

## [4] The Liposomal Formulation of Doxorubicin

By SHEELA A. ABRAHAM, DAWN N. WATERHOUSE, LAWRENCE D. MAYER,  
PIETER R. CULLIS, THOMAS D. MADDEN, and MARCEL B. BALLY

### Abstract

Doxorubicin is the best known and most widely used member of the anthracycline antibiotic group of anticancer agents. It was first introduced in the 1970s, and since that time has become one of the most commonly used drugs for the treatment of both hematological and solid tumors. The therapy-limiting toxicity for this drug is cardiomyopathy, which may lead to congestive heart failure and death. Approximately 2% of patients who have received a cumulative (lifetime) doxorubicin dose of 450–500 mg/m<sup>2</sup> will experience this condition. An approach to ameliorating doxorubicin-related toxicity is to use drug carriers, which engender a change in the pharmacological distribution of the drug, resulting in reduced drug levels in the heart. Examples of these carrier systems include lipid-based (liposome) formulations that effect a beneficial change in doxorubicin biodistribution, with two formulations approved for clinical use. Drug approval was based, in part, on data suggesting that beneficial changes in doxorubicin occurred in the absence of decreased therapeutic activity. Preclinical (animal) and clinical (human) studies showing that liposomes can preferentially accumulate in tumors have provided a rationale for improved activity. Liposomes represent ideal drug delivery systems, as the microvasculature in tumors is typically discontinuous, having pore sizes (100–780 nm) large enough for liposomes to move from the blood compartment into the extravascular space surrounding the tumor cells (Hobbs *et al.*, 1998). Liposomes, in the size range of 100–200 nm readily extravasate within the site of tumor growth to provide locally concentrated drug delivery, a primary role of liposomal formulation. Although other liposomal drugs have been prepared and characterized due to the potential for liposomes to improve antitumor potency of the encapsulated drug, the studies on liposomal doxorubicin have been developed primarily to address issues of acute and chronic toxicity that occur as a consequence of using this drug. It is important to recognize that research programs directed toward the development of liposomal doxorubicin occurred concurrently with synthetic chemistry programs attempting to introduce safer and more effective anthracycline analogues. Although many of these drugs are approved for use, and preliminary liposomal formulations of these analogues have been prepared, doxorubicin continues to be a mainstay of drug cocktails used

in the management of most solid tumors. It will be of great interest to observe how the approved formulations of liposomal doxorubicin are integrated into combination regimes for treatment of cancer. In the meantime, we have learned a great deal about liposomes as drug carriers from over 20 years of research on different liposomal doxorubicin formulations, the very first of which were identified in the late 1970s (Forssen *et al.*, 1979; Rahman *et al.*, 1980). This chapter will discuss the various methods for encapsulation of doxorubicin into liposomes, as well as some of the important interactions between the formulation components of the drug and how this may impact the biological activity of the associated drug. This review of methodology, in turn, will highlight research activities that are being pursued to achieve better performance parameters for liposomal formulations of doxorubicin, as well as other anticancer agents being considered for use with lipid-based carriers.

## Introduction

When considering the biological activity of liposomal formulations of doxorubicin, it is important to remember that (1) doxorubicin is released from the liposomes following intravenous administration, (2) the distribution of doxorubicin following administration of a liposomal formulation is dependent, in part, on the biodistribution characteristics of the liposomes, and (3) the biological activity of liposomal doxorubicin is dependent on when, where, and at what rate the drug is released. In consideration of the above, we can make two broad statements about all the different liposomal formulations described for doxorubicin. First, differences in doxorubicin-mediated toxicity and efficacy are dependent on liposomal lipid composition, which in turn influences drug release attributes and liposome biodistribution patterns. Second, the biologically active component of all formulations described is doxorubicin, and a general understanding of the chemical and physical properties, as well as the mechanism(s) of action of this drug, is necessary before considering liposomal formulations.

## Doxorubicin

### *Chemistry*

Doxorubicin is an anthracycline antibiotic originally isolated from *Streptomyces peucetius* var. *caesius*. This amphipathic molecule possesses a water-insoluble aglycone (adriamycinone: C<sub>21</sub>H<sub>18</sub>O<sub>9</sub>) and a water-soluble, basic, reducing amino-sugar moiety (daunosamine: C<sub>6</sub>H<sub>13</sub>NO<sub>3</sub>) (Fig. 1).

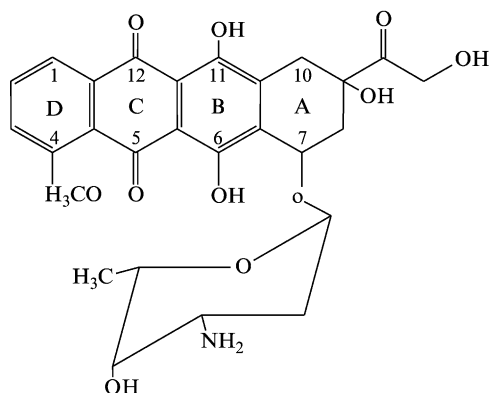


FIG. 1. The chemical structure of doxorubicin.

Of note, doxorubicin has three significant prototropic functions with associated  $pK_a$ s: (1) the amino group in the sugar moiety ( $pK_1 = 8.15$ ), (2) the phenolic group at C<sub>11</sub> ( $pK_2 = 10.16$ ), and (3) the phenolic group at C<sub>6</sub> ( $pK_3 = 13.2$ ) (Bouma *et al.*, 1986; Fiallo *et al.*, 1998).

Doxorubicin hydrochloride typically exists as a hygroscopic crystalline powder composed of orange-red thin needles. It has a melting point of 229–231° and absorption maximums (in methanol) of 233, 252, 288, 479, 496, and 529 nm due to the dihydroxyanthraquinone chromophore (Budavari *et al.*, 2000). Any variation of groups on the chromophore ultimately leads to changes in the absorption spectrum, therefore the spectrum depends on pH, binding ions and their concentration, drug concentration, solvent type, and ionic strength. Deprotonation of the chromophore results in a modification of the UV, visible, and circular dichroic (CD) spectra, causing a red shift due to the deprotonation of the phenolic functions of the chromophore. Doxorubicin is documented to appear orange at pH 7, violet at pH 11, and blue at pH 13 (Fiallo *et al.*, 1999).

It has also been well documented that doxorubicin has a propensity to self-associate (Chaires *et al.*, 1982; Menozzi *et al.*, 1984). Self-association constants in the literature vary dramatically, most likely due to differences in methods, experimental conditions, pH, and buffer compositions. This phenomenon can be detected in aqueous media at concentrations as low as 1  $\mu M$  and can be monitored by measuring the ratio of absorbance at 470/550 nm. Interactions between the  $\pi$ -electron systems have a measured thermodynamic value of  $\Delta H = -33$  kJ/mol (Bouma *et al.*, 1986), making self-association a favorable interaction, with the mechanism

of self-association being attributed to interactions between planar aromatic rings of individual molecules. The nonprotonated neutral anthracycline species is thought to be the dominant species involved in self-association. Increasing the ionic strength of the buffer solution increases the constant ( $K$ ) of association of doxorubicin, and increasing methanol concentration decreases  $K$  (Menozzi *et al.*, 1984).

### *Mechanism of Activity*

The precise mechanism of action of doxorubicin is not understood; however, it is known to intercalate between DNA base pairs, resulting in DNA and DNA-dependent RNA synthesis inhibition due to template disordering and steric obstruction. Intercalation leads to single and double strand breaks, as well as exchange of sister chromatids. Scission of DNA is believed to be mediated through the action of topoisomerase II or by the iron-catalyzed generation of free radicals, both hydrogen peroxide and hydroxyl, which are highly destructive to cells. Doxorubicin also induces the formation of covalent topoisomerase–DNA complexes, resulting in inhibition of the religation portion of the ligation–religation reaction in replicating DNA. Although it is active throughout the cell cycle, the maximal toxicity occurs during the DNA synthesis (S) phase. At low concentrations of drug, cells will continue through the S phase, and die in G<sub>2</sub> (Hortobagyi, 1997).

### *Toxicity*

The therapy-limiting toxicity for this drug is cardiomyopathy, which may lead to congestive heart failure (CHF) and death. Approximately 2% of patients who have received a cumulative (lifetime) doxorubicin dose of 450–500 mg/m<sup>2</sup> will experience this condition. Recently, this toxicity has been highlighted in clinical studies evaluating the use of doxorubicin with a humanized monoclonal antibody targeting the oncoprotein HER-2/*neu*, referred to as trastuzumab (Pegram *et al.*, 1999, 2000). Combinations of trastuzumab and doxorubicin provide particularly promising therapeutic effects, but the use of the combination exacerbated cardiac-related toxicities.

It is important to remember that doxorubicin, administered in free form, exhibits many side effects other than cardiotoxicity. Similar to other anticancer drugs targeting proliferating cell populations, doxorubicin causes significant gastrointestinal toxicity, with nausea, vomiting, and diarrhea being common soon after therapy, and stomatitis that occurs within 7–10 days after administration. The typical acute dose-limiting toxicity for doxorubicin is that of a high incidence of myelosuppression,

typically leukopenia and thrombocytopenia. In severe cases, this may lead to neutropenic fever and sepsis, requiring hospitalization ([Gabizon \*et al.\*, 1994](#)).

### Liposomal Formulations of Doxorubicin: General Considerations

The primary aim of doxorubicin encapsulation in liposomes has been to decrease nonspecific organ toxicity. Liposomes are able to direct the doxorubicin away from sites with tight capillary junctions such as the heart muscle. Instead, they distribute in areas where fenestrations or gaps exist in the vasculature (liver, spleen, and bone marrow, areas of inflammation, and neoplasms). Phagocytic cells that comprise the mononuclear phagocyte system (MPS)<sup>1</sup> can recognize these particulate carrier systems as “foreign”. Although distribution to these phagocytic cells is dependent on the physical (size) and chemical (charge) attributes of the liposomes used, it should be noted that when liposomes contain doxorubicin, the cells of the MPS are adversely affected. More specifically, following uptake into a phagocytic cell, release of doxorubicin causes these cells to die, thus reducing the capacity of the MPS to accumulate the injected liposomes. This is reflected, in turn, by significantly increased liposome circulation lifetimes ([Bally \*et al.\*, 1990](#)). Liposomal formulations containing poly(ethylene glycol) (PEG)-modified lipids are known to exhibit increased circulation time because of a reduced tendency to aggregate following iv administration ([Allen \*et al.\*, 2002](#)); however, these formulations also exhibit MPS toxicities ([Parr \*et al.\*, 1993](#)). Whether the enhanced circulation lifetime of a liposomal formulation of doxorubicin is due to subtle changes in lipid composition, inclusion of doxorubicin, which affects MPS cell function, or the use of PEG-modified lipids, increased circulation longevity of the liposomes allows enhanced extravasation across the leaky endothelium of solid tumors ([Papahadjopoulos \*et al.\*, 1991](#)).

Two liposomal doxorubicin formulations that have received clinical approval are Doxil (United States) or Caelyx (Canada and Europe) and Myocet, which was given community marketing authorization from the European Commission in August 2000 for the treatment of metastatic breast cancer. The Doxil/Caelyx liposomal formulation is composed of

<sup>1</sup>Abbreviations: A, absorbance; Chol, cholesterol; [<sup>3</sup>H]CHE, [<sup>3</sup>H]cholesteryl hexadecyl ether; cTEM, cryotransmission electron microscopy; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DOX, doxorubicin;  $\Delta\Psi$ , electrochemical gradient; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]; HBS, HEPES-buffered saline; iv, intravenous; LUV, large unilamellar vesicle; MLV, multilamellar vesicle; MPS, mononuclear phagocyte system; SHE, sucrose-HEPES-EDTA.

hydrogenated soya phosphatidylcholine, cholesterol (Chol) and PEG-modified phosphatidylethanolamine (55:40:5 molar ratio) whereas Myocet is composed of egg phosphatidylcholine (EPC) and Chol (55:45 molar ratio). The EPC–Chol formulation releases drug fairly rapidly and has a relatively short circulation lifetime (approximately three times that of free doxorubicin) but has been shown clinically to reduce doxorubicin-induced cardiotoxicity and gastrotoxicity. In contrast, Doxil/Caelyx has a much longer circulation lifetime due to the steric barrier provided by the surface-grafted PEG, which leads to large changes in biodistribution and particularly increased amounts of drug being delivered to the skin. This has advantages for the treatment of skin localized cancers such as Kaposi's sarcoma but disadvantages in the observation of new dose-limiting toxicities such as the hand and foot syndrome (reviewed in [Waterhouse \*et al.\*, 2001](#)).

### General Description of Materials and Techniques

When generating a pharmaceutically viable liposomal doxorubicin formulation, several important factors must be considered. The methodology must be straightforward and conceptually easy, using the most economically available materials possible. Liposomes must be uniformly generated with reproducible size distributions; an optimal loading procedure would approach 100% trapping efficiency at the desired drug-to-lipid ratio, thereby negating the need to remove unencapsulated doxorubicin from the sample. The drug-loaded sample must also exhibit drug release rates that are conducive to improvements in drug activity through decreases in toxicity or increases in efficacy ([Mayer \*et al.\*, 1994](#)). In the extreme, rapid (instantaneous) doxorubicin release from liposomal formulations following iv administration will result in a drug that is substantially no different from the free, unencapsulated, drug. Such a formulation would typically not be classified as a drug carrier. In the opposite extreme, complete drug retention (no drug release) should, theoretically, result in a formulation that is neither toxic nor efficacious. Such a formulation may exhibit substantial improvements in drug delivery to sites of tumor growth and substantial reductions in drug delivery to cardiac tissue, but such a formulation would be of little therapeutic value or interest. Thus *in vivo* drug release parameters between these extremes has become the guiding light through which liposomal formulations of doxorubicin have been developed.

The lipid composition selected, when preparing liposomal formulations of doxorubicin, largely dictates rates of doxorubicin binding, partitioning, and retention in the liposomes, as well as the elimination rate of the liposomal formulation. In general, the chemical attributes of the specific

lipid molecules chosen affect bilayer permeability in a manner that is somewhat predictable on the phase transition temperature of the bulk lipid component (Table I). Liposomes prepared of phospholipids with short acyl chains (e.g., C14:0, DMPC) release doxorubicin more rapidly than phospholipids with longer acyl chains (e.g., C18:0, DSPC). Cholesterol is a common constituent of liposomal doxorubicin formulations due to its ability to modulate membrane permeability and biological stability. Membrane permeability is thought to be dependent on the amount of cholesterol being incorporated and the transition temperature of the bulk phospholipid being used. For example, cholesterol at concentrations above 30 mol% lowers the amount of energy required to melt the acyl chains, eliminates the gel-to-liquid-crystalline phase transition (Cullis *et al.*, 1987), and causes an increase in acyl chain disorder at temperatures below the phase transition temperature of the bulk phospholipid component. In addition to the lipid components, the chemical interactions of doxorubicin with the carrier should be considered, particularly when charge-charge interactions with the phospholipid head groups or partitioning into the lipid hydrocarbon chains may affect drug loading and release attributes.

Further, although this chapter describes what are believed to be the most efficient and versatile methods for preparing liposomal formulations of doxorubicin, other methods could be used. In general, methods of encapsulation are categorized into two procedures, passive and active. Passive encapsulation involves the hydration of a dried lipid film with an

TABLE I  
EXAMPLES OF SOLUTIONS AND THEIR CONCENTRATIONS IN ACTIVE LOADING

Active encapsulation procedures	Concentration of hydrating buffer	pH	Hydrating buffer osmolarity (mOsm/liter)	Concentration of external buffer	pH	Osmolarity of external buffer
Citrate	C <sub>6</sub> H <sub>8</sub> O <sub>7</sub> 300 mM	4.0	550	(HEPES buffered saline) 150 mM NaCl 25 mM HEPES	7.5	326
Ammonium sulfate	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> 120 mM	5.5	276	NaCl 145 mM	5.5	268
Manganese sulfate	MnSO <sub>4</sub> 300 mM	3.5	319	300 mM sucrose 30 mM HEPES	7.5	380
Manganese sulfate +A23187	MnSO <sub>4</sub> 300 mM	3.5	319	300 mM sucrose 20 mM HEPES 15 mM EDTA	7.5	517

aqueous solution of doxorubicin. This method takes advantage of the fact that during the preparation of liposomes, a certain aqueous volume is obtained within each liposome. Drug and liposomes are codispersed with a certain fraction of the drug entrapped directly, resulting from the combination of the hydrophilic, hydrophobic, and ionic interactions. As an amphipathic drug, doxorubicin, depending on the pH, has the potential to reside in the aqueous core, as well as partition into the lipid bilayer. Typically, passive trapping methods are not efficient (maximum efficiencies of 80%; Cullis *et al.*, 1989), and the maximum drug-to-lipid ratio achievable is low and dependent on the maximum solubility of doxorubicin (<10 mM). Passive encapsulation methods also require removal of the unencapsulated drug. Although this can be accomplished easily in a laboratory setting, the methods for passive encapsulation are not well suited to scaled production of pharmaceutical batches for use in clinical trials.

Active trapping procedures involve adding doxorubicin to preformed liposomes that possess a trans-bilayer ion gradient. Under appropriate conditions, when the drug is added to liposomes possessing such a gradient, a redistribution of the drug occurs such that the drug crosses the liposomal bilayer and is subsequently trapped within the core of the liposome. Trapping occurs for reasons that involve internal aqueous pH and induced drug precipitation along with associated chemical reactions that affect the nature of the precipitate formed. The active trapping procedure results in drug-to-lipid ratios as high as 0.3:1 (wt:wt), which corresponds to approximately 48,000 doxorubicin molecules per 100-nm-diameter liposome. Active trapping methods can be used with any lipid composition that is able to form a bilayer and maintain a transmembrane ion gradient. These methods have been used to prepare liposomal doxorubicin formulations approved for clinical use, and, thus, the techniques for preparing these formulations are the focus of this chapter.

### Encapsulation of Doxorubicin Using Active Loading Methods

Until recently (Abraham *et al.*, 2002; Cheung *et al.*, 1998), active loading methods have been dependent on an established and/or a created transmembrane pH gradient, as well as the accumulation of doxorubicin to levels within the liposome that exceed the solubility of the drug (Madden *et al.*, 1990). Transmembrane pH gradients across liposomes result in doxorubicin precipitation through drug self-association or through interaction with salts present in the aqueous core of the liposome. There are two principal ways to achieve a pH gradient across the liposome bilayer: directly or indirectly. To directly establish the trans-bilayer pH gradient, liposomes are prepared in the presence of an acidic buffer, and the exterior



buffer of the liposomes is then adjusted to a desired pH using either added bases that increase the pH or, alternatively, exchanging the outside buffer using column chromatography or dialysis. Obviously, it is critical that the pH gradient established across the bilayer is stable. Interestingly, protons ( $H^+$ ), relative to other cations, are highly permeable. As  $H^+$  diffuse outward, they create an electrochemical gradient ( $\Delta\Psi$  inside negative) (Harrigan *et al.*, 1992), and this prevents/limits further  $H^+$  efflux. If, for example, there is a  $\Delta pH$  of three units, this translates to  $\Delta\Psi$  of  $-177$  mV. Cullis *et al.* (1991) suggested that only 150 protons must diffuse outwardly to maintain this potential and allow maintenance of a stable  $\Delta pH$  and  $\Delta\Psi$ . A commonly used procedure to prepare liposomes with a transmembrane pH gradient relies on the entrapment of a 300 mM citrate buffer, pH 4.0 (Fig. 2A). Citrate is a triprotic buffer and possesses a large buffering capacity in the range of pH 3–6.5 ( $pK_{a1} = 3.13$ ,  $pK_{a2} = 4.76$ ,  $pK_{a3} = 5.41$  or 6.4) (Budavari *et al.*, 2000).

There are several ways to indirectly establish a pH gradient, including the use of electrochemical gradients and ionophores. For example, liposomes can be synthesized in a potassium ( $K^+$ )-based buffer and the external buffer replaced with a sodium-containing buffer by column chromatography or dialysis. In the presence of the created  $K^+$  gradient, the addition of an ionophore such as valinomycin (an  $H^+/K^+$  exchanger) will shuttle  $K^+$  to the liposomal exterior, thus creating an electrochemical gradient ( $\Delta\Psi$ ), with the interior of the liposomes being negative. Stable electrical potentials in excess of 150 mV can be generated in large unilamellar vesicles (LUVs) using this technique (Redelmeier *et al.*, 1989).  $H^+$  readily cross the lipid bilayer into the interior in response to this  $\Delta\Psi$  gradient, thus establishing a pH gradient (Cullis *et al.*, 1991). It has been shown that this pH gradient is smaller than theoretically predicted for electrochemical equilibrium and is sensitive to the ionic composition of the external buffer.

By virtue of being able to exchange cations, other ionophores can also be used to form pH gradients from chemical gradients. Several researchers have documented that both nigericin ( $K^+$  ionophore) and A23187 (divalent cation ionophore) exchange specific cations for either one or two protons, respectively (Fenske *et al.*, 1998) (Fig. 2B). Liposomes possessing a transmembrane salt gradient (the liposome interior containing either  $K_2SO_4$  or  $MnSO_4$ ) can be incubated with the specific ionophore and drug. The ionophores are able to transport the outward movement of cations for the inward movement of protons, thus creating a pH gradient, which in turn promotes drug loading.

Another method capable of causing formation of a pH gradient is one that relies on encapsulation of ammonium sulfate (Lasic *et al.*, 1992)

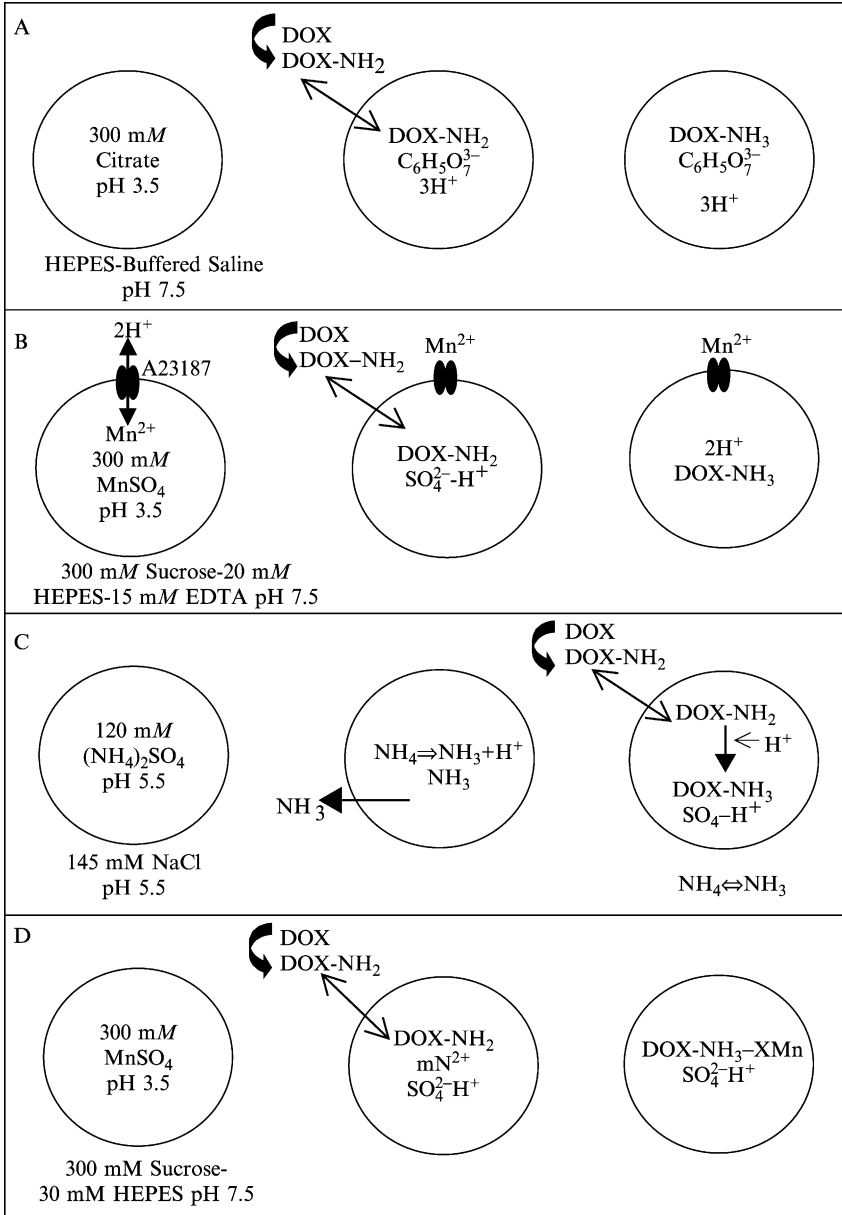


FIG. 2. Methods of doxorubicin encapsulation into liposomes exhibiting the indicated gradients. (A) The citrate loading procedure. Liposomes are prepared in 300 mM citrate buffer, pH 3.5, and outside buffer exchanged to HEPES-buffered saline at pH 7.5. (B) The

(Fig. 2C). Following encapsulation of  $(\text{NH}_4)_2\text{SO}_4$ , the external buffer is exchanged to a solution such as 145 mM NaCl to establish an  $(\text{NH}_4)_2\text{SO}_4$  gradient. Due to the high permeability of  $\text{NH}_3$  ( $1.3 \times 10^4$  cm/s), it readily crosses the liposome bilayer, leaving behind one proton for every molecule of  $\text{NH}_3$  lost (Bolotin *et al.*, 1994). This creates a pH gradient, the magnitude of which is determined by the  $[\text{NH}_4^+]_{\text{in}}/[\text{NH}_4^+]_{\text{out}}$  gradient. This approach differs from other chemical methods in that the liposomes are not prepared in an acidic buffer nor is there a need to alkalize the exterior liposome solution (Haran *et al.*, 1993).

The choice of aqueous buffer in which liposomes are formed has direct effects on the subsequent loading of doxorubicin. The osmolarity, ionic strength, pH, counterions (e.g., citrate, sulfate, or glutamate), and their concentrations interplay to generate different precipitated structures, but it is unclear whether this affects the biological behavior of the resulting product. As indicated earlier, doxorubicin (Menozzi *et al.*, 1984) self-associates with stacking of the planar aromatic rings under specific conditions (Chaires *et al.*, 1982). It has been suggested that doxorubicin forms a precipitate when encapsulated in liposomes in response to a pH gradient, an observation that has been confirmed by several research groups (Lasic, 1996; Lasic *et al.*, 1992; Li *et al.*, 1998). Cryotransmission electron microscopy (cTEM) reveals doxorubicin precipitates as fibrous-bundle aggregates in both citrate- and sulfate-containing liposomes (Fig. 3). The planar aromatic anthracycline rings are thought to stack longitudinally to form linear fibers. These fibers are aligned in a hexagonal arrangement to form bundles, with approximately 12–60 fibers per bundle. Doxorubicin aggregates in the presence of sulfate typically have rigid linear fiber bundles (interfiber spacing is approximately 27 Å) compared with the doxorubicin–citrate aggregates in the presence of citrate, which appear mostly linear or curved (interfiber spacing is approximately 30–35 Å) (Li *et al.*, 1998). These results suggest that the sulfate anion, being smaller than the citrate anion, may allow a tighter packing arrangement, resulting in a decreased flexibility of fiber bundles.

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manganese sulfate loading procedure with the A23187 ionophore. Liposomes are prepared in 300 mM manganese sulfate, pH 3.5, and outside buffer is exchanged to 300 mM sucrose/20 mM HEPES/15 mM EDTA at pH 7.5 with the A23187 added to the liposomes prior to doxorubicin addition. (C) The ammonium sulfate loading procedure. Liposomes are prepared in 120 mM ammonium sulfate, pH 5.5, and outside buffer is exchanged to 145 mM sodium chloride, pH 5.5. (D) The manganese sulfate loading procedure. Liposomes are prepared in 300 mM manganese sulfate, pH 3.5, and outside buffer is exchanged to 300 mM sucrose/20 mM HEPES/15 mM EDTA at pH 7.5.

Cheung *et al.* (1998) described a novel encapsulation procedure that takes advantage of the ability of doxorubicin to complex or coordinate with manganese (Fig. 2D). Work from our laboratory and others suggests that manganese can theoretically bind doxorubicin at three sites: (1) the anthraquinone (hydroxyketone) moiety at the C<sub>5</sub>–C<sub>6</sub> position, (2) the anthraquinone (hydroxyketone) moiety at the C<sub>11</sub>–C<sub>12</sub> position, and (3) the

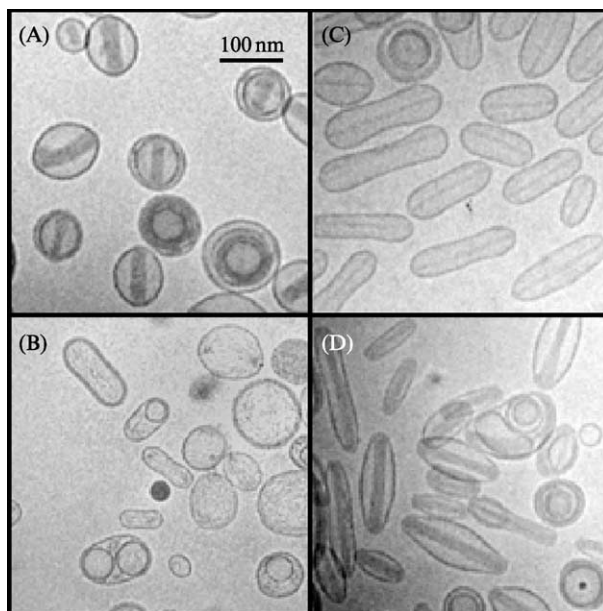


FIG. 3. Cryotransmission electron microscopy (cTEM) images of either DMPC/Chol (55/45 mol ratio) or DSPC/Chol (55/45 mol ratio) liposomes after drug loading achieving a final drug-to-lipid ratio of 0.1/0.2:1 (wt:wt). The liposomes were prepared and loaded with doxorubicin as illustrated in Fig. 2. Briefly, the samples were incubated at 60° for 30 min to facilitate >95% doxorubicin encapsulation. All images are representative of the entire sample. (A) DSPC/Chol liposomes prepared in 300 mM citrate, pH 3.5, and with the outside buffer changed to HEPES-buffered saline, pH 7.5, as the exterior buffer followed by the encapsulation of doxorubicin. (B) DMPC/Chol liposomes prepared in 300 mM MnSO<sub>4</sub>, pH 3.5, and with the outside buffer changed to 300 mM sucrose–30 mM HEPES, pH 7.5, followed by the encapsulation of doxorubicin. (C) DMPC/Chol liposomes prepared in 120 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, pH 5.5; with the outside buffer changed to 145 mM NaCl, pH 5.5, and followed by the addition of doxorubicin. (D) DMPC/Chol liposomes prepared in 300 mM MnSO<sub>4</sub>, pH 3.5, with the outside buffer changed to 300 mM sucrose–20 mM HEPES–15 mM EDTA, pH 7.5, with the addition of the ionophore A23187, followed by the encapsulation of doxorubicin. The bar in (A) is equivalent to 100 nm, and all micrographs are shown at the same magnification. (Electron micrographs courtesy of Katarina Edwards and Göran Karlsson, Uppsala University, Uppsala, Sweden.)

side chain at C<sub>9</sub>, possibly in combination with the C<sub>9</sub> hydroxyl position and the amino-sugar group at the C<sub>3</sub>–C<sub>4</sub> position (Abraham *et al.*, 2002; Bouma *et al.*, 1986;). Several studies using spectroscopic methods, circular dichroism (CD), electron paramagnetic resonance (EPR), proton nuclear magnetic resonance (<sup>1</sup>H-NMR), and infrared spectroscopy (IR) have concluded that the deprotonated hydroxy-anthraquinone moieties are the predominant metal-binding sites. Complexation is favored between pH 2 and 8; at pH < 2, no complexation occurs, and at pH > 8, metal hydroxide formation is highly favored. Protonation of the amine group inhibits complex formation, even though this group is not directly involved in metal ion binding.

#### Specific Description of Techniques: Preparation of Liposomal Doxorubicin through the Use of Ion Gradients

There are four principal stages involved in the preparation of liposomal doxorubicin: (1) preparation of the multilamellar vesicles (MLVs), (2) formation of LUVs from MLVs, (3) establishment of an ion gradient, and (4) encapsulation of doxorubicin. Over the course of this process, a number of critical parameters should be routinely checked, including liposomal lipid concentrations, drug concentrations, drug-to-lipid ratio, encapsulation efficiency, liposomal size, and pH gradient, both before and after loading. The following methods should serve as a guide through this process.

##### *Stage I: Preparation of Multilamellar Vesicles*

*Weighing and Dissolving Lipids.* In general, lipids (purchased from Avanti Polar Lipids, Alabaster, AL, or Northern Lipids Inc., Vancouver, BC, Canada, or other companies specializing in lipids) should be stored at –80° for optimal stability. Prior to weighing, lipids should be brought to room temperature in a closed container containing a desiccant [e.g., calcium chloride (anhydrous) desiccant 20-mesh, catalog no. C77-500/500 g, purchased from Fisher Scientific Company, Pittsburgh, PA]. This decreases the likelihood of contamination by condensation of the lipid stock and ensures accuracy when dispensing the lipid. Lipids are weighed using an analytical balance, e.g., 71.4 mg of DSPC and 28.6 mg of cholesterol would be weighed into a 16 × 100-mm glass test tube and mixed together to prepare 100 mg of DSPC/cholesterol liposomes at a molar ratio of 55:45. All lipids are initially dissolved in organic solvent, e.g., chloroform, methylene chloride, or a chloroform–methanol solution (95:5 v/v). High-performance liquid chromatograph (HPLC) grade solvents should be used

to avoid contamination by impurities. Approximately 50–200 mg of total lipid dissolves well in 1 ml of solvent. When using lipids such as sphingomyelin and lysolipid, the addition of several drops of methanol aids in solubilization.

*Quantitation of Lipids.* At this point it must be decided which method will be used to subsequently quantitate the liposomal lipid concentration. Lipid can be quantitated by measuring the total concentration of phosphorus and calculating the phospholipid concentration. This can be done at any stage during the processing of the liposomes or lipid mixtures; however, if the concentration is to be determined at the stage when solvents are present, exhaustive steps must be taken to remove all traces of solvents prior to performance of the phosphate assay. The phosphate assay is described below in detail (see [Stage IV: Doxorubicin Encapsulation](#)).

An alternative method of lipid quantitation involves the addition of a radioactive marker to the dissolved lipid. The ideal marker must be non-exchangeable and nonmetabolizable if it is to be used as a tracer of liposomal lipid *in vivo*. In this regard, we utilize cholesteryl hexadecyl ether (CHE), a lipid that can be bought in a  $^3\text{H}$  or  $^{14}\text{C}$  form from PerkinElmer Life Sciences Inc. (Boston, MA). The specific activity of  $^3\text{H}$  is typically 40–60 Ci/mmol (which we prefer), and the specific activity of  $^{14}\text{C}$  is typically 40–60 mCi/mmol. For *in vitro* experiments, typically 1  $\mu\text{Ci}$  of [ $^3\text{H}$ ]CHE is added per 100 mg of lipid, and for *in vivo* experiments, approximately 10  $\mu\text{Ci}$  of lipid marker is added to 100 mg of lipid. For accuracy, we recommend determining the  $^3\text{H}$  or  $^{14}\text{C}$  disintegrations per minute (dpm) to bulk lipid ratio at the stage when the weighed lipids are dissolved in solvents. The lipids and [ $^3\text{H}$ ]- or [ $^{14}\text{C}$ ]CHE are transferred to a 1-ml volumetric flask and topped with organic solvent to the 1-ml mark. Three 10- $\mu\text{l}$  samples are taken using a 50- $\mu\text{l}$  Hamilton syringe and transferred to three separate scintillation vials. The solvent is then evaporated completely using a steady stream of nitrogen gas, as even the smallest amounts of chloroform can cause significant quenching of radioactivity. Five milliliters of scintillation cocktail is added to each vial, and the dpm are obtained using a scintillation counter such as the Packard 1900TR Liquid Scintillation Analyzer. Knowing the amount of lipid weighed and dissolved per 1 ml of solvent allows calculation of the average dpm per mg or  $\mu\text{mol}$  lipid, which will serve as the specific activity to be subsequently used to calculate all lipid concentrations.

*Solvent Removal by Evaporation.* Once the lipid has been fully dissolved with the specific activity determined, the solvent must be removed completely to create a lipid film. It is advantageous to slowly evaporate the bulk of the solvent with a stream of nitrogen gas at a constant temperature of 37–45° until a thick viscous lipid slurry remains. This is followed by a

quick transfer to a vacuum pump, where the sample remains under a pressure of  $\sim 76$  cm Hg for several hours to ensure the removal of residual solvent. Solvent must be uniformly removed to prevent certain lipid components from crystallizing, leading to a less homogeneous lipid mixture. In particular, care must be taken in removing chloroform–methanol solvent mixtures from cholesterol-containing formulations. As the solvent is being evaporated, the cholesterol can precipitate in alcohols, leading to cholesterol microcrystals that will not incorporate well into the lipid bilayer after hydration and can block the polycarbonate filters during the extrusion process (described in [Stage II](#), under Extrusion). The lipid sample should eventually assume a dried white fluffy appearance to obtain maximal surface area for hydration. All of the organic solvent must be removed from the lipid sample, as any residual solvent has the potential to intercalate within the lipid bilayer and disrupt the ability of the liposomes to retain the ion gradient or the doxorubicin itself.

Solvents can be removed by other methods, including rotary evaporation (Waterhouse *et al.*, this volume), lyophilization (benzene–methanol solvents) (Mayer *et al.*, 1984), or even reverse-phase evaporation to directly prepare LUVs (Szoka and Papahadjopoulos, 1978, 1980).

*Lipid Hydration.* The dried lipid, prepared as described above, is then hydrated with the desired buffer to form MLVs. If using an extruder as a secondary processing technique to generate unilamellar vesicles, and in consideration of the concentration of the final lipid sample, a good rule is to aim for a minimum final volume of 1 ml if 100 mg total lipid is used. [Table I](#) outlines the hydrating buffers, their concentrations, and pHs for the various loading procedures. For saturated acyl chain phospholipids, the hydration buffer should be initially heated for 5 min at a temperature approximately  $5^\circ$  above the phase transition temperature of the highest melting lipid component ([Table II](#)). The heated buffer is added to the lipid to achieve the desired lipid concentration (50–200 mg/ml final lipid concentration is recommended), and the lipid sample is intermittently vortexed during the 5-min hydration process to ensure maximal hydration. The resulting MLVs are very heterogeneous and range from 500 nm to several micrometers in size.

*Freeze and Thaw Cycles.* Mayer *et al.* (1985) documented that upon hydration of the lipid film, the buffer solute does not equally distribute between the aqueous core of the liposome and the outside buffer. Cyclic dehydration and rehydration of the MLV lipid head groups are thought to assist in increasing the aqueous trapped volume without changing the overall vesicle size distribution. Dehydration can be accomplished by evaporation, lyophilization, or freezing (the formation of ice crystals in this case also physically disrupts the multilamellar structure). Rehydration

TABLE II  
LIPIDS COMMONLY USED IN LIPOSOMAL DOXORUBICIN FORMULATIONS

Phospholipids	Carbon chain	Phase transition temperatures	Molecular weight	Charge
1,2-Dilauroyl- <i>sn</i> -glycerol-3-phosphocholine	12:0	-1	621.89	Neutral
1,2-Dimyristoyl- <i>sn</i> -glycerol-3-phosphocholine	14:0	23	677.95	Neutral
1,2-Dipalmitoyl- <i>sn</i> -glycerol-3-phosphocholine	16:0	41	734.05	Neutral
1,2-Distearoyl- <i>sn</i> -glycerol-3-phosphocholine	18:0	55	790.15	Neutral
1,2-Distearoyl- <i>sn</i> -glycerol-3-[phospho-rac-(1-glycerol)]	18:0	55	801.06	Negative
1,1',2,2'-Tetramyristoyl cardiolipin	14:0	59	1284.97	Negative
1,2-Dioleoyl- <i>sn</i> -glycerol-3-[phospho-L-serine]	18:1	-11	810.03	Negative

is achieved by hydrating (for the evaporated and the lyophilized sample) or thawing (for freezing), respectively. The freeze and thaw method, in general, allows efficient distribution of solute. In this method, the hydrated lipid sample is subjected to five repeated freezing and thawing cycles. The lipid sample is transferred to a 5-ml cryogenic vial (e.g., Nalgene cryoware, Nalge Company, Rochester, NY) and is immersed for 5 min into a Dewar jar containing liquid nitrogen. The sample is then immersed in a water bath kept at approximately 5° above the transition temperature of the highest melting lipid component for an additional 5 min. This freeze and thaw cycle is repeated four more times. It is important to wear safety goggles during the freeze and thaw cycles due to the potential hazards of working with liquid nitrogen. At the completion of this stage, the frozen MLVs can be either stored in a -20° freezer or can be carried to the next step of the formation of LUVs.

### *Stage II: Formation of Unilamellar Vesicles*

*Extrusion.* After producing the MLVs, several processing methods are available to produce LUVs. The repetitious physical extrusion of MLVs, under moderate pressures of 400–600 psi through polycarbonate filters of a defined pore size, results in a homogeneous population of single bilayer vesicles of decreased particle size. The extrusion of liposomes is a



procedure based on the work of Olson *et al.* (1979), who described a technique involving extruding MLVs under low pressure (<50 psi) through filters with sequentially decreasing pore size in order to obtain small liposomes. This methodology has been improved and developed further through the efforts of our research group (Hope *et al.*, 1985; Mayer *et al.*, 1986), and commercial devices are available for extrusion of lipids under high pressures, such as the Thermobarrel Extruder System, which can be purchased from Northern Lipids Inc. (Vancouver, BC, Canada).

The extrusion process is started by making sure the extruder is scrupulously clean. While wearing gloves, a typical cleaning cycle consists of removing all grease and dirt from each extruder component with a brush and phosphate-free soap followed by rinsing with hot tap water, isopropanol, and finally distilled water. All components without rubber or plastic parts may be subsequently sonicated in chloroform in a chemical hood for 30–60 s; each component is sonicated separately to avoid scratching or rubbing. The chloroform is allowed to evaporate completely before assembling the unit. Prior to assembling the extrusion unit, a new support drain disk is placed on the metal mesh filter, followed by polycarbonate filters with pore sizes selected to produce a defined size liposome. To prepare liposomes with mean diameters between 100 and 120 nm, two stacked 0.1- $\mu\text{m}$  pore-sized filters are typically used; however, some investigators use a combination of a 0.08- $\mu\text{m}$  pore-size filter and a 0.1- $\mu\text{m}$  pore-size filter. Care must be taken to ensure that the drain disk and the filters adhere well to the metal mesh. This can be accomplished by the use of one or two drops of the hydrating buffer applied directly to the positioned filters.

Once assembled, the extruder should be positioned in a fume hood (a radioactive aerosol may be produced during the extrusion process) and allowed to equilibrate to the desired extrusion temperature ( $5^\circ$  higher than the phase transition temperature of the lipid possessing the highest melting temperature). The MLV sample is added through the extruder inlet port with a  $5\frac{3}{4}$ -inch glass Pasteur pipette, taking care that the sample is not added close to the extruder cap but more toward the bottom of the barrel without piercing the filters. Once the cap is securely fastened and the pressure release valve is closed, the nitrogen gas (at 300–600 psi) is slowly released into the extruder chamber. This will force the lipid sample out through the rubber tubing and into a glass collection tube making one extrusion pass. This is repeated nine more times. Ideally, the collection tube is kept at approximately the same temperature as the extruder using a heat block. This extrusion process consistently yields unilamellar vesicles exhibiting a narrow size dispersity of 100–120 nm. The resulting lipid sample should have an increased opalescence and a more transparent appearance compared with the opaque chalky white color of the MLVs.

Unilamellar vesicles can be stored at 4° for 1–2 weeks depending on the susceptibility of the specific lipid composition to the pH of the buffer. For liposomes to be used *in vivo*, it is recommended that the LUVs are used within 24 h after preparation.

*Determination of Liposome Size.* Size serves as an important determinant in the characteristics of the liposomal doxorubicin formulation. Particle size is an important indicator of batch-to-batch reproducibility, and changes may indicate liposome instability. The most accurate technique available to determine individual particle size is the electron microscope (Woodle and Papahadjopoulos, 1989). Because equipment associated with this technique may not be readily available to most laboratories, a less arduous and simpler way of determining the average particle size and the relative distribution for sample particles involves using a particle sizer, e.g., NICOMP 380/DLS Submicron Particle Sizer (Santa Barbara, CA). This instrument employs a laser-based technique using quasielastic light scattering to determine the hydrodynamic equivalent diameter of particles. Samples are simply prepared by diluting the liposomes, for example 20–25  $\mu\text{l}$  of a 100 mg/ml lipid solution is added to 400  $\mu\text{l}$  filtered saline in a borosilicate tube of the correct dimensions for the particular particle sizer. Size is determined using the software program accompanying the equipment. If the particle size distribution is greater than anticipated, the sample may be reextruded to obtain the desired size range.

### *Stage III: Establishing an Ion Gradient*

The buffer employed during lipid hydration establishes the chemical conditions of the interior aqueous compartment of the liposomal preparation. For the creation of an ion gradient, the exterior buffer of the liposomes must be exchanged. The external buffer can be exchanged by several techniques. Creating a transmembrane pH gradient with an acidic interior requires an increase in pH of the exterior liposomal environment. One method involves the addition of an alkalinizing reagent [e.g., a concentrated base or an alkaline buffer such as dibasic phosphate (0.1–1 *M*) or sodium carbonate] to neutralize the extravesicular pH. Creating an ion (or again a pH) gradient can also be accomplished by exchanging the exterior media using size-exclusion column chromatography. A gel media such as Sephadex G-50 (medium), which has a dry bead size of 50–150  $\mu\text{m}$  and a globular protein fractionation range of 1500–30,000 MW, is suitable for this purpose. Sephadex G-50 is hydrated in an excess amount of the desired exterior buffer (75% of settled gel) for approximately 3 h at 20°. The suspension is fully degassed for approximately 20 min prior to pouring the column. The volume of the column is dependent on the sample size; a

sample volume of 0.5–5% of the bed volume is recommended. To stabilize the column bed and equilibrate with the exterior buffer, two or three volumes of eluent should be passed through the column. The lipid sample should be applied carefully and evenly to the column with the least amount of dilution. The fractions containing the lipid can be clearly identified and will appear opaque white compared with the eluent buffer-containing fractions. The liposomes are always collected in the void volume of the column. This technique allows the freedom of choice for a specific external buffer. The extravascular buffer can also be exchanged using dialysis techniques. The lipid sample is placed within dialysis bags of a molecular cut-off range of 12–14 kDa and placed in a large volume of the extravascular buffer. The buffer is replaced two or three times over the course of 24–48 h in a 4° cold room. This technique also provides flexibility when choosing an external buffer.

#### *Stage IV: Doxorubicin Encapsulation*

Following the establishment of an ion gradient, doxorubicin must be encapsulated within a time frame that does not compromise the gradient; this is dependent on both the lipid composition and the buffer. Drug uptake requires incubation at specific elevated temperatures. The time of incubation and the temperature are dependent on the lipid composition and the desired drug-to-lipid-ratio and, therefore, must be determined experimentally (see Fig. 4 for an example).

The liposome sample concentrations are calculated prior to doxorubicin encapsulation. If using a radiolabeled lipid marker, liposome lipid concentrations are determined by adding a small aliquot to 5 ml of scintillation cocktail, where the radioactivity of the sample is subsequently determined by scintillation counting with the liquid scintillation analyzer. If using the phosphate concentration to calculate the total liposome concentration, the phosphate assay initially described by Fiske and Subbarow (1925) can be followed. This procedure requires all equipment in contact with the lipid sample to be phosphate free, which necessitates that all glassware be acid washed. The glassware can be soaked overnight in Fisher's chromic/sulfuric acid mix (Fisher Scientific Company, Pittsburgh, PA). Briefly, 700  $\mu$ l of 70% perchloric acid is added to lipid samples and standards in 16  $\times$  125-mm-thick walled borosilicate heat-resistant tubes (e.g., Pyrex or Kimax Brand, Fisher Scientific Company, Pittsburgh, PA). Standards can be prepared in the 0–200 nmol total phosphate range using a 2 mM sodium phosphate solution. Tubes are capped with acid-washed marbles to minimize evaporation and allow the venting of pressure. Samples and standards are heated to approximately 180–200° using a block

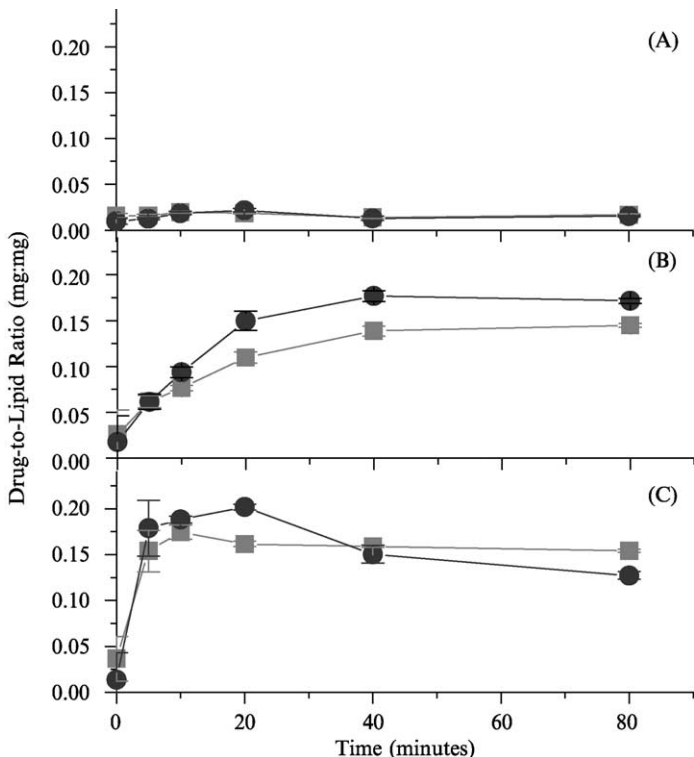


FIG. 4. Doxorubicin encapsulation in DMPC/Chol (55/45 mol ratio) liposomes using either the manganese chloride-loading procedure (■) or the manganese chloride-loading procedure with the A23187 ionophore (●). Liposomes were prepared in the presence of 300 mM  $\text{MnCl}_2$  at pH 3.5, and the outer buffers were exchanged using column chromatography to create a pH or an  $\text{Mn}^{2+}$  gradient. For the  $\text{MnCl}_2$  loading procedure with the A23187 ionophore, the A23187 was added and incubated 5 min prior to the addition of drug. Doxorubicin was added to the liposomes to achieve a 0.2:1 drug-to-lipid ratio (w:w) and incubated at 20° (A), 40° (B), or 60° (C). At the indicated time points, aliquots were fractionated on 1-ml spin columns to separate the encapsulated drug (collected in the void volume) from the unencapsulated drug. Lipid concentrations were determined using [ $^3\text{H}$ ]CHE, and doxorubicin was quantitated by reading the  $A_{480}$  of a detergent solubilized sample as described in Section IV. Data points represent the mean drug-to-lipid ratios of at least three replicate experiments, and the error bars indicate the standard deviation.

heater for 2 h or until the samples are colorless. The block heater is typically set up in a fume hood with constant hydration (the water faucet in the fume hood is left on during the entire procedure). The tubes must not be allowed to completely dry, as this is an explosion hazard. Great care must be taken when using perchloric acid; refer to appropriate Material

Safety Data Sheets prior to use. Samples are cooled, and 700  $\mu\text{l}$  of Fiske reagent (as described below) is added to each tube. Seven milliliters of a 5% ammonium molybdate solution is also added, and samples are gently vortexed and subsequently reheated again at 100° for 20 min until the samples are at a full boil. Samples are then cooled to room temperature, and the absorbance is read at 820 nm. Phospholipid concentrations may then be calculated based on standard curve results. The Fiske and Subbarow reagent can be prepared by weighing 150 g of  $\text{NaHSO}_3$ , 5 g  $\text{Na}_2\text{SO}_3$ , and 2.5 g 1-amino-2-naphthol-4-sulfonic acid and dissolving in 1 liter of distilled water with slight warming to assist in dissolution. The solution should be stored in an amber bottle at 4° and discarded within 6 months. The solution should be filtered using a 0.45- $\mu\text{m}$  filter before use.

Doxorubicin can be purchased in a pure powdered form (e.g., Hande-tech USA, Inc., Houston, TX) or in a powder injectable form of 10, 50, and 150 mg of doxorubicin-hydrochloride with 52.6, 263.1, and 789.4 mg lactose, respectively, in each vial [e.g., Faulding (Canada) Inc., Kirkland, QC, Canada, or Faulding Pharmaceuticals, South Paramus, NJ). As with the handling of any cytotoxic agent, extreme care must be taken when weighing doxorubicin; gloves and masks must be used if handling doxorubicin powder directly. Direct handling of doxorubicin in powdered form is not recommended, and the injectable powder, for example, can be dissolved in injectable water, as specified by the manufacturer, prior to any dispensing of the drug.

The desired quantities of doxorubicin and the liposomes are individually incubated at the determined temperature for optimal loading; this temperature is typically selected to give greater than 95% loading within 30 min following mixing of drug with the liposomes. Once lipid and drug have reached the specific temperature, the doxorubicin is rapidly added to the prepared liposomes and vortexed intermittently during the incubation period. An example of typical loading conditions is provided in [Table III](#).

The accumulation of doxorubicin into liposomes can be determined by measuring the drug-to-lipid ratio as a function of time. This is determined

TABLE III  
DOXORUBICIN LOADING

Component	Amount	Volume (total = 2.0 ml)
Lipid (20 mg/ml)	10 mg	500 $\mu\text{l}$
Doxorubicin (10 mg/ml)	2 mg	200 $\mu\text{l}$
External buffer	N/A	1300 $\mu\text{l}$ [2.0-(0.5+0.2)]

by removing small (e.g., 100  $\mu$ l) aliquots from a loading sample and separating unencapsulated drug from encapsulated drug on 1-ml Sephadex G-50 (medium) spin columns equilibrated with the appropriate buffer. One-milliliter spin columns can be purchased commercially or can be easily made for a fraction of the price. Sephadex G-50 (medium) beads are hydrated in the external buffer used for the liposomes. The solution is degassed as in a typical column preparation. One-milliliter tuberculin syringes without needles are used, with the plunger removed. A small portion of glass wool is rolled into a small ball (the size of a pea) and firmly inserted into the empty syringe using the plastic plunger (with the sealing rubber tip removed), thus creating a plug for the column. This should be done in a fume hood to avoid inhalation of the glass particles that can arise when working with glass wool. Columns are placed into empty 16  $\times$  100-mm glass tubes, and each syringe is filled with the Sephadex, making sure no air bubbles are present. Columns are gently shaken to compact the solution, and more Sephadex is added until the tops of the syringes are almost overflowing. Excess buffer is removed from the glass tube, and columns are centrifuged at 760g for 1 min. Columns should typically pack to the 1 ml mark. The columns are then put into clean test tubes and are ready to be used within 1 h of preparation. Before adding the liposome sample, the columns are always "prewet" with 50–100  $\mu$ l of external buffer, and the sample is immediately added. Columns are recentrifuged at 760g for 3 min, and the liposomes and associated drug are collected in the voided fraction.

The concentration of doxorubicin in the excluded fraction is easily determined using either spectrophotometric or fluorescent assays. Doxorubicin absorbance is typically read at 480 nm from a solution consisting of the sample or standard. Standards are prepared using diluted doxorubicin in the 10–60  $\mu$ g/ml range. Samples or standards are adjusted to 100  $\mu$ l with extravesicular buffer, to which 900  $\mu$ l of 1% Triton X-100 is added. Prior to assessing absorbance at 480 nm, the sample is placed in a water bath at  $>90^\circ$  until the cloud point of the detergent is observed. The amount of doxorubicin-associated fluorescence can be determined by first solubilizing the liposomes using a suitable detergent devoid of contaminating fluorescent chemicals that may interfere with doxorubicin fluorescence. Typically, our laboratory uses *n*-octyl  $\beta$ -D-glucopyranoside, a high-quality nonionic detergent. Doxorubicin fluorescence can be read using a luminescence spectrometer with excitation/emission wavelengths depending on an initial prescan of doxorubicin to obtain optimal values. The fluorescence readings are compared with a standard curve of doxorubicin prepared using known amounts of doxorubicin in the 0–500 ng range in a solution similar to that used for the unknowns.

## Conclusions

This chapter has described active loading methods for the encapsulation of doxorubicin into unilamellar liposomes for use *in vitro* and *in vivo*. It is hoped that sufficient insight has been provided into the physicochemical properties of doxorubicin and how this drug interacts with lipids and with the specific buffers used to promote encapsulation of the drug. This chapter has not, however, defined how the physicochemical properties of the formulation influence biological activity. It is believed that the *in vivo* pharmacokinetics and biodistribution of iv-injected liposomal doxorubicin are dependent on a fine balance between the rate of doxorubicin release from the lipid carrier and the elimination rate of the carrier itself. The rate of doxorubicin release from the lipid carrier is dictated by several factors. The internal pH of the liposome dictates the proportion of drug in the neutral or charged form, and as the proportion shifts to the neutral form (e.g., as the interior pH increases), the rate of drug dissociation increases. Some investigators believe that the initial stage of drug release involves dissolution of the drug itself from the precipitated form adopted within the liposome; however, results from our laboratory suggest that this is not a rate-limiting step (Abraham *et al.*, 2002).

For doxorubicin to move from the inside of the liposome to the outside, it must pass through the lipid bilayer, a process that involves interactions with the membrane interface on the inside of the liposome, the lipid headgroups, the lipid acyl chains, and the membrane interface on the outside of the liposome. Obviously, the pH within the aqueous core of the liposome and the pH at the interface dictate the proportion of drug in the neutral and in the charged form. It is believed that both neutral and charged drugs can permeate the bilayer, but it is acknowledged that the rate of permeation of the neutral form of the drug is far faster than the charged form. Evidence in support of membrane partitioning of the protonated form of doxorubicin arises from studies evaluating the effects of charged lipid species, which increase hydration at the interface and, in turn, will increase drug release. More specifically, the presence of anionic lipids likely increases the concentration of charged doxorubicin at the interface, and this results in an increased rate of drug release even under conditions where the internal pH is kept below 5. It is also established that inclusion of PEG-modified phosphatidylethanolamine (an anionic lipid) increases doxorubicin release rates, an effect that could be attributed to surface-grafted PEGs increasing hydration at the interface or to the fact that the PEG lipid is anionic.

Assuming that the lipid composition consists primarily of the zwitterionic lipid phosphatidylcholine and cholesterol (55:45 mol%), drug release

is primarily a consequence of drug partitioning through the bilayer. This statement is not intended to minimize the important parameters such as trapping efficiency, drug-to-lipid ratios, and their link to maintenance of a large pH gradient (Mayer *et al.*, 1989). Rather it acknowledges that under conditions in which the loading parameters are identical and the interior pHs are comparable, the most important parameter controlling doxorubicin release is the liposomal lipid composition. The importance of this was best illustrated in a review published in 1994 (Mayer *et al.*, 1994), where the drug release rates and acute toxicity parameters of five different liposomal formulations of doxorubicin were compared. All formulations were comparable on the basis of drug-to-lipid ratio, liposome size, and liposome elimination rates following iv administration, yet there was more than a 10-fold range in the dose that could be tolerated in mice. Using the maximum tolerated dose (MTD) of free drug (20 mg/kg) as a comparator, the MTD of doxorubicin encapsulated in DMPC/Chol liposomes was less than 5 mg/kg, while the MTD of doxorubicin encapsulated in DSPC/Chol liposomes was greater than 80 mg/kg. The rate of drug release from liposomes in the plasma compartment was shown to be linear over a 24-h time course for both liposomal formulations, but the rate of drug release from the DMPC/Chol liposomes was significantly faster.

As a final note, we strongly believe that the lessons learned while developing liposomal formulations of doxorubicin are valuable when considering the development of other liposomal drug formulations. There are several therapeutic applications being considered that will benefit from continued research on liposomal formulations of doxorubicin. In particular, we and other investigators are considering the use of liposomal anticancer drug formulations that are designed to retain a drug well in the plasma compartment but to release it rapidly following accumulation at sites of disease. The rationale for these formulations is simple. An ideal liposomal anticancer drug would exhibit little or no drug release while in the plasma compartment, thus ensuring limited exposure of the drug to healthy tissue. This feature would also maximize drug delivery to disease sites, as mediated by the movement of the drug-loaded liposomes from the plasma compartment to the extravascular space at disease sites, such as a region of tumor growth. Following localization, however, the drug-loaded liposome must transform itself from a stable carrier to an unstable carrier. This would ensure that the drug, which has localized in the diseased site, is bioavailable. In this regard, our research team has placed specific emphasis on the development of liposomal anthracycline formulations that meet these design attributes, including the use of programmable fusogenic liposomes (Adlakha-Hutcheon *et al.*, 1999) and thermosensitive liposomes combined with use of hyperthermia (Needham *et al.*, 2000).



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## [5] Preparation and Characterization of Taxane-Containing Liposomes

By ROBERT M. STRAUBINGER and  
SATHYAMANGALAM V. BALASUBRAMANIAN

### Abstract

Drug carriers such as liposomes provide a means to alter the biodisposition of drugs and to achieve concentration–time exposure profiles in tissue or tumor that are not readily accomplished with free drug. These changes in biodisposition can improve treatment efficacy. For hydrophobic drugs, incorporation in liposome carriers can increase drug solubility markedly. The taxanes paclitaxel (taxol) and docetaxel (Taxotere) are members of one of the most important new classes of oncology drugs. However, their poor solubility presents pharmaceutical challenges, and