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Gelation of liposome interior

A novel method for drug encapsulation

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Liposomes can be loaded with weak acids and bases, which exist in solutions in equilibrium with membrane permeable uncharged form, using various gradients across their membranes. Because in some cases the estimated drug concentration in the loaded liposomes exceeds their aqueous solubility we investigated the physical state of the liposome encapsulated anticancer drug Doxorubicin. X-Ray diffraction, electron microscopy, and test tube solubility experiments have shown that upon encapsulation the drug molecules form a gel-like phase

Liposome; Drug loading; $(NH_4)_2SO_4$ gradient; Doxorubicin; Gel

1. INTRODUCTION

Several reports have shown that weak acids and bases can be loaded into preformed liposomes by the application of ionic gradients across the liposome bilayer [1–4]. For example, high concentrations of particular molecules can be accumulated into liposomes upon their addition into the external medium in the presence of a pH gradient between the inside and outside of the liposome [1–3]. Similar distributions of various fluorescent probes have been used to measure the internal pH of various cells and organelles and results were explained in terms of the Henderson–Hasselbach equation [1–3,5,6].

These same methods can be applied also for the loading of drugs into liposomes. For instance, doxorubicin, an anticancer drug, which is a weak amphiphatic base (pK_a = 8.25), has been loaded into liposomes where the pH of the inside was 4 and the pH of the outside solution was 7 [3]. Unfortunately the gradients are not very stable and the drug molecules leak out, with half-lives ranging from minutes to hours, depending on the bilayer composition. Moreover, lowering the outside pH results in re-equilibration and further drug leakage [3].

In order to overcome these shortcomings, we have used a method of ammonium sulfate gradient loading. With this method, the drug is not released after liposomes are loaded and its encapsulation is quite stable [7]. In fact, the drug remains encapsulated even after relaxation of the ammonium sulfate gradient and its release is not sensitive to the external pH. The internal drug concentration, estimated from measurements of liposome internal volume, exceeded the solubility of the drug severalfold. We attributed this phenomenon, by analogy with the concept of chemical potential gradient loading with a molecular sink on one side of the semipermeable membrane (so-called 'black-hole' loading), to the formation of a (semi)solid phase in the liposome interior.

In this paper, we further investigate the reasons for the unusually efficient loading of doxorubicin into liposomes with a salt gradient across the bilayer membrane. In particular, we use cryo-electron microscopy and X-ray diffraction to study the physical phase of doxorubicin in the interior of vesicles loaded by ammonium sulfate gradients.

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2. MATERIALS AND METHODS

Small unilamellar vesicles (SUV), with homogeneous size distribution around 85 nm, were prepared by microfluidization (5 passes at 1,000 atm and 50°C) or by extrusion [4] of a dispersion of multilamellar vesicles (MLV, composition: hydrogenated soy lecithin/cholesterol/PEG–lipid in a mol ratio 57:38:5) in 155 mM ammonium sulfate, pH 5.5. The PEG–lipid consists of polyethylene glycol (M_w = 2,000 Da) covalently attached to distearoyl phosphatidylethanolamine. We have chosen a lipid composition containing polymer-bearing lipid to increase liposome stability against aggregation [8,9] which is often a problem in work with uncharged lipid bilayers. This, the so-called steric stabilization, as opposed to charge stabilization, was extensively described elsewhere [9,10]. Because the ammonium sulfate gradient loading can operate at nominally the same pH inside and outside of liposomes, the external medium was exchanged for isotonic sucrose (pH 5.5) and liposomes were mixed with drug solution: doxorubicin-

HCl at 6 mg/ml in 10% sucrose at pH = 5.5. After 1 h incubation at 60°C the samples were cooled and assayed for encapsulation efficiency. Typically, loading efficiencies of around 95%, as measured by gel chromatography (desalting Pierce Excellulose GF5 columns, spectrophotometric detection of doxorubicin), were achieved and drug concentration was 3 mg/ml at a lipid concentration of 30 mM.

The loading efficiency did not depend on the presence of the PEG–lipid, negative charge, or the acyl chains composition of the lecithin. The apparent concentration of the doxorubicin in the liposome aqueous phase was almost independent of liposome size and lamellarity. The drug-to-lipid ratio, however, was dependent on liposome size and lamellarity (data not shown).

For cryo-electron microscopy, thin films were prepared from the suspensions by dipping a bare 700 mesh hexagonal grid in the suspension and blotting with filter paper after withdrawal, and the thin films were vitrified by plunging into melting ethane [11]. The vitrified thin films were stored under liquid nitrogen and observed in a Gatan 626 cryo-holder at −170°C with a Philips CM12 microscope. Micrographs were taken at 120 kV under low-dose conditions.

For X-ray diffraction experiments, empty and loaded liposomes were pelleted (230,000 × *g*, 10 h) and sealed in thin walled glass capillary tubes. Specimens were mounted in a point collimation X-ray camera and diffraction patterns were recorded on Kodak DEF-5 X-ray film in a flat plate film cassette as described previously [12,13]. Liposome pellets could be redispersed with no change in particle size, as measured by dynamic light scattering (Coulter N 4SD) or loss of drug encapsulation. X-ray patterns were also recorded from doxorubicin-HCl powder and mixtures of doxorubicin at 30 mg/ml with appropriate salts at concentrations in the range 0.1–0.5 M.

3. RESULTS AND DISCUSSION

Several doxorubicin–salt mixtures were prepared in a test tube and studied visually and in an optical microscope.

Flocculation occurred immediately upon mixing a doxorubicin solution (30 mg/ml in 10% sucrose) with ammonium sulfate crystals. Moreover, after heating to 60°C to liquify the system, a homogeneous gel formed upon cooling. Because this phase was optically isotropic, quite viscous, and exhibited thixotropic behavior, we refer to this semi solid phase as a 'gel'. At a salt concentration of 0.155 M the ammonium sulfate gel melted at 55°C. An observed hysteresis of 7–8°C, at cooling rate 10°C/min, indicates that this is a strong first-order phase transition. Similar behavior was observed also when Na_2SO_4 was used. As the ammonium sulfate concentration was increased from 0.1 to 0.5 M, the phase transition temperature linearly increased from 54 to 60°C.

At room temperature and pH 5.5 the maximum amount of doxorubicin–HCl that could be dissolved in sucrose solution was about 35 mg/ml. However, from liposome internal volume measurements and from the encapsulation efficiency we estimated that the doxorubicin concentration in liposomes was over 100 mg/ml and this probably indicates the presence of a phase change. Therefore X-ray and EM experiments were performed to determine the structure of the doxorubicin trapped in the liposomes.

Cryo-electron microscopy [11] of empty liposomes showed round structures with pale membranes, typical of SUV's (Fig. 1). In contrast SUV's containing doxorubicin were ellipsoidal and contained dark stripes, usually aligned with the major semiaxis of the ellipse (Fig. 2). These core structures were mostly present as a single rod, giving the appearance of a coffee bean. Occasionally two or more core structures were observed in a single liposome or alternatively 'V'- and 'Y'-shaped cores were observed. The core structure seemed to be composed of longitudinal rods or sheets (periodicity of the order of 27 Å) but this can only be observed in a few areas where parts of a core have a favourable orientation to the electron beam.

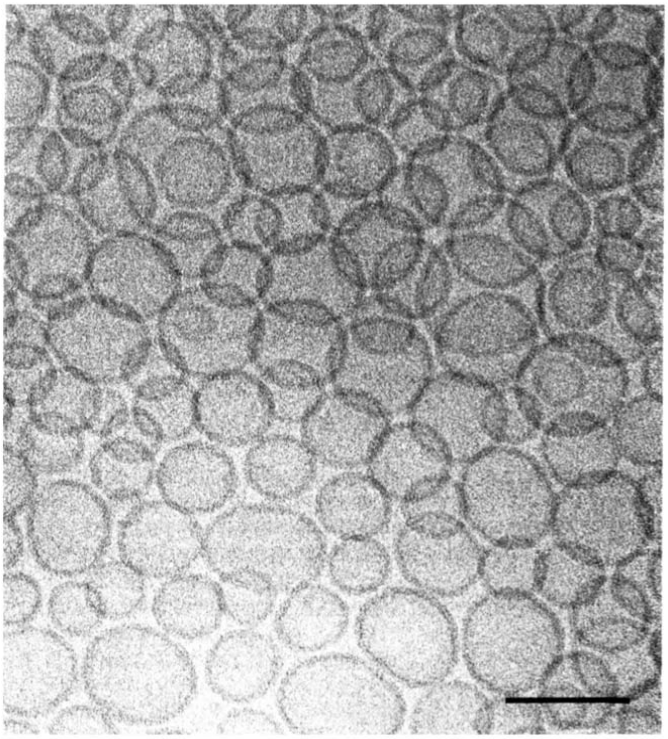

Fig. 1. Cryo-electron micrograph of extruded vesicles without loaded drug. Vesicles vitrified in a thin film can be observed superimposed due to the 'through vision' of the method. The lipid bilayer can be resolved (arrowheads) using a defocus of 1.5 μm. Bar = 100 nm.

X-ray diffraction patterns were recorded from several forms of doxorubicin: dry powder, in sucrose solutions with and without ammonium sulfate and sodium sulfate, and loaded and empty liposomes. X-ray patterns from the dry doxorubicin powder contained a series of sharp diffraction rings. The spacings of the darkest of these rings were: 20.0, 10.1, 8.5, 7.6, 6.6, 5.9, 5.3, 4.8, 4.5, and 4.3 Å.

Diffraction patterns from SUV's containing doxorubicin, loaded by the ammonium sulfate gradient method, contained a single sharp reflection at 27 Å, similar to the periodicity observed by EM for the core structure within the drug loaded liposomes (Table I). In control experiments, diffraction patterns from SUV's made in the absence of doxorubicin gave 3 weak reflections at 83, 55, and 41.5 Å. These reflections index as

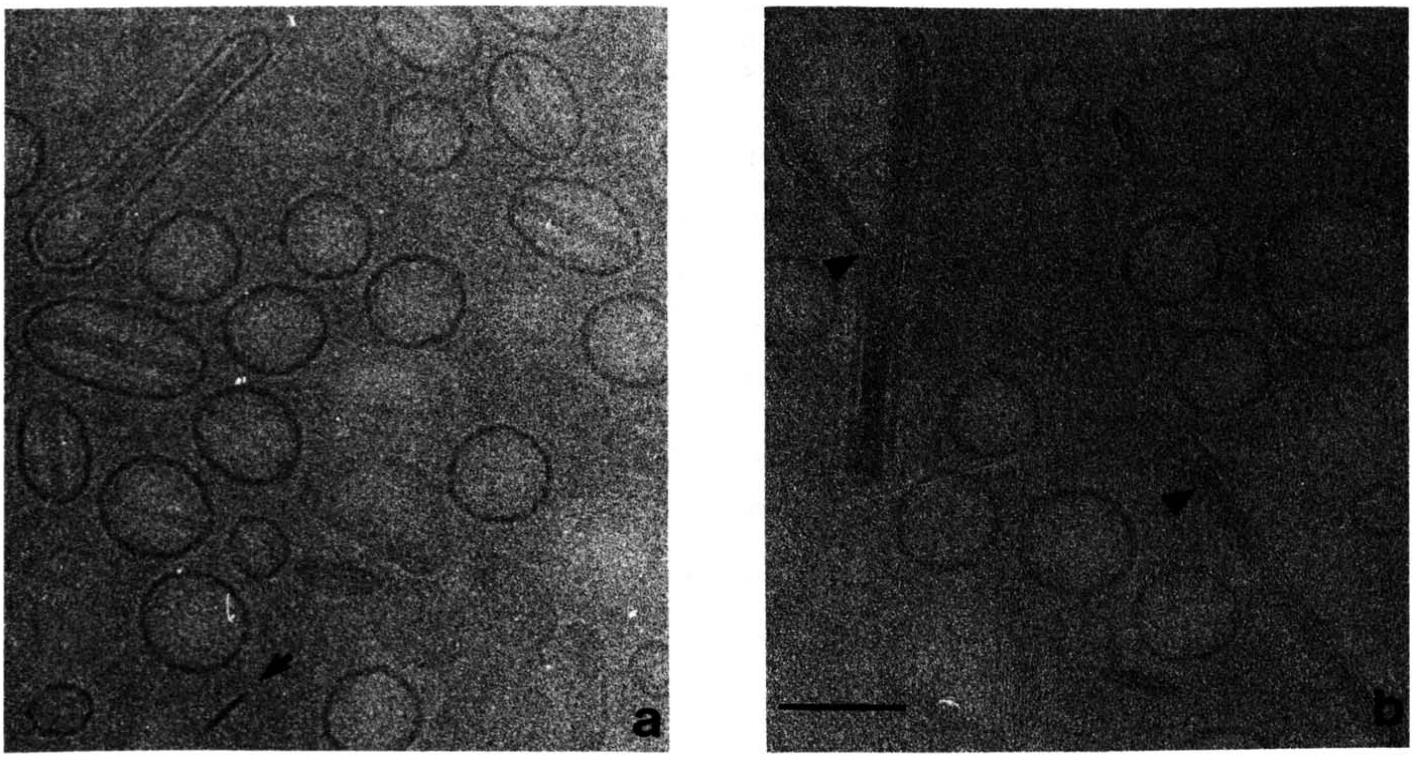

Fig. 2. Cryo-electron microscopy of drug-loaded vesicles. Vesicles with a core can be observed as well as 'empty' vesicles (a). In larger core structures (b) cross-striations are visible with a periodicity of about 26 Å. These liposomes were prepared by microfluidization which causes the formation of a small fraction of disc-like micelles which can be seen as dark rods (Frederik and Lasic, in preparation). Bar = 100 nm.

orders 2, 3, and 4 of a lamellar repeat period of about 166 Å. This lamellar repeat period was similar to that recorded from fully hydrated MLV of stearoyloleyl phosphatidylcholine/cholesterol (2:1) containing 4 mol% PEG 2000 lipid [9] and thus undoubtedly arose from a small amount of MLVs in the sample.

Diffraction patterns of 30 mg/ml doxorubicin in 10% sucrose with either 250 mM Na_2SO_4 or $(NH_4)_2SO_4$ gave a single sharp reflection at 27 Å. In contrast, 30 mg/ml solution of doxorubicin in 10% sucrose with no added salt gave no discrete reflections.

All of these samples were optically isotropic and no birefringence was observed. Calculations of shape changes at constant surface area and taking into account heterogeneity of the vesicles indicate that the ellipsoidal structures are prolate and not oblate ellipsoids. This implies that the gel particles are one-dimensional rods rather than two-dimensional sheets, in agreement with EM observations.

Table I

Small angle X-ray reflections from different samples

Sample	Reflections (Å)
Doxorubicin-HCl	20.0, 10.1, 8.5, 7.6, 6.6
Crystalline	5.9, 5.3, 4.8, 4.5, 4.3
Empty liposomes	83, 55, 41.5
Loaded liposomes	27
3% Dox solution	none
3% Dox-$(NH_4)_2SO_4$	27
3% Dox-Na_2SO_4	27

The doxorubicin aggregation is also supported by complete quenching of its fluorescence and the increase in the 550/470 nm absorbance ratio in the electron absorption spectra [14]. This ratio increased from 0.19 in the solution of μM doxorubicin in 0.15 M NaCl to 0.53 in the liposome suspension. Both changes are related to the interactions of π electrons of the planar aromatic anthracycline rings which overlap when stacked in the aggregated phase [14].

The EM images indicate that the ammonium sulfate gradient procedure loads doxorubicin into SUV's, producing an electron-opaque band within the liposome. The X-ray diffraction experiments indicate that the contents of the liposome have a similar structure to that of 'gels' obtained by the addition of ammonium sulfate or sodium sulfate to doxorubicin solution. That is, our results provide direct evidence that the doxorubicin is incorporated into liposomes in a gel-like phase, and is not adsorbed into/onto the bilayer. The stability of these doxorubicin-containing SUV's was demonstrated by their ability to withstand very large centrifugal forces without changing their average size. This result is consistent with previous observations of 'gelosomes', liposomes with a gelatinized interior [15], as well as with theoretical predictions [16].

In conclusion, we have shown that the high encapsu-

lation efficiency of doxorubicin into liposomes, obtained by the ammonium sulfate gradient loading method, is due to the formation of a gel-like precipitate in the liposome interior.

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