUVs

LUVs (diameter = 100 nm) are prepared from SPM/Chol (55:45) using 50 μ mol lipid as described above ([Lipid] = 25 mM). It has been shown that liposomal vincristine prepared from SPM/Chol exhibits greater efficacy than systems prepared from DSPC/Chol (Webb *et al.*, 1995). The hydration solution is 300 mM $MnSO_4$ (2 ml).

Spin columns for monitoring drug uptake with time are hydrated in 300 mM sucrose. A Mn^{2+} gradient is formed by running an aliquot (200 μ l) of the LUVs down a column (1.5 \times 15 cm) of Sephadex G-50 eluted in HEPES-buffered sucrose (20 mM HEPES, 300 mM sucrose, pH 7.5) containing 3 mM Na_2EDTA . Alternatively, the gradient can be formed using spin columns prepared in the same buffer (spin 2 \times 100 μ l) and pooling the fractions.

Vincristine sulfate (commercially available from Eli Lilly Canada at 1 mg/ml) is added to give a D/L ratio of 0.03 mol:mol. Five micromoles of lipid and 0.15 μmol of vincristine are combined in a glass test tube, with addition of HEPES-buffered sucrose containing 3 mM Na_2EDTA to give a final volume of 1 ml (5 mM lipid concentration). The mixture is incubated at 60° for 10 min, saving an aliquot (50 μl) for determination of the initial D/L ratio. An aliquot (50–100 μl) is applied to a spin column to assess any drug uptake prior to addition of ionophore. There may be a small amount of uptake if the salt solution is acidic (as MnSO_4 solutions tend to be), but the majority of uptake will begin with addition of the ionophore.

At time $t = 0$, the A23187 (Sigma-Aldrich) (in ethanol) is added in a volume of approximately 5 μl to give a concentration of 0.1 $\mu\text{g}/\mu\text{mol}$ lipid. If necessary, the concentration of A23187 can be increased 10 fold. Aliquots (100 μl) are withdrawn at appropriate time points (0, 5, 15, 30 min) and applied to spin columns (2000 rpm for 2 min). The initial mixture and each time point are then assayed for vincristine and lipid. Lipid can be quantified as described above. Vincristine is quantified by an absorbance assay. A standard curve is prepared consisting of four or five tubes containing 0–150 nmol vincristine in a volume of 0.2 ml. After addition of 0.8 ml of 95% ethanol, the absorbance of the standard curve and assay samples are read at 295 nm.

A representative uptake experiment is shown in Fig. 5, where the effects of ionophore, external pH, and EDTA are apparent. Under the

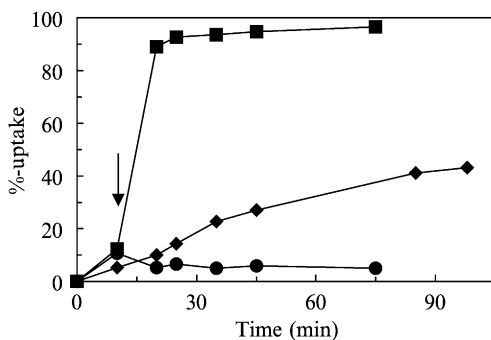


FIG. 5. Effect of external pH and EDTA on the uptake of vincristine in 100 nm SPM/Chol LUVs containing 300 mM MnSO_4 . The external medium was 20 mM HEPES, 300 mM sucrose, 3 mM EDTA, pH 7.5 (■), 300 mM sucrose, 3 mM EDTA, pH 6 (◆), or 20 mM HEPES, 300 mM sucrose, pH 7.5 (●). The addition of A23187 (0.1 $\mu\text{g}/\mu\text{mol}$ lipid) is indicated by the arrow. The uptake temperature was 60°, and the initial D/L ratio was 0.03 (mol:mol). [Reprinted from Fenske, D. B., Wong, K. F., Maurer, E., Maurer, N., Leenhouts, J. M., Boman, N., Amankwa, L., and Cullis, P. R. (1998). *Biochim. Biophys. Acta* **1414**, 188–204, with permission.]

conditions described above, >95% uptake is observed within 20 min of addition of ionophore. However, if the external pH is reduced to 6.0, the uptake reaches 40% only after 90 min of incubation. If EDTA is absent, no uptake occurs.

If the toxicity of Mn^{2+} is of concern, equally effective loading can be achieved using $MgSO_4$ solutions. In this case, the lipids are hydrated with 300 mM $MgSO_4$, pH 6.5, and the Mg^{2+} gradient is formed on a Sephadex column eluted with 20 mM HEPES, 300 mM sucrose, pH 6.0, containing 15 mM Na_2EDTA . Due to the lower affinity of Mg^{2+} for the ionophore relative to Mn^{2+} , a higher concentration of A23187 is required, usually in the range of 0.5–1 $\mu g/\mu mol$ lipid.

Encapsulation of Doxorubicin within LUVs in Response to Transmembrane Manganese Gradients

During our studies on ionophore loading, it was observed that doxorubicin uptake could occur in the presence of a $MnSO_4$ gradient but in the absence of a pH gradient. The driving force for drug uptake was found to be the formation of a doxorubicin– Mn^{2+} complex, which can be observed by eye as the characteristic red color of doxorubicin changes to an intense purple and which is monitored by absorption spectroscopy by a shift in absorbance maximum from 480 to 580 nm (Cheung *et al.*, 1998). The method was very efficient, allowing D/L ratios of 0.5 (mol:mol) to be achieved with 100% efficiency and ratios as high as 0.8 at lower loading efficiencies. Interestingly, the formation of Mn^{2+} –drug complexes does not appear to play a role in A23187-mediated uptake of doxorubicin, as no evidence of complex formation was observed (Cheung *et al.*, 1998). This formulation may have pharmaceutical potential in that complexes of doxorubicin with other cations (such as Fe^{3+} and Cu^{2+}) have been found to increase drug cytotoxicity (Gutteridge, 1984; Gutteridge and Quinlan, 1985; Hasinoff, 1989a,b; Hasinoff *et al.*, 1989; Muindi *et al.*, 1984). In addition, the high D/L ratios achievable will permit a higher payload of drug to be delivered to cells. As the methodology is similar to that of ionophore loading, only a brief description will be provided here.

Experimental Procedure: Manganese-Mediated Loading of Doxorubicin into SPM/Chol LUVs

LUVs composed of SPM/Chol (55:45 mol:mol), containing a trace of [3H]cholesterol hexadecyl ether, are prepared as described above using lipid mixtures lyophilized from *t*-butanol (lyophilization guards against phase separation during solvent evaporation). The hydration solution is 300 mM $MnSO_4$ prepared in 30 mM HEPES, pH 7.4. A transmembrane

Mn²⁺ gradient is formed using spin columns equilibrated in 300 mM sucrose, 30 mM HEPES, pH 7.4, and drug uptake is accomplished by addition of doxorubicin (D/L ratio = 0.5 mol:mol) followed by incubation at 60° for 70 min. Drug uptake is monitored as described above.

Encapsulation of Genetic Drugs within LUVs: Long-Circulating Vectors for the Systemic Delivery of Genes and Antisense Oligonucleotides

The development of genetic drugs, such as antisense oligonucleotides or plasmid DNA carrying a therapeutic gene, holds great promise in the treatment of acquired diseases such as cancer and inflammation. Much current effort is directed at development of gene delivery vehicles capable of accessing distal disease sites following systemic (intravenous) administration. While numerous methods exist for effective *in vitro* gene delivery, the situation *in vivo* is very different. Many current systems, such as viral vectors, lipoplexes, and lipopolyplexes, have limited utility for systemic applications (Fenske *et al.*, 2001). Viral vectors cannot carry plasmids that exceed a certain size and often elicit strong immune responses. Lipoplexes and lipopolyplexes tend to be cleared rapidly from the circulation due to their large size and positive charge characteristics and suffer from toxicity issues (see Fenske *et al.*, 2001, and references therein). Unfortunately, liposomal carriers of genetic drugs that possess the optimized characteristics of conventional drug carriers (small size, serum stability, and long circulation lifetimes) have been difficult to achieve (Maurer *et al.*, 1999) due to the technical challenges involved in encapsulating large, highly charged molecules within relatively small vesicles. Recently, though, we have developed two very different methods for the generation of liposomes capable of carrying either antisense oligonucleotides or plasmid DNA. The first of these employs a detergent-dialysis approach for the formation of liposomal DNA carriers known as stabilized plasmid–lipid particles (SPLP) (Fenske *et al.*, 2002; Tam *et al.*, 2000; Wheeler *et al.*, 1999). The second method involves entrapping polynucleotides, via electrostatic interactions, within preformed ethanol-destabilized cationic liposomes (Maurer *et al.*, 2001; Semple *et al.*, 2000, 2001). Both methods are discussed below.

Stabilized Plasmid–Lipid Particles (SPLP): Vectors for Systemic Gene Delivery

SPLP are small (~70 nm), monodisperse particles consisting of a single plasmid encapsulated in a unilamellar lipid vesicle. The lipid composition can be varied and can include additional components, but the basic system is composed of DOPE, a cationic lipid [usually *N,N*-dioleoyl-*N*,

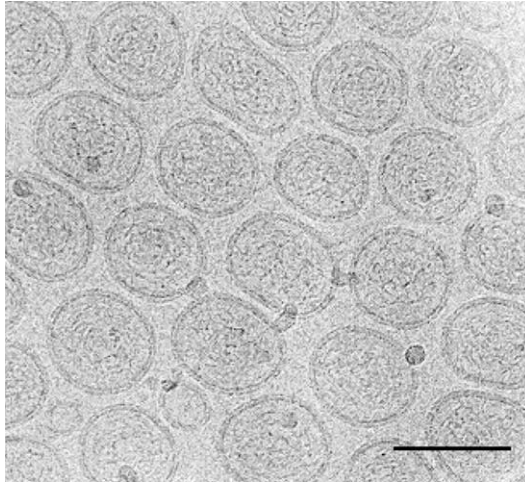


FIG. 6. Cryoelectron micrographs of purified SPLP prepared from DOPE:DODAC:PEG-CerC₂₀ (83:7:10; mol:mol:mol) and pCMVluc as described in the text. The bar indicates 100 nm. [Reprinted from Tam, P., Monck, M., Lee, D., Ludkovski, O., Leng, E. C., Clow, K., Stark, H., Scherrer, P., Graham, R. W., and Cullis, P. R. (2000). *Gene Ther.* **7**, 1867–1874, with permission.]

N-dimethylammonium chloride (DODAC)] and PEG-ceramide (PEG-Cer) (Tam *et al.*, 2000; Wheeler *et al.*, 1999). The plasmid DNA can be visualized within the particles by cryo-EM (Fig. 6). SPLP are formed from mixtures of plasmid and lipid by a detergent-dialysis procedure involving octyl-glucopyranoside (OGP). SPLP protect plasmid DNA from DNase I and serum nucleases (Wheeler *et al.*, 1999), possess extended circulation half-lives (6–7 h) (Tam *et al.*, 2000), and have been shown to accumulate in distal tumor sites with subsequent gene expression in mouse tumor models following *iv* injection (Fenske *et al.*, 2001). These vectors are clearly capable of disease-site targeting, making them promising vectors for *in vivo* gene transfer. The experimental procedures discussed below outline the production of SPLP for smaller-scale *in vitro* or *in vivo* studies. A detailed protocol for the production of a large-scale SPLP batch, suitable for animal studies, has been published elsewhere (Fenske *et al.*, 2002).

Experimental Procedure: A Detergent Dialysis Approach to the Encapsulation of Plasmid DNA within LUVs

The basic procedure involves solubilizing lipid and plasmid (200 μ g) in a detergent solution of appropriate ionic strength and removing the

detergent by dialysis to form SPLP and empty vesicles. Unencapsulated DNA is removed by ion-exchange chromatography and empty vesicles by sucrose density gradient centrifugation.

Due to the relatively large quantities of buffer required, it is convenient to initially prepare 2 liters of 10× HBS (1× = 20 mM HEPES, 150 mM NaCl, pH 7.4) and to dilute as required. Lipid stock solutions can be prepared in chloroform or in methanol, at (approximate) concentrations of 100 mM for DOPE (Avanti Polar Lipids; Northern Lipids), 25 mM for DODAC (Inex Pharmaceuticals), 10 mM for PEGCerC₂₀ (Northern Lipids; Inex Pharmaceuticals), and 0.78 mM (1 mg/ml) for rhodamine-PE [1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(Lissamine Rhodamine B Sulfonyl)] (Avanti Polar Lipids). These are combined in the proportions DOPE:DODAC:PEGCerC₂₀:Rho-PE (81.5:8:10:0.5) for a total of 10 μmol lipid. Chloroform is dried under a stream of nitrogen gas and then under high vacuum as described above.

The 10 μmol of dry lipid is solubilized by addition of 200 μl of OGP (1 M in water) (Sigma-Aldrich), with heating (in a 60° waterbath) and vortexing until the lipid is mostly dissolved and no longer sticking to the glass tube. The addition of 600 μl HBS is followed by vortexing with heating until clear. The addition of 200 μg plasmid DNA (from a 1 mg/ml stock solution), with gentle vortexing, will give a 1 ml solution with 200 μg plasmid DNA, [lipid] = 10 mM, and [OGP] = 200 mM.

Encapsulation of plasmid and formation of SPLP is highly sensitive to the cationic lipid content and the salt concentration of the dialysis buffer. In general, a salt concentration is selected that gives high levels of encapsulation (50–70%), a small diameter (80–100 nm), and a monodisperse particle distribution (a χ^2 value of 3 or less obtained from QELS). In practice, it is often necessary to test a range of salt concentrations to achieve optimal encapsulation. This is particularly critical when preparing SPLP with high DODAC content (14–24 mol%), which requires inclusion of a polyanionic salt such as citrate or phosphate in addition to NaCl (Saravolac *et al.*, 2000; Zhang *et al.*, 1999). For SPLP containing 8 mol% DODAC, particle formation should occur for 20 mM HEPES, pH 7.5, containing 140–150 mM NaCl. As it may be necessary to test several salt concentrations for a given lipid formulation, it is advisable to prepare more SPLP than will be required, taking into account final particle yields.

Optimal encapsulation conditions can be assessed by dialyzing 250-μl aliquots of the above preparation in 1-cm-diameter dialysis tubing overnight against 1 liter of 20 mM HEPES, pH 7.5, containing 140, 145, and 150 mM NaCl. For dialysis, Spectrum Spectra/Por Molecular porous 10-mm membrane tubing with 12,000–14,000 MWCO (Spectrum Laboratories, Rancho Dominguez, CA) gives good results. The aliquots are

analyzed for size (via QELS analysis using a Nicomp Model 370 Submicron Particle Sizer or equivalent unit) and encapsulation efficiency (picogreen assay). The picogreen assay involves pipetting 5- μ l aliquots of each formulation into disposable 1-cm fluorescence cuvettes and adding 1.8 ml of picogreen buffer [containing 1 μ l of picogreen (Molecular Probes, Eugene, OR) per 2 ml HBS]. The fluorescence is read in the absence and presence of Triton X-100 (Sigma-Aldrich), using excitation and emission wavelengths of 480 and 520 nm, respectively (with slit widths set to 5 nm). After reading the fluorescence in the absence of detergent (– Triton value), 30 μ l of 10% (v/v) Triton X-100 (in water) is added to the cuvette with thorough mixing, after which the fluorescence is read again (+ Triton value). The % encapsulation = $(([+ \text{Triton}] - [- \text{Triton}]) \times 100) / [+ \text{Triton}]$.

The optimal NaCl concentration is determined by the percent of plasmid encapsulation and particle size. The optimal NaCl provides for 50–80% encapsulation and a single population with a particle size of ≤ 100 nm. Particle sizes greater than 120 nm indicate the presence of vesicle aggregation. Once the optimal NaCl concentration has been selected, 4 \times 2 liters of dialysis buffer is prepared for a 48-h dialysis. The dialysis bags should be transferred to fresh dialysis buffer (2 liters) every 12 h. Following dialysis, both a picogreen assay and QELS analysis are performed on the resulting material. The percent encapsulation should be in the range of 50–80%. If this is not achieved, another salt curve should be performed before proceeding to the next step (the lipid–DNA mixture can be resolubilized in OGP and redialyzed with a different buffer).

Following formation of SPLP, unencapsulated DNA is removed by DEAE-Sepharose chromatography. Up to 0.64 mg of DNA can be loaded per 1 ml volume of the DEAE-Sepharose CL-6B (Sigma) column. The column can be poured in a small plastic holder (such as a 3-ml disposable syringe stopped with a small plug of glass wool) and washed with 10 column volumes of 1 \times HBS. Once the column has settled, the formulation is slowly loaded onto the resin and eluted with HBS. The final volume equal to 1.5 times the sample volume is collected to completely elute all of the formulation from the column.

The final step in the purification of SPLP is removal of empty vesicles from those containing plasmid DNA. This is accomplished by stepwise sucrose density gradient ultracentrifugation in a Beckman ultracentrifuge using an SW41 rotor. Solutions containing 1.0, 2.5, and 10.0% w/v sucrose are prepared in 1 \times HBS, filter sterilized into a sterile container, and stored at 4°. Prior to pouring the gradients, a long glass pipette is pulled to a small point using forceps and a Bunsen burner and is broken at the tip to give a slow flow rate. The elongated pipette is placed in the ultracentrifuge tube (Beckman Ultra-clear polycarbonate centrifuge tubes, 14 \times 89 mm,

#344059), and 3.6 ml of a 1.0% sucrose solution is poured into the pipette using a second (regular) pipette, avoiding air bubbles in the narrow part of the pipette. The sucrose layers are poured in order of increasing density; i.e., 3.6 ml of 1.0%, 3.6 ml of 2.5%, and 3.6 ml of 10.0%. One milliliter of SPLP is then gently layered on top of the gradients. All the tubes are then balanced with $1 \times$ HBS to within 0.01 g and placed in the SW41 buckets. The tubes are centrifuged for 2 h at 36,000 rpm at 20°, ensuring that the brake is off during deceleration.

The SPLP will be visible as a pink (rhodamine) band layered at the interface of the 2.5 and 10% sucrose solutions (lower band). The empty vesicles will either be banded at the 1–2.5% interface or spread throughout the 1 and 2.5% solutions. To isolate the SPLP, the tube is punctured 2 mm below the SPLP band using an 18G1/2 needle with a 3-ml syringe, and the SPLP band is slowly aspirated. All of the SPLP bands are pooled and placed into a dialysis bag overnight in $1 \times$ HBS to remove the sucrose. A picogreen assay is performed to determine the percent encapsulation and DNA concentration, and the size is measured by QELS. Ideally, the SPLP concentration should be in the range of 0.1 mg DNA/ml. If necessary, the sample can be concentrated using an Amicon filtration device or by using Aquazide. The final sample should be filter sterilized through a sterile Millipore 0.22- μ m filter unit in a Biological Safety Cabinet. SPLP may be stored in sterile vials at 4° for up to 2 years. If desired, the lipid concentration can be determined from a phosphate assay, and agarose gel electrophoresis can be used to verify the integrity of the plasmid.

Stabilized Antisense–Lipid Particles (SALP): Vectors for Regulation of Gene Expression

Antisense oligonucleotides are a class of genetic drugs that inhibits gene expression by virtue of the ability to bind to specific mRNA sequences and interfere with protein synthesis (Stein and Cohen, 1988). These molecules may have clinical utility in the treatment of cancer. For example, the proliferation of human colon carcinoma cell lines expressing the protooncogene *c-myb* could be inhibited by antisense oligodeoxynucleotides directed against *c-myb* (Melani *et al.*, 1991). Similar effects have been observed for leukemia cell lines expressing either the *c-myc* protooncogene (Wickstrom *et al.*, 1988) or the proliferative protein BCR-ABL (Szczylik *et al.*, 1991) and for human glioma cell lines expressing basic fibroblast growth factor (Morrison, 1991). As with conventional drugs, the major problems hindering clinical applications of antisense therapy center on drug stability, transport, and uptake by cells *in vivo*. Recently, it has been shown that antisense molecules directed against the epidermal growth

factor receptor (EGFR) of KB cells could be efficiently delivered to cells by encapsulation within folate-targeted liposomes (Wang *et al.*, 1995). Cells treated with the liposome-delivered antisense displayed growth inhibition, likely due to reduced EGFR expression (Wang *et al.*, 1995). This study, in which encapsulation of antisense was achieved by passive entrapment during extrusion of the liposomes, highlights the potential benefits of encapsulation within a liposomal carrier and the need for methods to achieve higher D/L ratios. Progress in this direction includes the development by Allen and co-workers of a novel system in which charge-neutralized cationic lipid–antisense particles are coated by a layer of neutral lipids. These coated cationic liposomes had an average diameter of 188 nm, entrapped 85–95% of the antisense particles, and exhibited an extended circulation half-life of >10 h compared with <1 h for free antisense (Stuart and Allen, 2000; Stuart *et al.*, 2000). A different approach has been reported by Madden and co-workers, who have developed a liposomal antisense formulation, involving the use of programmable fusogenic vesicles (PFV), that has been observed to cause down-regulation of bcl-2 mRNA levels in 518A2 melanoma cells (Hu *et al.*, 2001). Recently, we described a novel formulation process that utilizes an ionizable aminolipid [1,2-dioleoyl-3-dimethylammonium propane (DODAP)] and an ethanol-containing buffer for encapsulating large quantities of polyanionic antisense oligonucleotide in lipid vesicles (Maurer *et al.*, 2001; Semple *et al.*, 2000, 2001). The resulting particle is known as a “stabilized antisense–lipid particle” or SALP. Initially, an ethanolic liposome solution is formed by addition of lipids (DSPC/Chol/PEGCer₁₄/DODAP) in ethanol to an aqueous buffer with subsequent extrusion (Semple *et al.*, 2001) or by addition of ethanol to preformed vesicles of the same composition (Maurer *et al.*, 2001). A citrate buffer is used to acidify the ethanol-containing buffer (pH 4) to ensure that the cationic lipid is protonated. The addition of oligonucleotide to the ethanolic liposome solution leads to the formation of multilamellar liposomes (as well as some unilamellar and bilamellar vesicles) that trap oligonucleotides between the bilayers. Subsequent dialysis against acidic and then neutral buffers removes the ethanol and causes release of any externally bound oligonucleotide from the uncharged liposome surface. Unencapsulated ODN is then removed by anion-exchange chromatography. The end result is a multilamellar vesicle with a small diameter (70–120 nm) and a maximum entrapment of 0.16 mg ODN/mg lipid, which corresponds to ~2200 oligonucleotide molecules per 100 nm liposome (Maurer *et al.*, 2001) (Fig. 7). The SALP exhibit extended circulation half-lives, ranging from 5–6 h for particles formed with PEGCer₁₄ to 10–12 h for particles formed with PEGCer₂₀ (Semple *et al.*, 2001). The combination of high entrapment efficiencies, small size, and extended

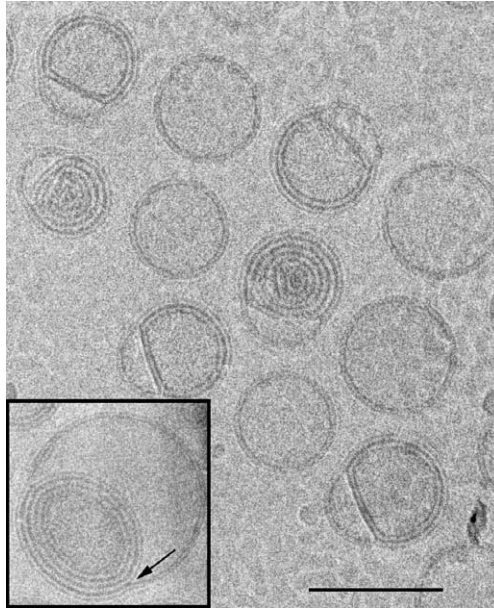


FIG. 7. Cryoelectron micrographs of DSPC/Chol/PEGCerC₁₄/DODAP (20:45:10:25 mol%) liposomes entrapping oligonucleotides (SALP). The inset is an expanded view of a multilamellar liposome showing two initially separated membranes forced into close apposition by bound oligonucleotides (arrow). The entrapped antisense-to-lipid weight ratio was 0.125 mg/mg. The bar represents 100 nm. [Reprinted from Maurer, N., Wong, K. F., Stark, H., Louie, L., McIntosh, D., Wong, T., Scherrer, P., Semple, S. C., and Cullis, P. R. (2001). *Biophys. J.* **80**, 2310–2326, with permission.]

circulation lifetimes suggests that the SALP system should prove of utility for the liposomal delivery of antisense drugs. The following procedure describes the loading of antisense molecules via electrostatic attraction into preformed vesicles destabilized by ethanol.

Experimental Procedure: Spontaneous Entrapment of Antisense Oligonucleotides upon Electrostatic Interaction with Ethanol-Destabilized Cationic Liposomes

Stock solutions of DODAP (Inex Pharmaceuticals), DSPC, Chol, and PEGCerC₁₄ (Northern Lipids; Inex Pharmaceuticals) are prepared in chloroform or ethanol. Oligonucleotide (ODN) stock solutions are prepared at 20 mg/ml in distilled water or buffer [e.g., human *c-myc* (16-mer), 5'-TAACGTTGAGGGGCAT-3'] (Inex Pharmaceuticals).

A lipid mixture containing 25 mg total lipid is prepared, with a molar ratio of DSPC/Chol/PEG-CerC₁₄/DODAP of 20:45:10:25. Solvent is removed under a stream of N₂ followed by high vacuum for 1 h. The lipid film is hydrated in 1 ml of 300 mM citrate buffer, pH 4, and subjected to five freeze/thaw cycles. LUVs are formed by extrusion at 60° as previously described.

Absolute ethanol (≥99%) is slowly added to the LUVs with rapid vortexing up to a concentration of 40% (to avoid high local concentrations). The total lipid concentration should be 10 mg/ml. Slow addition of ethanol and rapid mixing are important as liposomes become unstable and coalesce into large lipid structures as soon as the ethanol concentration exceeds a certain upper limit, which depends on the lipid composition. The final liposomes should be around 100 nm in diameter. If necessary, the ethanolic liposome dispersions can be additionally extruded (2×) to reduce size.

LUVs can also be prepared by slow addition of the lipids dissolved in ethanol (total volume of 0.4 ml) to citrate buffer at pH 4 (0.6 ml) followed by extrusion through two stacked 100-nm filters (two passes) at room temperature. Typical liposome diameters obtained from QELS should be 75–100 nm. The extrusion step can be omitted if ethanol is added very slowly under vigorous mixing to avoid high local concentrations of ethanol.

Entrapment of antisense is achieved by slow addition of the oligonucleotide solution (1–2 mg ODN) with vortexing to the ethanolic liposome dispersion (10 mg lipid/ml) to give an ODN-to-lipid ratio of 0.1–0.2 mg/mg.

The ethanolic ODN–lipid mixture is then incubated at 40° for 1 h, followed by dialysis for 2 h against 2 liters of citrate buffer (to remove most of the ethanol) and twice against 2 liters of HBS (20 mM HEPES, 145 mM NaCl, pH 7.5). At pH 7.5, DODAP becomes neutral, and oligonucleotides bound to the external membrane surface are released from their association with the cationic lipid.

Unencapsulated ODNs are removed by anion-exchange chromatography on DEAE-Sephacrose CL-6B (Sigma-Aldrich) columns (3–4 ml of DEAE-Sephacrose per 1 ml of ODN/liposome dispersion) equilibrated in HBS, pH 7.5.

To determine trapping efficiencies, the SALP is solubilized by addition of chloroform/methanol at a volume ratio of 1:2.1:1 chloroform/methanol/SALP. The oligonucleotide concentrations are determined from the absorbance at 260 nm on a Shimadzu UV160U spectrophotometer. If the solution is not completely clear after mixing, an additional 50–100 μl of methanol can be added. Alternatively, the absorbance can be read after solubilization of the samples in 100 mM OGP. The antisense concentrations are calculated according to $c [\mu\text{g}/\mu\text{l}] = A_{260} \times 1 \text{ OD}_{260} \text{ unit}$

$[\mu\text{g}/\text{ml}] \times \text{dilution factor} [\text{ml}/\mu\text{l}]$, where the dilution factor is given by the total assay volume [ml] divided by the sample volume [μl]. OD_{260} units are calculated from pairwise extinction coefficients for individual deoxynucleotides, which take into account nearest-neighbor interactions. One OD corresponds to $30.97 \mu\text{g}/\text{ml}$ anti-*c-myc*. Lipid concentrations are determined by the inorganic phosphate assay (Fiske and Subbarow, 1925) after separation of the lipids from the oligonucleotides by a Bligh and Dyer extraction (Bligh and Dyer, 1959) (initial aqueous volume = $250 \mu\text{l}$).

Conclusions

The rapid development of liposome 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. While liposomal systems for genetic drugs have not been developed to the same extent, several systems are available for both plasmid DNA and antisense oligonucleotides that have systemic potential. Further advances in this field are anticipated as tissue targeting and controlled drug release capabilities are included in future liposome designs.

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[2] Preparation, Characterization, and Biological Analysis of Liposomal Formulations of Vincristine

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Abstract

Vincristine is a dimeric Catharanthus alkaloid derived from the Madagascan periwinkle that acts by binding to tubulin and blocking metaphase in actively dividing cells. While vincristine is widely used in the treatment of a number of human carcinomas, its use is associated with dose-limiting neurotoxicity, manifested mainly as peripheral neuropathy. It is known that the therapeutic activity of vincristine can be significantly enhanced after its encapsulation in appropriately designed liposomal systems. Enhanced efficacy is also associated with a slight decrease in drug toxicity. Thus, the therapeutic index of vincristine can be enhanced significantly through the use of a liposomal delivery system. Vincristine may be