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Loading of doxorubicin into liposomes by forming Mn^{2+}-drug complexes

Benny C.L. Cheung [a], Tyler H.T. Sun [a], Johanna M. Leenhouts [a], Pieter R. Cullis [a,b,*]

[a] *University of British Columbia, Department of Biochemistry and Molecular Biology, 2146 Health Sciences Mall, Vancouver, BC V6T 1Z3, Canada*

[b] *Inex Pharmaceuticals Corporation, 100-8900 Glenlyon Parkway, Glenlyon Business Park, Burnaby, BC V5J 5J8, Canada*

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Abstract

A new procedure for loading doxorubicin into large unilamellar vesicles (LUVs) is characterized. It is shown that doxorubicin can be loaded into LUVs composed of sphingomyelin/cholesterol (55:45 mole/mole) in response to a transmembrane $MnSO_4$ gradient in the absence of a transmembrane pH gradient. Complex formation between doxorubicin and Mn^{2+} is found to be a driving force for doxorubicin uptake. Uptake levels approaching 100% can be achieved up to a drug-to-lipid molar ratio of 0.5 utilizing an encapsulated $MnSO_4$ concentration of 0.30 M. In vitro leakage assays show excellent retention properties over a 24 h period. The possible advantages of a liposomal formulation of doxorubicin loaded in response to entrapped $MnSO_4$ are discussed.

Keywords: Adriamycin; Anthracycline; Ion gradient; Large unilamellar vesicle; ^{14}C-Methylamine; ^{14}C-Mevalonic acid; Manganese(II) sulfate

1. Introduction

Doxorubicin is a potent antineoplastic agent active against a wide range of human cancers. However, treatment with doxorubicin is associated with severe toxic side effects which include dose-limiting cardiotoxicity and myelosuppression [1–5]. When administered by intravenous injection, the use of liposomes to encapsulate doxorubicin can reduce toxic side effects without decreasing drug potency [3,4,6–12].

Doxorubicin is an amphipathic weak base consisting of an anthraquinone moiety (the aglycone part) and an amine sugar. Previous work has shown that doxorubicin and other weak bases can be accumulated into vesicles with an acidic interior in response to the transmembrane pH gradient [13–15]. Recently, a new method for drug loading has been developed using LUVs containing Mn^{2+} in the presence of the ionophore A23187 [35]. The ionophore A23187 translocates one Mn^{2+} ion to the outside of the LUV in exchange for the inward movement of two protons, thereby generating a pH gradient (interior acidic) across the bilayer, which drives drug uptake. Efficient loading of vincristine and ciprofloxacin has been demonstrated using this method. In the present study, the ionophore method was used to load doxorubicin. However, it was found that doxorubicin can be efficiently loaded into liposomes with a transmembrane Mn^{2+} gradient in the absence of ionophore via the formation of intravesicular doxorubi-

Abbreviations: ^{3}H-CHE, ^{3}H-cholesteryl hexadecyl ether; LUV, large unilamellar vesicle; ΔpH, pH gradient; pH_o, extraliposomal pH; pH_i, intraliposomal pH

* Corresponding author. Fax: (604) 822-4843;
E-mail: pieterc@unixg.ubc.ca

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cin-Mn^{2+} complexes. It is shown that complex formation between doxorubicin and Mn^{2+} provides a strong driving force for doxorubicin accumulation inside liposomes.

2. Materials and methods

2.1. Materials

Egg sphingomyelin was purchased from Northern Lipids (Vancouver, BC). Cholesterol and the calcium ionophore A23187 were obtained from Sigma (Mississauga, ON). Doxorubicin hydrochloride (adriamycin) was manufactured by Pharmacia and Upjohn (Don Mills, ON). ^{14}C-Mevalonic acid (*RS*-[2-^{14}C]mevalonic acid, DBED salt) was purchased from Amersham Canada (Oakville, ON). ^{3}H-Cholesteryl hexadecyl ether (^{3}H-CHE) was produced by NEN Life Science Products (Boston, MA). ^{14}C-Methylamine (^{14}C-methylamine hydrochloride) was purchased from DuPont (Boston, MA). Sterile mouse serum was obtained from Cedarlane Laboratories (Hornby, ON). Nucleopore polycarbonate filters (25 mm, 0.1 μm pore size) were obtained from Costar Scientific (Toronto, ON). All chemicals were reagent grade.

2.2. Preparation of 100 nm large unilamellar vesicles (LUVs)

Liposomes were prepared by the freeze-thaw extrusion method as described previously by Hope et al. [16]. Throughout this study, 100 nm diameter large unilamellar vesicles consisting of sphingomyelin/cholesterol (55:45 molar ratio) were used, because they have been shown to exhibit excellent retention properties [17]. Briefly, mixtures of sphingomyelin and cholesterol (55:45 mole ratio) were dissolved in 2-methylpropan-2-ol at 60°C. A trace amount of ^{3}H-CHE was added to achieve a final activity of 0.05 μCi/μmole lipid, and the solution was then frozen in liquid nitrogen. This frozen material was lyophilized for at least 4 h under high vacuum to remove the organic solvent. Unless specified otherwise, the lyophilized lipid mixture was then hydrated at 50 mM with a buffer containing 0.30 M $MnSO_4$ and 30 mM HEPES (pH 7.4) in a 60°C water bath. This suspension was subjected to five freeze-thaw cycles by alternating between liquid nitrogen and a 60°C water bath with vigorous vortexing between cycles. Subsequently, the hydrated lipid mixture was extruded 10 times through two layers of polycarbonate filters with pore size of 0.1 μm at 60°C using a water-jacketed extruder (Lipex Biomembranes, Vancouver, BC). Phospholipid content of the LUVs (which accounts for 55% of the total lipid) was measured employing Fiske-Subbarow phosphate assay [18]. Subsequently, the specific activity of the liposomes (expressed as dpm/nmole total lipid) was determined by liquid scintillation counting.

2.3. Doxorubicin uptake experiments

A transmembrane Mn^{2+} gradient was generated across the LUVs by exchanging the extraliposomal buffer using Sephadex G-50 spin columns [15]. Subsequently, the lipid concentration was determined by liquid scintillation counting. Unless specified otherwise, all experiments involving the ionophore A23187 used buffer containing 0.30 M sucrose, 20 mM HEPES and 15 mM EDTA at pH 7.4; experiments performed in the absence of the ionophore employed buffer containing 0.30 M sucrose and 20 mM HEPES at pH 7.4. The transition temperatures of sphingomyelins range from 40.5 to 57°C [19]; as a consequence, all uptake experiments were performed at 60°C in order to permit efficient partitioning of the drug into and across the lipid bilayer. A23187 was used at a concentration of 0.1 μg ionophore/μmole lipid and LUVs were incubated with ionophore at 60°C for 5 min prior to the addition of doxorubicin. The amount of doxorubicin used in each experiment varied depending on the desired drug-to-lipid ratio. After the addition of doxorubicin, the lipid concentration was 5 mM. Samples of this mixture were taken at specific time points, applied onto spin columns and centrifuged at $760 \times g$ for 2 min. This step removes unencapsulated doxorubicin, leaving liposomes with encapsulated doxorubicin in the eluant. In order to determine the drug-to-lipid ratio, part of the eluant was subjected to liquid scintillation counting to assay for the amount of lipid, while another part was added to a solution of 1.0% Triton X-100, 2.0 mM EDTA and 20 mM HEPES (pH 7.5) for the

quantification of doxorubicin by absorption spectroscopy at 480 nm ($\varepsilon = 1.06 \times 10^4$ M^{-1} cm^{-1} [20]). The total drug-to-lipid ratio (100% value) in the uptake mix was determined at specific time points by the same procedure but omitting the spin column centrifugation step.

2.4. Determination of pH gradients

To determine the pH gradient (inside acidic) present across the lipid bilayer, a trace amount of ^{14}C-methylamine [21] was added to the uptake cocktail (final activity = 0.2 μCi/ml). The doxorubicin uptake experiments were performed as described above, except dual ^{3}H- and ^{14}C-radioisotope liquid scintillation counting was used. Assuming a trapped volume of 1.5 μl per μmole lipid for 100 nm diameter LUVs [16,22], intraliposomal and extraliposomal methylamine concentrations could be determined. The pH gradient (ΔpH) can then be calculated by [21,23]:

$$\Delta pH = \log \{[H^+]_{inside}/[H+]_{outside}\} =$$

$$c\log \{[methylamine]_{inside}/[methylamine]_{outside}\}$$

For the determination of ΔpH in LUVs with a basic interior, ^{14}C-mevalonic acid was used instead of ^{14}C-methylamine [23].

2.5. Absorption spectra

The effect of Mn^{2+} on the doxorubicin absorption spectrum was determined by mixing various concentrations of $MnSO_4$ with doxorubicin (54 μM final concentration) in 100 mM HEPES (pH 7.4). Absorption spectra from 350 to 700 nm were taken using a Shimadzu UV160U spectrophotometer. At saturating concentration of Mn^{2+}, the pH dependence of the doxorubicin-Mn^{2+} complex was studied. 400 mM $MnSO_4$, 24 μM doxorubicin and 100 mM of various buffers were used: MES as the buffer for pH 5.2 and 5.6, PIPES buffer for pH 6.0, 6.4 and 6.8 and HEPES was used at pH 7.2, 7.5, 7.8 and 8.0. Spectra of doxorubicin loaded in the presence or absence of A23187 were compared with the spectrum of unencapsulated doxorubicin in the presence of empty liposomes.

2.6. In vitro leakage assay

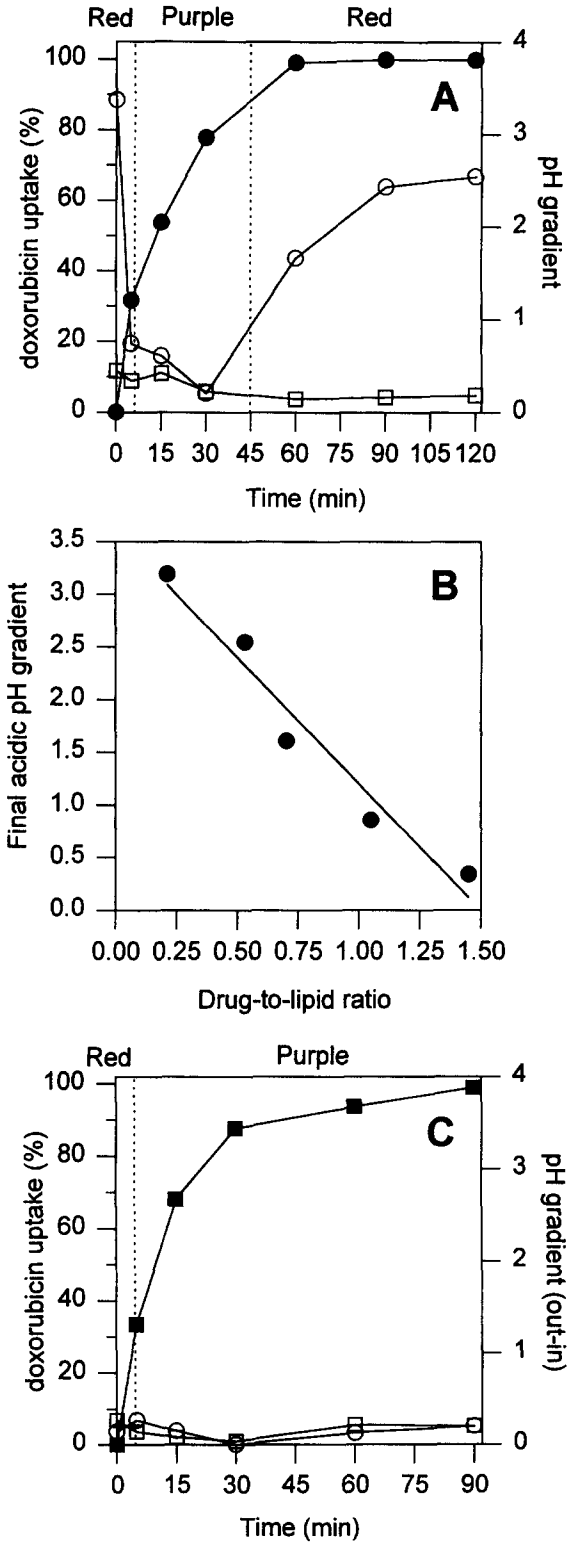
Doxorubicin was loaded at a drug-to-lipid ratio of 0.5 (mole/mole) in the presence or absence of A23187. Each uptake mixture was incubated for 70 min at 60°C after which it was applied onto spin columns to remove unencapsulated doxorubicin. After centrifugation, an equal volume of sterile mouse serum was added followed by incubation at 37°C for up to 24 h. The final concentration of liposomes was 1 mM. Aliquots were taken out at specified time points for the determination of doxorubicin retention.

3. Results

3.1. Doxorubicin is accumulated into Mn^{2+} containing LUVs in the absence of a pH gradient

Previous studies [35] have shown that amino-containing drugs such as vincristine and ciprofloxacin can be loaded into liposomes exhibiting a Mn^{2+} gradient in the presence of external EDTA and the ionophore A23187. This method was also found to load doxorubicin efficiently. The kinetics of doxorubicin loading into 100 nm diameter LUVs at 60°C is shown in Fig. 1A. The transbilayer pH gradient was also measured, demonstrating an initial pH gradient of 3.4 units (inside acidic). Upon drug addition, this gradient was quenched within 5 min. However, the ΔpH was later re-established, rising to 2.4 units at t=90 min. The magnitude of the re-established ΔpH varied depending on the drug-to-lipid ratio used in the experiment (Fig. 1B).

The disappearance and subsequent recovery of the acidic gradient was accompanied by marked color changes: the solution was initially red in color ($t = 0$) and then turned purple within 5 min. After 45 min, the color changed back to red. The transient purple color could not be due to the change of the internal pH, since doxorubicin turns purple only at pH values higher than 8 (results not shown). The ^{14}C-mevalonic acid data show that no basic pH gradient was generated throughout the entire uptake process (Fig. 1A). This means that the highest intraliposomal pH at any point during drug uptake was equivalent to the extraliposomal pH (i.e. pH 7.4).



Interestingly, doxorubicin uptake continued between 5 and 30 min despite the absence of any acidic gradient (Fig. 1A). This suggested that the pH gradient was not required for doxorubicin uptake. To test this hypothesis, uptake of doxorubicin was examined in the absence of the ionophore A23187. The kinetics of doxorubicin uptake in the absence of A23187 (Fig. 1C) are very similar to those in the presence of A23187 (Fig. 1A). Only background lev-

Fig. 1. (A) Time course of doxorubicin uptake in the presence of A23187 (●) into LUVs containing $MnSO_4$ (0.30 M). The acidic (○) or basic (□) nature of the LUV interior was determined during the uptake process using ^{14}C-methylamine and ^{14}C-mevalonic acid as ΔpH probes, respectively. The color of the uptake mixture is indicated on top of the diagram. Uptake was performed at a drug-to-lipid molar ratio of 0.53 with an A23187 concentration of 0.1 μg/μmole lipid. (B) Final pH gradient (interior acidic) re-established after loading at various drug-to-lipid ratios in the presence of A23187. Final pH gradients were determined using ^{14}C-methylamine after an equilibrium interior pH had been established. (C) Time course of doxorubicin uptake in the absence of A23187 (■). The pH of the LUV interior was monitored using ^{14}C-methylamine (○) and ^{14}C-mevalonic acid (□). The color of the uptake mixture is indicated on top of the diagram. Uptake was performed at a drug-to-lipid ratio of 0.45.

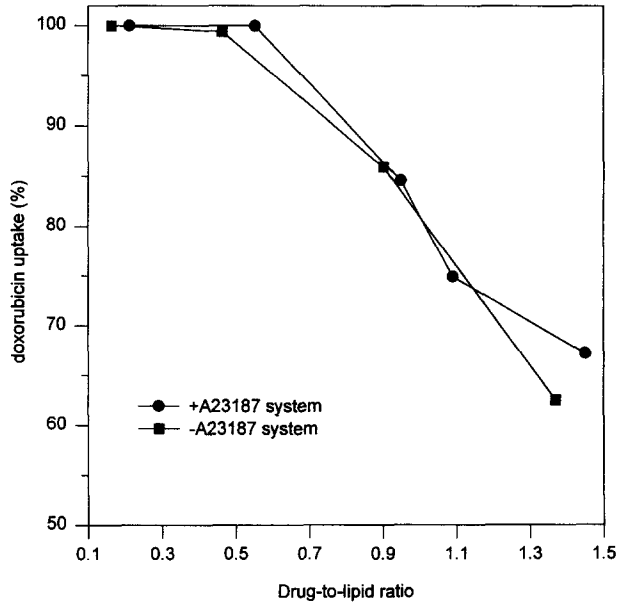


Fig. 2. Equilibrium doxorubicin uptake into LUVs containing $MnSO_4$ in the presence (●) or absence (■) of A23187 at various drug-to-lipid ratios. All uptake experiments were performed at 60°C and were continued until the uptake levels became constant (60–120 min). LUVs used for both systems were composed of sphingomyelin/cholesterol (55:45 molar ratio) and contained 0.30 M $MnSO_4$ titrated to pH 7.4 by 30 mM HEPES.

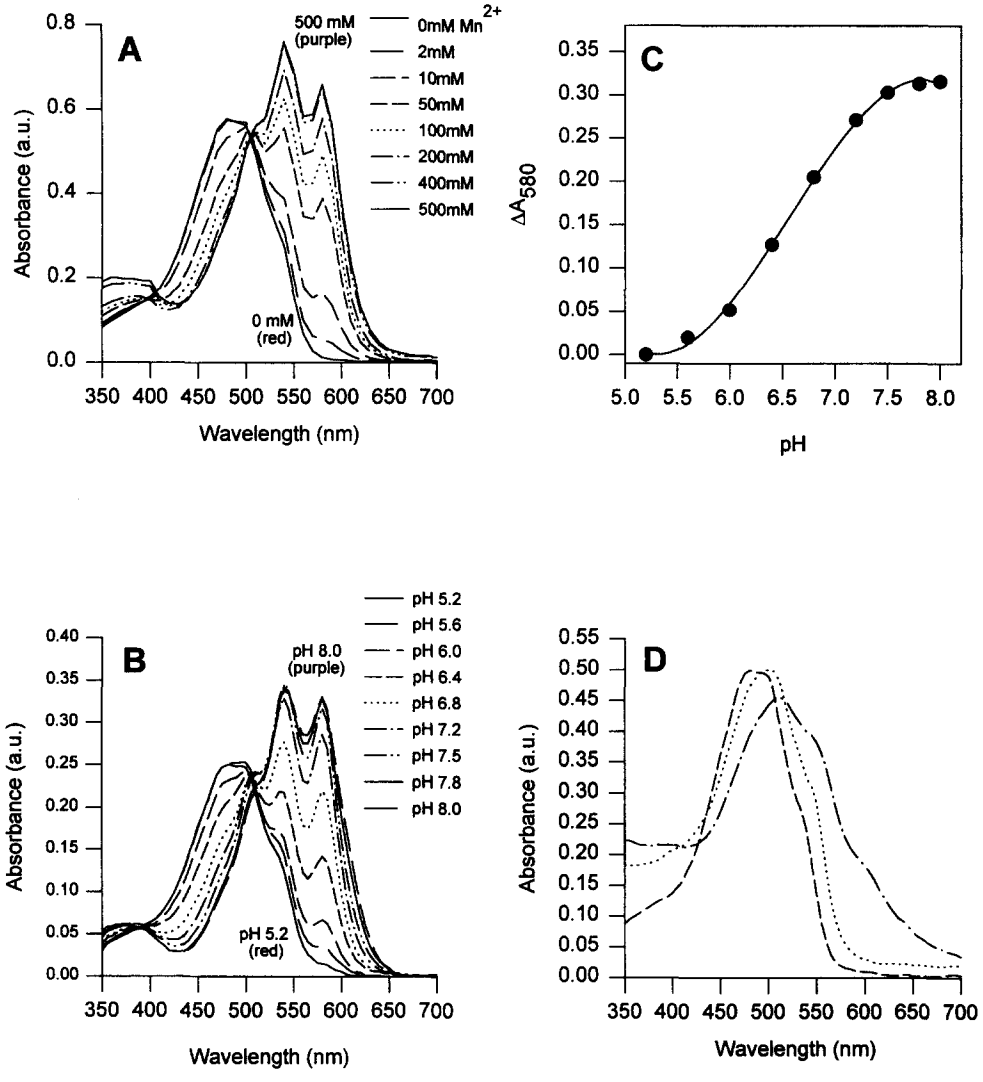


Fig. 3. (A) Absorption spectra of doxorubicin in the presence of various concentrations of $MnSO_4$. Doxorubicin (54 μM) was mixed with 0, 2, 10, 50, 100, 200, 400 or 500 mM $MnSO_4$. All solutions were buffered at pH 7.4 by 100 mM HEPES. The color of the uptake mixture is indicated in parentheses. (B) Absorption spectra of doxorubicin-Mn^{2+} complexes at various pH values. $MnSO_4$ (400 mM) and doxorubicin (24 μM) were buffered using 100 mM concentrations of the following buffers: MES pH 5.2–5.6; PIPES pH 6.0–6.8 and HEPES pH 7.2–8.0. The color of the uptake mixture is indicated in parentheses. (C) Plot of absorbance (A_{580}) vs. pH. The pK_a of the doxorubicin-Mn^{2+} complex was determined from the inflection point of the curve ($pK_a = 6.6$). (D) Absorption spectra of unencapsulated doxorubicin in the presence of empty liposomes (—), and of encapsulated doxorubicin, loaded either in the presence of A23187 (···) or the absence of A23187 (– · –). All spectra were obtained at a drug-to-lipid molar ratio of 0.5.

els of methylamine or mevalonic acid were found entrapped inside liposomes during uptake in the absence of A23187, indicating that no pH gradient was generated during the uptake process. The intraliposomal and the extraliposomal pH values were stable at pH 7.4 throughout the uptake process. The solution turned from red to purple within 5 min after uptake had begun and it stayed purple thereafter.

Doxorubicin was loaded at various drug-to-lipid ratios in the presence or absence of A23187 (Fig. 2). Both systems demonstrated similar doxorubicin loading efficiencies. Nearly 100% drug uptake could be achieved up to a drug-to-lipid ratio of approx. 0.5. At higher drug-to-lipid ratios, the efficiency of drug uptake decreased. A drug-to-lipid ratio of approx. 1 was therefore used in experiments designed to detect possible differences in uptake levels between different systems.

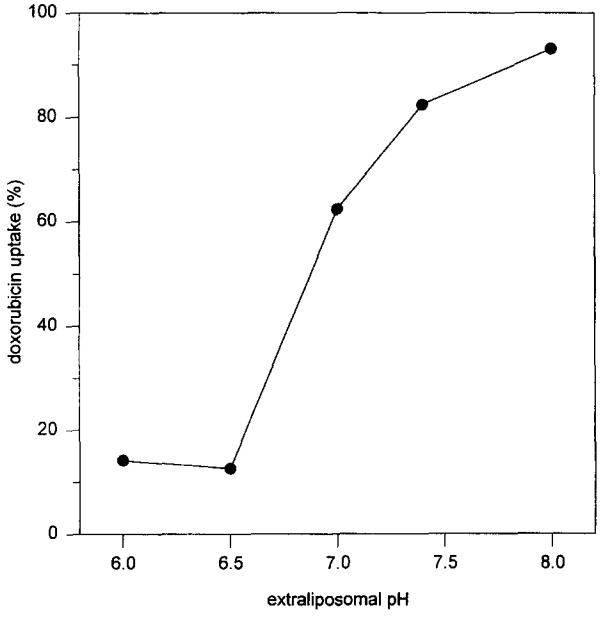


Fig. 4. Effects of extraliposomal pH on doxorubicin uptake in the presence (●) or in the absence of A23187 (■). All uptake experiments employed 0.30 M $MnSO_4$+30 mM HEPES (pH 7.4) containing liposomes and doxorubicin was loaded at a drug-to-lipid ratio of 1.1. In the extraliposomal buffers, HEPES was used to buffer at pH 7.0, 7.4 and 8.0 while HEPES and PIPES were used to buffer at pH 6.0 and 6.5.

3.2. *Doxorubicin forms complexes with entrapped Mn^{2+} ions*

In order to explore the mechanism of doxorubicin uptake into liposomes exhibiting a transmembrane Mn^{2+} gradient but no pH gradient, the formation of doxorubicin-Mn^{2+} complexes was investigated using absorption spectroscopy. Absorption spectra of doxorubicin at pH 7.4 in the presence of various concentrations of $MnSO_4$ are shown in Fig. 3A. A gradual red shift was observed in the spectra upon the addition of Mn^{2+}, and the color of the solution turned from red to purple at higher Mn^{2+} concentrations. Doxorubicin (54 μM) was found to be saturated with Mn^{2+} at approx. 400 mM $MnSO_4$. Fig. 3B shows the change in absorption spectra of the doxorubicin-Mn^{2+} complex (at the saturating concentration of Mn^{2+}) when titrated from pH 5.2 to 8.0. At pH 5.2, most of the complex is dissociated, resulting in an absorption spectrum resembling the characteristic absorption peak for free doxorubicin that has a peak at approx. 480 nm. Between pH 6.0 and 7.8 the spectrum was red-shifted, resulting in a spectrum characteristic of the doxorubicin-Mn^{2+} complex. A further increase in pH beyond 8.0 caused precipitation of $Mn(OH)_2$. The absorbance of the doxorubicin-Mn^{2+} complex varies significantly between pH 5.2 and 8.0, with the most drastic changes at 580 nm. When plotting the change in A_{580}

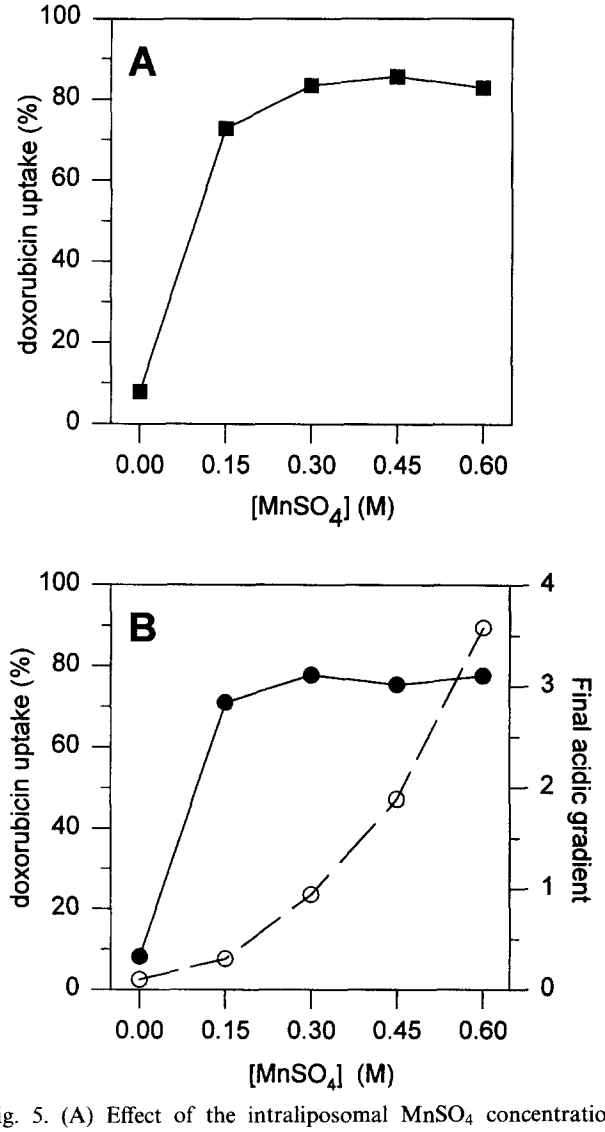


Fig. 5. (A) Effect of the intraliposomal $MnSO_4$ concentration on doxorubicin uptake achieved in the absence of A23187. (B) The effect of intraliposomal $MnSO_4$ concentration on doxorubicin uptake (●) and on the final acidic gradient (○) in the presence of A23187. Doxorubicin uptake and the final acidic gradient were determined after their readings became constant (60–120 min). All concentrations of $MnSO_4$ solutions were titrated to and buffered at pH 7.4 with 30 mM HEPES. When no $MnSO_4$ was present, the LUVs contained 0.30 M Na_2SO_4 and 30 mM HEPES (pH 7.4). Uptake in both panels was performed at a drug-to-lipid ratio of 1.0 at 60°C.

versus the pH, the apparent pK_a of complex formation was determined to be approx. 6.6 (Fig. 3C).

The characterization of the liposomal doxorubicin loaded in the presence or absence of A23187 was also performed using absorption spectroscopy. Absorption spectra were taken after doxorubicin had been loaded into liposomes (Fig. 3D). As a control, a spectrum of free doxorubicin in the presence of empty liposomes was taken. All spectra were obtained from doxorubicin and liposomes at a drug-to-lipid ratio of approx. 0.5. In the absence of A23187, the (loaded) doxorubicin spectrum shifted to higher wavelengths resembling a non-saturating doxorubicin-Mn^{2+} complex spectrum at pH 7.4. In the A23187 containing system, the spectrum of liposomal doxorubicin was identical to that of the unencapsulated doxorubicin, indicating that the doxorubicin was not complexed to Mn^{2+}.

3.3. *Effects of external pH and entrapped Mn^{2+} levels on complex dependent doxorubicin accumulation*

To investigate the influence of the external pH on doxorubicin uptake, the extraliposomal pH (pH_o) in uptake experiments was varied while keeping the intraliposomal pH (pH_i) constant at pH 7.4. Fig. 4 shows the effect of the pH_o on the loading of doxorubicin into Mn^{2+}-containing liposomes in the absence of A23187 using a drug-to-lipid ratio of 1.1. Doxorubicin uptake remained constant from pH 6.0 to 6.5 and gradually increased as the pH_o rose from pH 6.5 to 8.0. By measuring the pH gradients during these uptake processes, it was found that the pH_i became equal to the pH_o as soon as drug uptake had begun (data not shown), regardless of the initial pH gradient.

The effect of the Mn^{2+} concentration encapsulated inside the liposomes on the level of doxorubicin uptake was investigated both in the presence and absence of A23187. Liposomes with no Mn^{2+} (employing 0.30 M Na_2SO_4-containing liposomes), 0.15 M, 0.30 M, 0.45 M and 0.60 M $MnSO_4$ (buffered at pH 7.4 with 30 mM HEPES) were used in the uptake experiments. The results in Fig. 5A show that 0.30 M Mn^{2+} was sufficient to achieve a high level of drug uptake at a drug-to-lipid ratio of 0.96 in the absence of ionophore. A sharp increase in drug uptake was

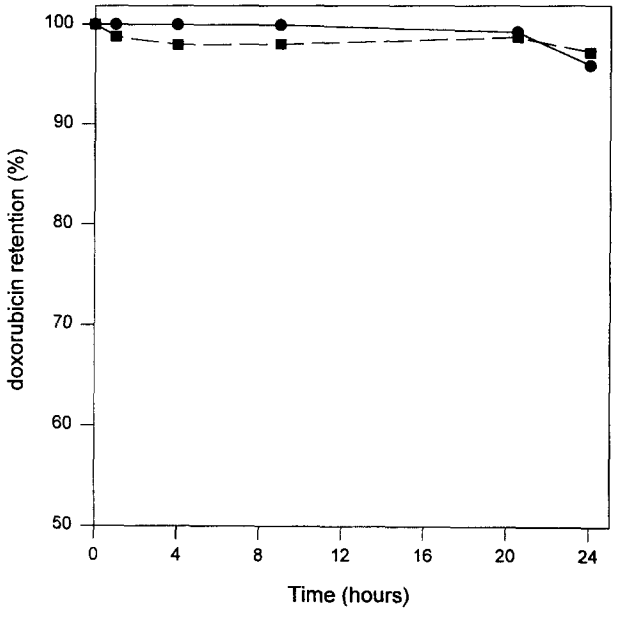


Fig. 7. In vitro leakage assay of liposomal doxorubicin using 50% mouse serum. Liposomal doxorubicin was loaded at a drug-to-lipid ratio of 0.5 in the presence (●) or in the absence (■) of A23187 by incubating doxorubicin with liposomes at 60°C for 70 min. Subsequently, the liposomal doxorubicin was centrifuged through spin columns before incubating for 24 h with mouse serum at 37°C. The lipid concentration in the mixture containing 50% mouse serum during the leakage assay was 1 mM.

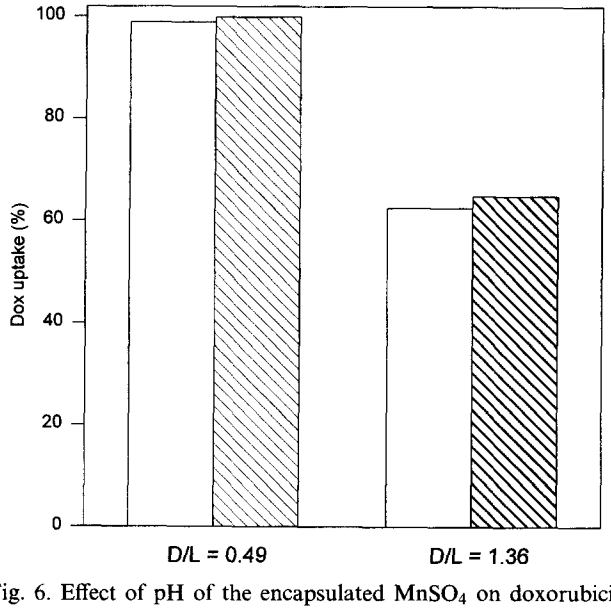


Fig. 6. Effect of pH of the encapsulated $MnSO_4$ on doxorubicin uptake in the absence of A23187. Encapsulated $MnSO_4$ solutions were either untitrated (pH 4.0, dashed bars), or titrated with 30 mM HEPES (pH 7.4, open bars). The drug-to-lipid ratios administered were 0.49 and 1.36.

observed between 0 M and 0.15 M Mn^{2+} and a further but moderate increase was found between 0.15 M and 0.30 M. The level of drug uptake then leveled off at 85% at Mn^{2+} concentrations higher than 0.30 M. At a drug-to-lipid ratio of 1.0, doxorubicin uptake in the presence of A23187 showed very similar results (Fig. 5B).

The reason we employed entrapped $MnSO_4$ at pH 7.4 was to eliminate any effects of an initial pH gradient on doxorubicin uptake in the Mn^{2+} containing system, thus simplifying data interpretation. To test whether liposomes with unbuffered $MnSO_4$ (pH 4.0) could be used for doxorubicin loading instead of the titrated $MnSO_4$ solution at pH 7.4, doxorubicin uptake into liposomes containing $MnSO_4$ encapsulated at pH 4.0 or at pH 7.4 were performed and the results are illustrated in Fig. 6. Fig. 6 (left) (in the presence of A23187) and Fig. 6 (right) (in the absence of A23187) show no significant difference in drug loading effectiveness between the pH 4.0 and the pH 7.4 $MnSO_4$-entrapped liposomes.

3.4. *Doxorubicin is stably entrapped after accumulation in response to a Mn^{2+} gradient*

To assess the stability of the liposomal doxorubicin systems, in vitro leakage assays were performed employing 50% (v/v) mouse serum. Doxorubicin was loaded in the presence or absence of A23187 at a drug-to-lipid ratio of 0.5. After removing unencapsulated doxorubicin (and A23187: see [35]), the liposomes loaded with doxorubicin were diluted with mouse serum and incubated at 37°C for 24 h. The final lipid concentration in 50% mouse serum was 1 mM. Fig. 7 shows that virtually no drug leakage was observed over a 24 h period for LUVs loaded in the presence and absence of A23187.

4. Discussion

The results presented here demonstrate a novel method for the loading of doxorubicin into liposomes. It is shown that doxorubicin uptake can be driven by a transmembrane Mn^{2+} gradient, in the absence of any pH gradient. Loading procedures for doxorubicin described previously all rely on the presence of a transmembrane pH gradient to drive uptake [24]. Loading of doxorubicin in response to a transmembrane Mn^{2+} gradient relies on the formation of a membrane-impermeable complex with Mn^{2+}. In the following section, we discuss the mechanism and properties of this loading process and potential applications.

The formation of doxorubicin-Mn^{2+} complexes, with a high stability constant ($pK_d = 7.0$) has been previously described [25]. Complex formation is accompanied by the changes in the absorption spectrum of doxorubicin upon titrating with Mn^{2+} (Fig. 3A), which changes the color of doxorubicin from red to purple. This color change is also observed during doxorubicin loading into the liposomes in response to a Mn^{2+} gradient in both the presence and absence of A23187. In the presence of the ionophore A23187, however, the color changes back to red after the doxorubicin is loaded, indicating dissociation of

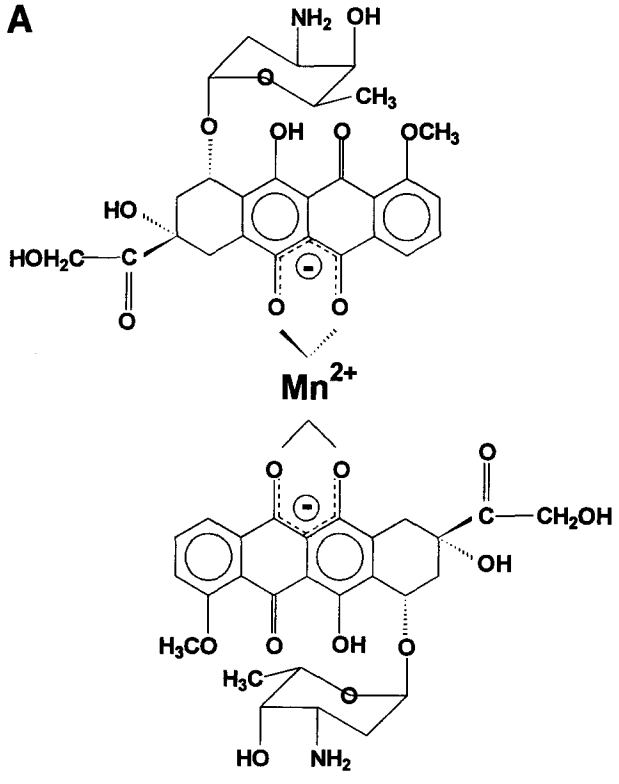


Fig. 8. (A) Chemical structure of the doxorubicin-Mn^{2+} (2:1) complex. (B) Schematic representation of reactions involved in the loading of doxorubicin in response to a transmembrane gradient Mn^{2+}. Hydroxyl (-OH) groups attached to the doxorubicin molecule are on the anthraquinone moiety of doxorubicin; amine (-NH_2 or -NH_3^+) group attached to the doxorubicin molecule corresponds to the amine on the sugar moiety.

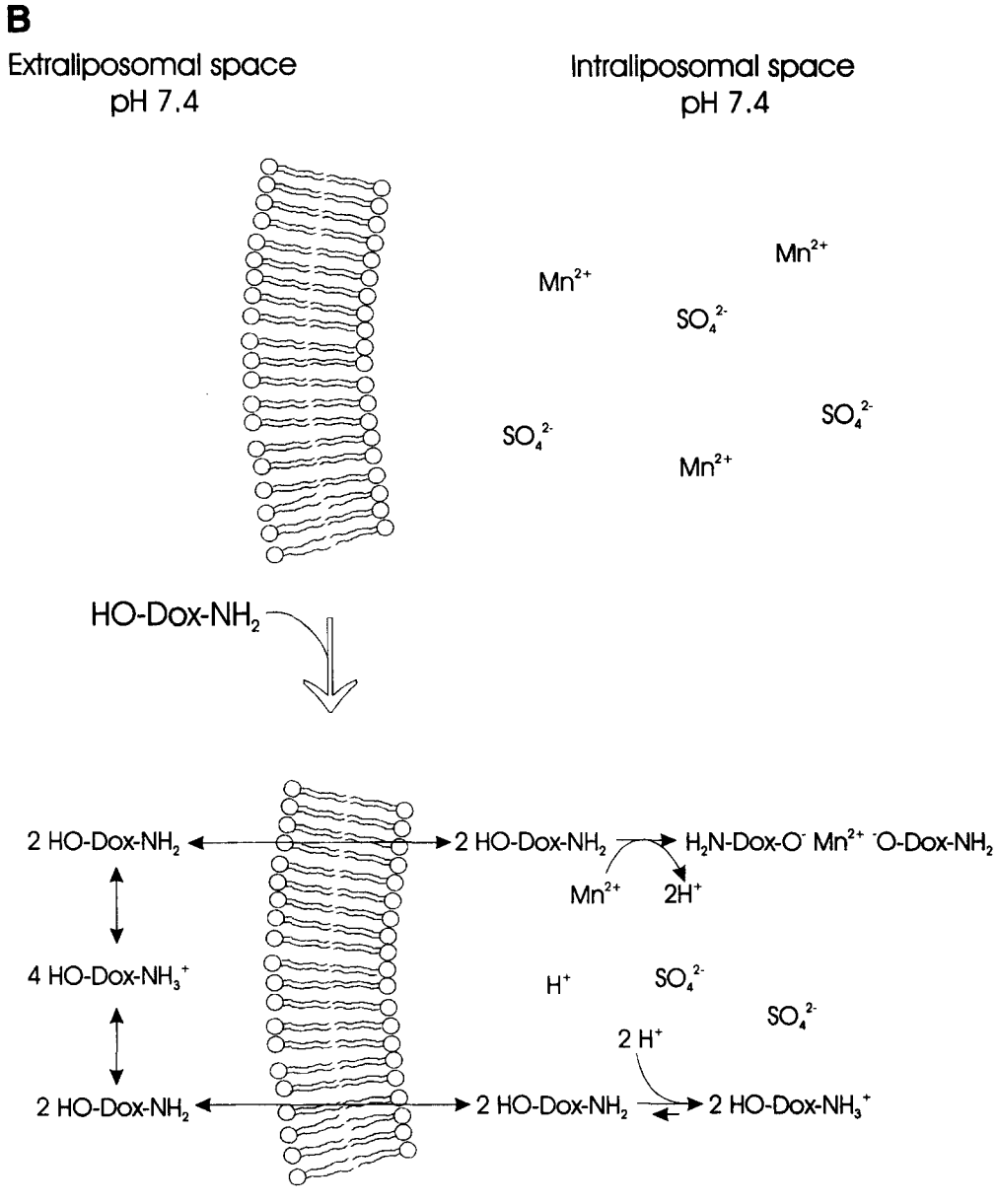


Fig. 8. (*continued*)

the doxorubicin-Mn^{2+} complex after re-establishment of the pH gradient (inside acidic) by the ionophore. This was confirmed by the absorption spectrum of the liposomal doxorubicin that was loaded in the presence of A23187 (Fig. 3D), which is characteristic of free (i.e. not complexed to Mn^{2+}) doxorubicin. The absorption spectrum of liposomal doxorubicin loaded in the absence of ionophore is red-shifted, indicating the formation of complexes with Mn^{2+}.

It has been reported that doxorubicin molecules complex with Cu^{2+} ions in a 1:1 or 2:1 ratio at pH 7.4, or as a polymeric complex [26], and one proton is released from the aglycone portion of each doxorubicin molecule upon complexation. Assuming that similar behavior is observed for Mn^{2+}, a model for doxorubicin complexation with Mn^{2+} is given in Fig. 8A (for the 2:1 complex). Upon loading into liposomes, complexation of two doxorubicin molecules with one Mn^{2+} ion will release two pro-

tons, thereby acidifying the intraliposomal medium. This will result in the protonation of the next two doxorubicin molecules that enter the liposome interior. The overall doxorubicin uptake scheme is outlined in Fig. 8B. A consequence of this loading scheme is that the pH_i will equilibrate to the pH_o, as was found in experiments where the external pH was varied (data not shown). As long as $pH_i < pH_o$, doxorubicin that enters the liposome interior will be protonated; protonation of doxorubicin depletes the intraliposomal protons and raises the pH_i. Protonation is the force for driving doxorubicin uptake until $pH_i = pH_o$. After that, uptake will take place by alternating between Mn^{2+} complexation and protonation as given in Fig. 8B. In the opposite case, when $pH_i > pH_o$ initially, doxorubicin that enters the liposome interior will complex with Mn^{2+}. This will result in the release of a proton from the aglycone region, which decreases the pH_i until $pH_i = pH_o$. Subsequently, uptake again will take place by alternating between complexation and protonation. In the experiments described in Fig. 4, the pH_i was rapidly equalized to the pH_o within the first 5 min of drug loading. Only background levels of doxorubicin uptake were observed at pH 6.5 or lower, and drug uptake increases at higher pH (Fig. 4). This can be explained by the fact that doxorubicin-Mn^{2+} complexation is pH dependent [25] (Fig. 3B,C). Half of the maximum level of doxorubicin uptake occurs at about pH 7.0, close to the pK_a of doxorubicin-Mn^{2+} complex formation ($pK_a = 6.6$; Fig. 3C).

Under the appropriate experimental conditions, doxorubicin loading in response to a transmembrane Mn^{2+} gradient is very efficient. Uptake levels of 100% can be reached up to a drug-to-lipid ratio of 0.5 (Fig. 2). When starting with a higher drug-to-lipid ratio (1.1), doxorubicin can even be loaded up to a final drug-to-lipid ratio of 0.8 (73% uptake). Overall, the efficiency of the loading procedure is comparable to that achieved employing the pH gradient loading of doxorubicin [27].

Although also for ciprofloxacin complex formation with divalent metal ions has been described [28] there was little uptake of this drug in response to a Mn^{2+} gradient [35]. The low levels of uptake in the absence of ionophore could be explained by the small pH gradient present, since unbuffered $MnSO_4$ solutions with a pH of 3–4 were used in those experiments. Apparently, metal ion-ciprofloxacin complex formation does not lead to drug uptake. This might be due to a less stable complex, a different pH optimum for complex formation, and/or membrane permeability of the complex.

Complexation of doxorubicin with several metal ions (such as Fe^{3+} and Cu^{2+}) has been reported to facilitate the binding of the anthracycline to DNA, promote the peroxidation of lipids and enhance the generation of reactive oxygen species [25,29–32]. These actions inside cells promote cell death through damaging intracellular organelles. For doxorubicin-Fe^{3+} and doxorubicin-Cu^{2+} complexes, enhanced cytotoxicity has been described [26,30,32–34]. Therefore, a liposomal formulation of doxorubicin-Mn^{2+} could be therapeutically advantageous. In this regard, a concentration of $MnSO_4$ (0.30 M) iso-osmotic to physiological saline solution was employed and was found to be sufficient to drive a high level of doxorubicin uptake at drug-to-lipid ratios as high as 1.0. Finally, in vitro leakage assays reveal that doxorubicin loaded in response to entrapped Mn^{2+} exhibits excellent retention over a 24 h incubation period at 37°C. These properties suggested that liposomal doxorubicin formulations loaded in response to entrapped $MnSO_4$ might have promise for in vivo applications.

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References

[1] R.H. Blum, S.K. Carter, Adriamycin: a new anticancer drug with significant clinical activity, Ann. Intern. Med. 80 (1974) 245–259.

[2] R.S. Benjamin, A practical approach to adriamycin (NSC-123127) toxicology, Cancer Chemother. Rep. 6 (1975) 191–198.

[3] E.H. Herman, A. Rahman, V.J. Ferrans, J.A. Vick, P.S. Schein, Prevention of chronic doxorubicin cardiotoxicity in beagles by liposomal encapsulation, Cancer Res. 43 (1983) 5427–5432.

[4] A. Rahman, A. Joher, J.R. Neefe, Immunotoxicity of multiple dosing regimens of free doxorubicin and doxorubicin entrapped in cardiolipin liposomes, Br. J. Cancer 54 (1986) 401–408.
[5] M.B. Bally, R. Nayar, D. Masin, P.R. Cullis, L.D. Mayer, Studies on the myelosuppressive activity of doxorubicin entrapped in liposomes, Cancer Chemother. Pharmacol. 27 (1990) 13–19.
[6] A. Rahman, A. Kessler, N. More, B. Sikic, E. Rowden, P. Woolley, P.S. Schein, Liposomal protection of adriamycin-induced cardiotoxicity in mice, Cancer Res. 40 (1980) 1532–1537.
[7] A. Gabizon, D. Goren, Z. Fuks, A. Meshorer, Y. Barenholz, Superior therapeutic activity of liposome-associated adriamycin in a murine metastatic tumor model, Br. J. Cancer 51 (1985) 681–689.
[8] L.D. Mayer, L.C. Tai, D.S. Ko, D. Masin, R.S. Ginsberg, P.R. Cullis, M.B. Bally, Influence of vesicle size, lipid composition, and drug-to-lipid ratio on the biological activity of liposomal doxorubicin in mice, Cancer Res. 49 (1989) 5922–5930.
[9] T.D. Madden, P.R. Harrigan, L.C.L. Tai, M.B. Bally, L.D. Mayer, T.E. Redelmeier, H.C. Loughrey, C.P.S. Tilock, L.W. Reinish, P.R. Cullis, The accumulation of drugs within large unilamellar vesicles exhibiting a proton gradient: a survey, Chem. Phys. Lipids 53 (1990) 37–46.
[10] F.C. Szoka, Liposome drug delivery, in: J. Wilschut, R. Hoekston (Eds.), Membrane Fusion, Marcel Dekker, New York, 1991, pp. 845–890.
[11] J. Vaage, D. Donovan, P. Uster, P. Working, Tumour uptake of doxorubicin polyethylene glycol-coated liposomes and therapeutic effect against a xenografted human pancreatic carcinoma, Br. J. Cancer 75 (1997) 482–486.
[12] T. Daemen, J. Regts, M. Meesters, M.T.T. Kate, I.A.J.M. Bakker-Woudenberg, G.L. Scherphof, Toxicity of doxorubicin entrapped with long-circulating liposomes, J. Control. Release 44 (1997) 1–9.
[13] L.D. Mayer, M.B. Bally, P.R. Cullis, Uptake of adriamycin into large unilamellar vesicles in response to a pH gradient, Biochim. Biophys. Acta 857 (1986) 123–126.
[14] L.D. Mayer, T.D, Madden, M.B. Bally, P.R. Cullis, pH gradient-mediated drug entrapment in liposomes, in: G. Gregoriadis (Ed.), Liposome Technology, vol. II, 1993, p. 27.
[15] P.R. Harrigan, K.F. Wong, T.E. Redelmeier, J.J. Wheeler, P.R. Cullis, Accumulation of doxorubicin and other lipophilic amines into large unilamellar vesicles in response to transmembrane pH gradients, Biochim. Biophys. Acta 1149 (1993) 329–338.
[16] M.S. Webb, T.O. Harasym, D. Masin, M.B. Bally, L.D. Mayer, Sphingomyelin-cholesterol liposomes significantly enhance the pharmacokinetics and therapeutic properties of vincristine in murine and human tumour models, Br. J. Cancer 72 (1995) 896–904.
[17] C.H. Fiske, Y. Subbarow, The colorimetric determination of phosphorus, J. Biol. Chem. 66 (1925) 375–400.
[18] D. Marsh, Handbook of Lipid Bilayers, CRC Press, Boca Raton, FL, 1990.
[19] F.A. De Wolf, R.W.H.M. Staffhorst, H. Smits, M.F. Onwezen, B. de Kruijff, Role of anionic phospholipids in the interaction of doxorubicin and plasma membrane vesicles: drug binding and structure consequences in bacterial systems, Biochemistry 32 (1993) 6688–6695.
[20] H. Rottenberg, The measurement of membrane potential and delta pH in cells, organelles, and vesicles, Methods Enzymol. 55 (1979) 547–569.
[21] M.J. Hope, M.B. Bally, G. Webb, P.R. Cullis, Production of large unilamellar vesicles by a rapid extrusion procedure. Characterization of size distribution, trapped volume and ability to maintain a membrane potential, Biochim. Biophys. Acta 812 (1985) 55–65.
[22] J.A. Veiro, P.R. Cullis, A novel method for the efficient entrapment of calcium in large unilamellar phospholipid vesicles, Biochim. Biophys. Acta 1025 (1990) 109–115.
[23] P.R. Harrigan, M.J. Hope, T.E. Redelmeier, P.R. Cullis, Determination of transmembrane pH gradients and membrane potentials in liposomes, Biophys. J. 63 (1992) 1336–1345.
[24] P.R. Cullis, M.J. Hope, M.B. Bally, T.D. Madden, L.D. Mayer, D.B. Fenske, Influence of pH gradients on the transbilayer transport of drugs, lipids, peptides and metal ions into large unilamellar vesicles, Biochim. Biophys. Acta 1331 (1997) 187–211.
[25] J. Bouma, J.H. Beijnen, A. Bult, W.J.M. Underberg, Anthracycline antitumour agents: A review of physicochemical, analytical and stability properties, Pharm. Weekbl. Sci. Edn. 8 (1986) 109–133.
[26] F.T. Greenaway, J.C. Dabrowiak, The binding of copper ions to daunomycin and adriamycin, J. Inorg. Biochem. 16 (1982) 91–107.
[27] J.M. Leenhouts, B. Cheung, T. Sun, D.B. Fenske, P.R. Cullis, New procedures for the loading of doxorubicin into liposomes, FASEB J. 11 (1997) A1430.
[28] C.M. Riley, D.L. Ross, D. Vander Velde, F. Takusagawa, Characterization of the complexation of fluoroquinolone antimicrobials with metal ions by nuclear magnetic resonance spectroscopy, J. Pharm. Biomed. Anal. 11 (1993) 49–59.
[29] N.R. Bachur, S.L. Gordon, M.V. Gee, Anthracycline antibiotic augmentation of microsomal electron transport and free radical formation, Mol. Pharmacol. 13 (1977) 901–910.
[30] J.M.C. Gutteridge, Lipid peroxidation and possible hydroxyl radical formation stimulated by the self-reduction of doxorubicin-iron (III) complex, Biochem. Pharmacol. 33 (1984) 1725–1728.
[31] J.W. Lown, H. Chen, J.A. Plambeck, Further studies on the generation of reactive oxygen species from activated anthracyclines and the relationship to cytotoxic action and cardiotoxic effects, Biochem. Pharmacol. 31 (1982) 575–581.
[32] J.R.F. Muindi, B.K. Sinha, L. Gianni, C.E. Myers, Hydroxyl radical production and DNA damage induced by anthracycline-iron complex, FEBS Lett. 172 (1984) 226–230.

[33] B.B. Hasinoff, Self-reduction of the iron(III)-doxorubicin complex, Free Radic. Biol. Med. 7 (1989) 583–593.
[34] B.B. Hasinoff, J.P. Davey, P.J. O'Brien, The adriamycin (doxorubicin)-induced inactivation of cytochrome *c* oxidase depends on the presence of iron or copper, Xenobiotica 19 (1989) 231–241.
[35] D.B. Fenske, K.F. Wong, E. Maurer, N. Maurer, J.M. Leenhouts, N. Boman, L. Amankwa, P.R. Cullis, Ionophore-mediated uptake of ciprofloxacin and vincristine into large unilamellar vesicles exhibiting transmembrane ion gradients, Biochim. Biophys. Acta 1414 (1998) 188–204.