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Physical biochemistry of a liposomal amphotericin B mixture used for patient treatment

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There seems little doubt now that intravenous liposomal amphotericin B can be a useful treatment modality for the management of immunocompromised patients with suspected or proven disseminated fungal infections. Interestingly, the very significant reduction in toxicity reported when amphotericin B is part of a bilayer membrane is closely tied to the physical characteristics of the liposomes involved, although these are poorly understood at the molecular level. We record here an examination by spectroscopy and freeze-etch electron microscopy of unsonicated amphotericin B multilamellar vesicles prepared along the lines that we and others have followed for samples used in clinical trials and preclinical in vivo or in vitro studies. Our study has focussed on liposomes of 7:3 dimyristoylphosphatidylcholine/dimyristoylphosphatidylglycerol (DMPC/DMPG) bearing 0–25 mol% amphotericin B, since this lipid mixture has been the choice for the first clinical trials. Phase transition behaviour of these liposomes was examined by electron paramagnetic resonance (EPR) spectroscopy of a nitroxide spin label partitioning into the bilayers. The same experiments were then performed on similarly prepared liposomes of the disaturated species, dipalmitoylphosphatidylcholine (DPPC), and the diunsaturated species, dielaidoylphosphatidylcholine (DEPC). Partial phase diagrams were constructed for each of the lipid/drug mixtures. Melting curves and derived phase diagrams showed evidence that amphotericin B is relatively immiscible with the solid phase of bilayer membranes. The phase diagram for DEPC/amphotericin B was very similar to that of DPPC/amphotericin B, and both exhibited less extensive temperature ranges of phase separation than did the 7:3 DMPC/DMPG mixture with amphotericin B. Between 25 and 37°C the measured fluidity of the 7:3 DMPC/DMPG liposomes was similar to that of the (unsaturated fatty acid) DEPC liposomes, and considerably higher than that seen for (saturated fatty acid) DPPC liposomes. Preparations of 7:3 DMPC/DMPG, DPPC, and DEPC containing 0–25 mol% amphotericin B were examined by freeze-etch electron microscopy at 35 and 22°C (to cover the temperature range of the mammalian body core and periphery). The same liposome features were present in all three liposome types studied. The appearance of individual liposomes at × 100 000 magnification reflected their molecular characteristics, which were found to be significantly heterogeneous within each batch. The lipid/drug structures were bilayer in nature, although liposomes showing considerable disruption were common, particularly at the highest drug concentrations. With increasing content of amphotericin B, liposome appearance became progressively more characteristic of disruption: discontinuous fracture faces, irregular liposome shapes, and loss of $P_{\beta'}$ crystal features. At very high drug concentrations (beyond those systematically studied here) liposome structure broke down. Dispersion of the drug into the bilayer seems to be less extensive than has recently been reported with 7:3 DMPC/DMPG preparations that have been sonicated (Janoff, A.S. et al. (1988) Proc. Natl. Acad. Sci. USA 85, 6122–6126).

Introduction

Although very much at the investigational stage, patient treatment programs involving liposomal drugs have begun to appear in a few research hospitals. A noteworthy example involves amphotericin B – the (intravenous) drug of choice for disseminated fungal infec-

Abbreviations: EPR, electron paramagnetic resonance; DMPC, dimyristoylphosphatidylcholine; DMPG, dimyristoylphosphatidylglycerol; DEPC, dielaidoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; TEMPO, 2,2,6,6-tetramethylpiperidine-*N*-oxyl; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

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tions in immunocompromised patients (Ref. 1 and references therein). Like other clinical applications of liposomes, it takes advantage of their ability to alter the kinetics and bioavailability of associated drugs. Amphotericin B by itself is insoluble in water. Hence the conventional (non-liposomal) commercial preparation, Fungizone®, relies upon 1 : 1 combination with the bile salt detergent, deoxycholate, to produce an injectable solution. The major problem with Fungizone® is toxicity – sufficient quite commonly to limit the dose to a range which is clinically inadequate [2,3]. In addition, the presence of deoxycholate makes the preparation phlebitic and results in difficulties in administration.

When dissolved in organic solvent with phospholipids, and then dried and hydrated, amphotericin B associates strongly with the lipid structures formed. It was noted some years ago that liposomes bearing amphotericin B had relatively little effect upon eucaryotic cells in culture, while retaining the toxicity of free drug and Fungizone® toward fungi, and subsequently a similar phenomenon was recorded in vivo [4–6]. The generally proposed mode of fungal organism killing by amphotericin B involves membrane destabilization as a result of a fairly specific interaction with the steroid, ergosterol (Ref. 7 and reviewed in Ref. 8). Amphotericin B can also induce leakiness in eucaryotic cell plasma membranes via a lesser interaction with the sterol, cholesterol. Although the mechanism of toxicity reduction to eucaryotes by incorporation of amphotericin B into liposomes remains incompletely understood [1,4,5,9–12], workers have experimented with various lipid mixtures in a successful search for preparations that reduce host toxicity while maintaining anti-fungal potency (Refs. 10, 13 and references therein). A very interesting recent study of an extensively sonicated preparation by Janoff et al. has shown a lower toxicity of very distorted structures bearing relatively higher proportions of the drug [12].

The source of lipid compositional effects on lethality of liposomal amphotericin B to fungal organisms vs. eucaryotic cells has attracted considerable interest, since specificity is the feature sought in this application. Presumably, the drug has to get from the liposome into the cell to cause damage. This may occur via one or more mechanisms including diffusion through the medium, collisional transfer between liposome and cell, and liposome association with or uptake by the cell. It is likely in each case that one would expect the rate of drug transfer to reflect liposome characteristics. However, liposome characteristics in general are sensitive to choice of lipid and method of preparation. Moreover, they vary with temperature and can be heterogeneous within a given batch. Further complicating the situation in the case of amphotericin B is the fact that the arrangement of amphotericin B in liposomes is unknown, although there is evidence that it and the related polyene antibiotic, nystatin, do not simply mix uniformly with phospholipids in a membrane [7,8,14–18]. In this article we investigate these questions with regard to preparations used by a variety of groups for in vitro or in vivo experimentation and clinical trials.

Juliano and Lopez-Berestein chose 7 : 3 (mole ratio) DMPC/DMPG containing 5 mol% amphotericin B when they introduced the liposomal drug for clinical trials [9,11]. Both of these phospholipids have only 14-carbon fully saturated fatty acids. Each one in pure bilayer form undergoes a phase transition at 23° $C_t$and they have the interesting property of mixing homogeneously to form bilayers with the same sharp melting temperature [19,20]. We were curious whether the physical characteristics of this lipid combination were special with regard to amphotericin B.

We describe here the results of thermodynamic and freeze-etch electron microscopy studies to characterize hand-shaken (i.e. unsonicated) amphotericin B liposomes over the temperature range of in vitro and in vivo assays. We have experimented with several different phospholipids and with drug concentrations up to 25 mol% in an attempt to better understand the influence of these parameters. One should note that while current clinical use has been with 5 mol% drug [11,21], liposomes with higher amphotericin B content are likely to see clinical trial soon. Some questions asked are as basic as: Is the drug mixed into the bilayer? Does the free drug coexist with liposomes? Do bilayer structures exist at high drug concentrations? The data permit these and more subtle questions to be answered. Previous reports from our lab and that of R. Juliano have described the physical nature of the amphotericin B liposomes with up to 5 mol% drug [13,22]. A ^{2}H-NMR report described the interaction of amphotericin B at 30 mol% with DMPC. Recently, Janoff et al. have recorded studies by physical techniques of extensively sonicated 7 : 3 DMPC/DMPG bilayers containing amphotericin B [12].

Materials and Methods

Pure amphotericin B for non-clinical work was obtained from Squibb Pharmaceuticals (Canada). Fungizone® (a dry mixture of deoxycholate and amphotericin B marketed by Squibb Pharmaceuticals for patient treatment) was obtained through our University Hospital pharmacy. The phospholipids L-α-dimyristoylphosphatidylcholine (DMPC), L-α-dimyristoylphosphatidylglycerol (DMPG), L-α-dipalmitoylphosphatidylcholine (DPPC) and L-α-dielaidoylphosphatidylcholine (DEPC) were purchased from Avanti Polar Lipids, Birmingham AL. Organic solvents were reagent grade. The spin label, TEMPO, was from Aldrich, Milwaukee, WI. Sucrose (Grade 1) was from Sigma, St. Louis, MO.

Liposome preparation

Preparation of liposomes by what we refer to as the

'standard' method involved dissolving pure amphotericin B in CH_3OH at 0.5 mg/ml to produce a slightly cloudy yellow solution. This was filtered through a tight glass wool plug to give a clear solution. Appropriate quantities of lipids were dissolved in $CHCl_3$ at 10–25 mg/ml and combined with the methanol solution of amphotericin B. The solution was taken to dryness in a glass vessel, pumped on in vacuum for 1–2 h to ensure removal of solvent traces, and hydrated with phosphate-buffered saline (some samples were hydrated with Hepes-buffered saline containing EDTA) using manual agitation at a temperature 10 C° higher than the lipid phase transition. Amphotericin B concentrations were quantitated by absorbance vs. standards in CH_3OH at 405 nm [23]. Drying of lipid/drug solutions from organic solvent was done in several ways with the same results: either solvent was removed by a stream of N_2 gas at room temperature using a test tube as a vessel, or it was removed at 40 °C under vacuum in a round-bottom flask on a rotary evaporator. This basic approach is by far the most common method of producing drug liposomes.

Preparation of liposomes by the dialysis method (i.e., our variant of the 'standard' method necessitated by unavailability of pure amphotericin B for patient treatment) involved dissolving Fungizone® at 1 mg/ml in CH_3OH to produce a slightly cloudy yellow solution. This was filtered through a glass wool plug to give a clear solution. The rest of the procedure followed the 'standard' protocol as described above, except that hydration was with distilled water (1 ml per 50 mg of lipid). In each case after hydration of the dry lipid/drug films, the lipid mixtures were warmed at least 10 C° above the lipid phase transition for 15 min to permit diffusional equilibrium. The samples prepared by our technique were subsequently dialysed exhaustively against phosphate buffer pH 7.8–8.0 to remove deoxycholate (nine changes of 20-fold excess buffer at 18–24 h intervals), and then against phosphate-buffered saline (pH 7.4) (one change 20-fold excess). Final deoxycholate content as determined by thin-layer chromatography (silica gel plates eluted with 15% methanol in chloroform) was less than 0.7 mol%. All samples bearing amphotericin B were protected from light exposure during preparation and storage.

Phase transition studies

EPR spectroscopy was used to measure the effect of incorporated amphotericin B upon its lipid host-matrix. Liposome suspensions in saline were mixed with spin label (TEMPO) at a lipid-to-spin-label molar ratio of 125 : 1 (spin label added from a $7 \cdot 10^{-3}$ M aqueous stock solution). These were sealed in Corning 50 µl microsampling pipettes and held in the Dewar insert of a Varian E12 EPR spectrometer equipped with TM_{110} cavity using an insert described by Gaffney and McNamee [24]. Sample temperature was monitored with a copper/constantan thermocouple in the Dewar insert. Data treated as was recommended by Shimshick and McConnell [25].

Electron microscopy

Samples of liposomes to be studied by freeze-etch electron microscopy were handled in normal saline. They were rapidly quenched from 22 or 35 °C by plunging suspension droplets on gold discs into liquid freon cooled in liquid nitrogen. Selected samples were also prepared by the slam freezing process using a Heusertype Cryopress (Med-Vac Inc., St. Louis, MO) to achieve more rapid freezing rates. Such specimens were processed in a room equilibrated at the temperature of interest (22 or 35 °C) and frozen on the aluminum diskettes with fixed rat lung overlay as recommended by the designers. The frozen specimens were fractured at −105 °C in a Balzers BAF 300 high-vacuum coating unit equipped with electron-beam guns. After the fracture step samples were 'etched' for 2 min to expose small areas of liposome outer surface prior to platinum shadowing. Replicas were cleaned initially in bleach, rinsed with distilled water, and picked up from 1 : 1 acetone/ethanol. Replicas were examined using a Phillips 300 electron microscope.

Results and Discussion

Liposome phase behaviour – implications for drug arrangement

The response of any compound to temperature variation reflects its molecular arrangement. This is particularly true when the temperature region scanned encompasses a change of phase. Bilayer membranes are crystal or liquid crystal in nature, and may undergo abrupt 'phase transitions' if they possess a high proportion of a single phospholipid type. The passage of a lipid bilayer through its phase transition is analogous to the melting of a pure solid: the change being from a rigid, crystalline lattice to a fluid, liquid crystal lattice, in which individual lipids are laterally mobile while retaining basic bilayer format. The phase transition temperatures for DEPC, DPPC, DMPC, DMPG and 7 : 3 DMPC/DMPG are generally recorded to be 12, 41.5, 23, 23 and 23 °C, respectively [19,20,25]. The presence of an impurity (amphotericin B in this case) alters host-matrix melting behaviour in ways that depend upon impurity arrangement. The method which we have used to monitor liposome thermal behaviour involves adding to samples trace quantities of the small spin label, TEMPO, that partitions reversibly between bilayer membrane and surrounding buffer [24,25]. The fraction of TEMPO spin label dissolved in membrane vs. aqueous phase can be measured directly from the EPR spectrum, which shows peaks assignable to each label pool. However, the solubility of TEMPO in the membrane is critically

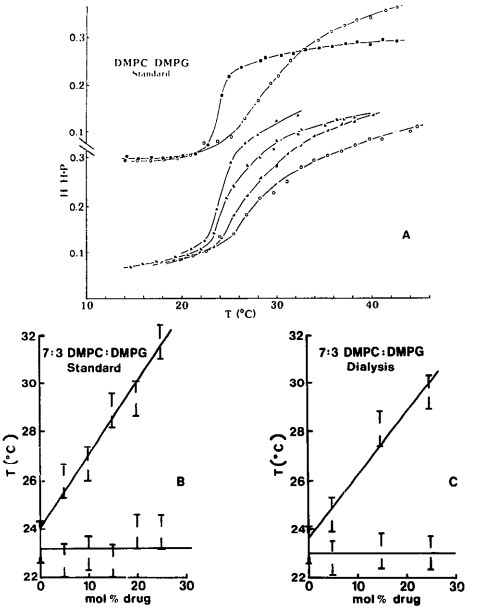

Fig. 1. (A) Membrane melting profiles for batches of amphotericin B liposomes. Data derived from EPR spectra of TEMPO spin label partitioning between buffer and membrane (the spectral parameter, $H/H+P$, represents the ratio of spin label dissolved in membrane to total spin label [22] and is directly related to membrane fluidity [21]). Curves shown are for the following drug concentrations: 0 (●), 5 (▲), 10 (◣), 20 (◢) and 25% (○) amphotericin B in 7:3 DMPC/DMPG liposomes prepared by the 'standard' protocol (see Materials and Methods). Samples were suspended in phosphate-buffered saline (pH 7.4) and scanned from low to high temperature at 0.4 C°/min. Each sample contained 1.6 mg lipid. (B) Partial phase diagram for amphotericin B in 7:3 DMPC/DMPG liposomes prepared by the 'standard' protocol (based on composite melting curve data as in (A) above using two to four experiments per point). Data are shown for liposome batches with up to 25 mol% amphotericin B. The lower curve (solidus) denotes the upper boundary of the solid phase. The upper curve (fluidus) denotes the lower boundary of the fluid phase. (C) Partial phase diagram as in (B) above, but for liposomes prepared via our modification of the 'standard' protocol (involving a dialysis step – see Materials and Methods).

dependent upon lipid fluidity, so that the spectral peak for TEMPO in the membrane shrinks and grows dramatically when the liposomes are being cooled or warmed respectively. The relative heights of the peaks for TEMPO in lipid and buffer can also provide a quantitative assessment of membrane fluidity [24].

TEMPO-derived melting curves typical of batches of liposomes prepared from 7:3 DMPC/DMPG using the 'standard' protocol with various lipid/amphotericin B ratios are illustrated in Fig. 1A. Note that as the temperature increases, the spectral parameter $H/H+P$ increases slowly, and then shows an abrupt elevation over the temperature region corresponding to the phase transition of the mixture before resuming its slow increase. The low-temperature onset of the abrupt rise is interpreted as the point at which fluid domains begin to appear in the membrane, while the point at which the curve plateaus indicates completion of the melting process. The curve features contain information relating to molecular arrangement. For example, the presence of an 'impurity' such as amphotericin B in an otherwise pure crystalline phospholipid membrane tends to shift the

onset and completion of melting farther apart along the temperature axis (i.e., to broaden the region of melting). This may often be the only effect of a small amount of drug in a bilayer. There may also be a slight drop in 'melting point' due to minor crystal lattice disruption. Failure of an 'impurity' to disperse amongst the molecules of the bilayer host matrix results in a lesser effect upon host melting behaviour in the temperature/composition region involved. Making the approximation of equilibrium among compositional domains [18], we have plotted the onset and completion temperatures for each ratio of drug to lipid, to arrive at partial 'phase diagrams' whose characteristics reflect the relative arrangement of the molecules. Fig. 1B shows the result of such a treatment of the data for the 7:3 mixture above. It shows features of solid phase immiscibility [26] over the temperature range studied. That is, the relative constancy of melting onset (solidus, lower curve) would be conventionally taken to indicate that amphotericin B does not mix uniformly into rigid phospholipid and leaves large amounts of DMPC/DMPG with little drug. The physical size and form of the phospholipid and amphotericin B domains involved cannot be known from such thermodynamic data, although domains smaller than some 30–60 lipid molecules would not show the cooperative melting behaviour seen here [26,27]. Within experimental error, the phase diagram generated using 7:3 DMPC/DMPG liposomes prepared according to our dialysis variant of the 'standard' protocol (Fig. 1C) is the same.

The 7:3 mixture of DMPC/DMPG referred to above comprises species with saturated fatty acids only. It has been observed that in general, saturated phospholipids form liposomes that are relatively protective with regard to amphotericin B toxicity (e.g., Refs. 10 and 13). Phospholipids with unsaturated fatty acids, on the other hand, are consistently reported to be relatively unprotective. We therefore performed identical studies on liposomal amphotericin B made using the saturated phospholipid, DPPC, or the diunsaturated phospholipid, DEPC, both of which have phase transition temperatures above 0°C permitting direct comparison. Partial phase diagrams derived from these experiments are shown in Fig. 2. The features seen are qualitatively similar to those already described in Fig. 1 for the phospholipids used clinically. In particular, the solidus is horizontal in each case, indicating relative immiscibility of amphotericin B with rigid phospholipid in this concentration range. Thus, once again, the drug does not seem to disperse uniformly into a rigid bilayer host matrix, but rather separates out, probably as a coexisting phase relatively enriched in amphotericin B. The temperature of completion of melting can be seen to increase measurably with amphotericin B content, demarcating melting of a phase relatively enriched in drug that melts at higher temperature. Note though that the

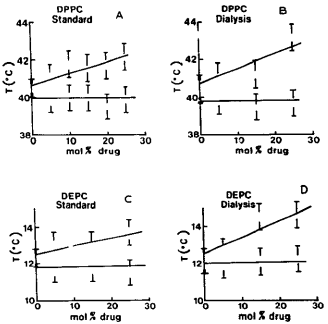

Fig. 2. Partial phase diagrams for amphotericin B in liposomes of a pure 16-carbon chain saturated phospholipid (DPPC) (A) and (B) and in liposomes of a pure 18-carbon chain unsaturated phospholipid (DEPC) (C) and (D). (A) and (C) are for liposomes prepared via the 'standard' protocol and (B) and (D) for liposomes from the dialysis protocol. Data have been recorded up to 25 mol% amphotericin B. Details in caption to Fig. 1.

quantitative appearance of the phase diagram for (protective) DPPC is more similar to that of DEPC than to that of the (protective) 7:3 DMPC/DMPG mixture. In vitro and in vivo assays of liposomal amphotericin B toxicity recorded in the literature are carried out between 20 and 37°C, which is above the transition temperature of unsaturated species such as DEPC. It will be seen from Fig. 1A that the *y*-axes of the melting curves provide a quantitative value for membrane fluidity as described by Gaffney and McNamee [24]. In our hands, the fluidity so measured for 7:3 DMPC/DMPG amphotericin B liposomes above 25°C ranged from 0.25–0.4, compared to 0.3–0.5 for DEPC and 0.02–0.05 for DPPC in comparable sample sizes over the same temperature range. Thus our phase studies provide no evidence of an intrinsic molecular difference in behaviour between amphotericin B in saturated vs. unsaturated host matrices and they also suggest that fluidity alone may not explain the protective nature of the host bilayer. One should caution though that DEPC has only *trans* double bonds vs. the *cis* double bonds and lower phase transition temperatures of most unsaturated species studied with amphotericin B.

Visualization of liposomal preparations to 2 nm resolution

Direct visualization of the systems studied here provides some important information for correlation with the temperature profiles and phase diagrams. The tech-

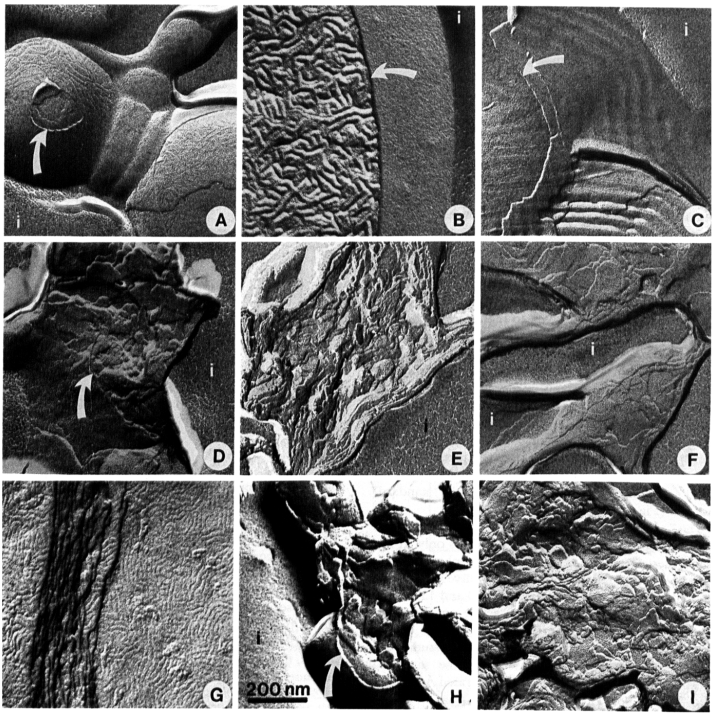

Fig. 3. Freeze-etch electron micrographs of liposomes representing features seen at 35° C in the preparations described in the text. The first row shows the bilayer membrane as it appears for lipid alone: (A) DEPC, (B) 7:3 DMPC/DMPG, (C) DPPC. Such liposomes were fairly homogeneously represented throughout batches without drug. Note the disordered ripple pattern on the fracture faces of fluid liposomes (A, B), while DPPC was below its phase transition temperature at 35° C and shows the relatively ordered ripples of the $P_{\beta'}$ phase (C). Arrows point across the etch face (liposome outer surface) to abut on the etch face/fracture face junction. The second and third rows illustrate effects created by incorporation of amphotericin B at 15 mol%. The host matrix was DEPC (D, G), 7:3 DMPC/DMPG (E, H), DPPC (F, I). Arrows indicate etch face/fracture face junctions as above. Ice is denoted by i. Note the less-extensive fracture planes due to bilayer disruption by drug, the irregular liposome shapes, and loss of crystal ripple pattern, e.g. (D)–(F) and (H) and (I). (G) is an example of a liposome with retained ripple pattern but has areas of focal disruption. (A)–(C) and (G)–(I) prepared via 'standard' protocol, (D)–(F) via dialysis protocol. All samples were in phosphate-buffered saline (pH 7.4). The magnification factor was originally ×80000 (in reproduction: ×72000). Shadow direction is from bottom to top.

nique of freeze-etch electron microscopy is a particularly appropriate one for bilayer model membrane samples. It does not require chemical fixatives or sample dehydration, but rather relies upon extremely rapid freezing of sample droplets to liquid nitrogen temperatures to preserve structural details that exist under the

original conditions of incubation. Fracture of frozen samples produces through-cuts of liposomes and also exposes bilayer hydrophobic interiors, while subsequent 'etching' displays liposome surfaces. The resultant platinum-shadowed preparations contain features not available by other techniques, and a resolution 100-times higher than that of light microscopy. We have described previously the freeze-etch electron microscopy features of unsonicated liposomal amphotericin B having up to 5 mol% drug in saturated and unsaturated phospholipid bilayer membranes [13,22], and more recently Janoff et al. have reported the appearance of sonicated 7:3 DMPC/DMPG with high drug content at 20°C [12]. Below we describe application of this technique to the higher ranges of amphotericin B concentration in our different types of unsonicated liposome. These studies have been performed at 22 and 35°C, temperatures that approximate the range experienced within a mammal (extremities tend to be as low as room temperature while the core is some 37°C). This is also the temperature range covered by in vitro assay systems reported to date. Results are summarized in the electron micrographs displayed in Figs. 3 and 4.

Figs. 3A–C illustrate, at high magnification, structural details of the three different liposome types without drug at 35°C. The presence or absence of these details in drug-containing liposomes provides information about the molecular nature of amphotericin B arrangement and distribution. Liposomes without drug show visual evidence of the so-called $P_{\beta'}$ phase [28], in which the membrane has a 'trough and peak' pattern due to rippling of the highly ordered phospholipid bilayer [28–32]. The pattern is especially regular in liposomes frozen ('quenched') from below their phase transition temperature as seen in Fig. 3C, since the phospholipid crystal packing that gives rise to it has had the opportunity to become well-established. Pure liposomes quenched from above their transition temperatures should in fact theoretically be smooth (the L_α phase) [28–32]. However, since the freezing process that is the first step in sample preparation for electron microscopy is not infinitely rapid, the lipid molecules have a brief opportunity to begin forming the ripples of the $P_{\beta'}$ phase as they are frozen. The result is 'jumbled ripples' as shown in Fig. 3A and B.

Figs. 3D–I show representative high-magnification structural detail of liposomes at 35°C having an overall amphotericin B concentration of 15 mol%. Their appearance was not a function of method of preparation within the limits described in Materials and Methods. Nor was it affected by slam freezing (rapid quenching) vs. conventional freezing, except that the former approach suppressed jumbled ripple formation in bilayers quenched from above their phase transition. However, each batch of drug-containing liposomes manifested a range of the liposome features shown here, i.e., each batch was appreciably heterogeneous. The presence of high concentrations of other molecules (amphotericin B in this case) in an otherwise homogeneous PC bilayer tends to prevent the formation of very highly ordered phases such as $P_{\beta'}$ and yields smooth bilayers above and below the phase transition. This can provide an important clue to lipid bilayer composition. Bilayer ripples related to the $P_{\beta'}$ phase discussed above were much more prevalent in preparations containing only 5 mol% drug as reported previously [13,22]. A somewhat related clue is the ability of bilayer inclusions (amphotericin B) to disrupt the long-range continuity of bilayer sheets as we have reported previously for integral proteins [33]. Note for instance that the fracture planes in Figs. 3A–C of pure lipid bilayers extend over the entire liposome, since the hydrophobic interior of a bilayer sheet is a smoothly continuous fracture plane. In liposomes whose bilayers are interrupted by high concentrations of amphotericin B, the fracture plane hops erratically from one bilayer to the next. Another feature to note is the presence of misshapen liposomes. These multilobed, deformed structures are evident for instance in Fig. 3F. They still appear sealed, but lack the ripple pattern of the $P_{\beta'}$ phase and seem likely to represent liposomes with a high concentration of amphotericin B. In keeping with this interpretation, no such structures appear in preparations without the drug, and the relative proportion of these structures is directly related to the amount of drug in the preparations. As expected from earlier comments regarding the effect of impurities such as amphotericin B upon continuity of bilayer lamellar sheets, the fracture plane in these misshapen liposomes jumps erratically amongst neighbouring lamellae, exposing the hydrophobic fracture faces of numerous adjacent bilayer sheets. The multilamellar nature of the liposomes is obvious from the layered platelike appearance of the fracture faces.

Fig. 4 illustrates some remaining noteworthy features, including the appearance of drug on its own (Fig. 4A and B), the three different lipids with 15 mol% drug as in Figs. 3D–I but quenched from 22°C (Figs. 4D–F), possible 'unincorporated' drug in liposomal preparations (Figs. 4C, G), and 'liposomes' with 25 mol% drug (Figs. 4H, I). To generate the samples without lipid (Figs. 4A, B), the drug was handled as if liposomes were being prepared (films dried down, hydrated with warming, and dialysed where appropriate) but in the absence of phospholipid. The result in each case is a suspension of amorphous material with no similarity to lipid bilayers. Cut surfaces of the resultant amorphous masses (fracture faces) are generally smooth and featureless. Etching to expose their outer surfaces reveals the microspherular appearance seen, with grains 6.25–25 nm in size. Material similar to that seen in Figs. 4A, B (i.e., traces of what may be free drug) can often be found in liposomal drug preparations produced by the 'standard'

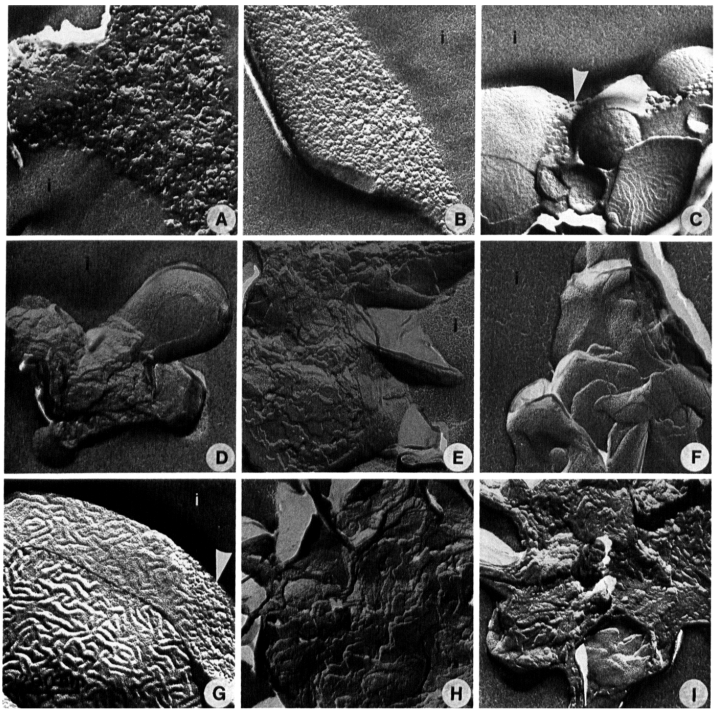

Fig. 4. Freeze-etch electron micrographs of features seen in preparations of liposomal drug. (A) and (B) show pure amphotericin B after passage through the liposome preparation protocols but without phospholipid: (A) quenched from 22°C and (B) quenched from 35°C. The amorphous masses are formed of granules 6.25–25 nm in diameter. Similar granules (arrowheads) appear in (C) and (G) which are 15 mol% amphotericin B in DEPC and 7:3 DMPC/DMPG, respectively, quenched from 35°C. Note the disordered ripple pattern in both (C) and (G). The row of micrographs, (D)–(F), displays preparations quenched from 22°C (as opposed to 35°C in Fig. 3) for 15 mol% amphotericin B in DEPC (D), 7:3 DMPC/DMPG (E), and DPPC (F). (H) and (I) show that at the highest concentrations of drug studied here, the structures still have bilamellar features, although highly disrupted: 25 mol% amphotericin B in 7:3 DMPC/DMPG (H) at 22°C and (I) at 35°C. (B), (C), (G), (H) via 'standard' protocol; (A), (D)–(F), (I) via dialysis protocol. All samples were in phosphate-buffered saline (pH 7.4). Ice is denoted by i. The magnification factor was originally ×80000 (in reproduction: ×72000). Shadow direction is from bottom to top.

protocol (e.g., Figs. 4C, G). Figs. 4D, E, F illustrate liposomes from preparations having 15 mol% overall amphotericin B concentration, but examined at 22°C. They have the same features already described for 35°C in Fig. 3. Figs. 4H, I show liposomes from batches of 7:3 DMPC/DMPG liposomes with 25 mol% drug content at 22 and 35°C, respectively. Overall, liposomes made from the three different lipid mixtures showed similar features.

Certain aspects of Figs. 3 and 4 require some discussion. Heterogeneity within a given liposome batch is likely due to the basic approach of preparing liposomes

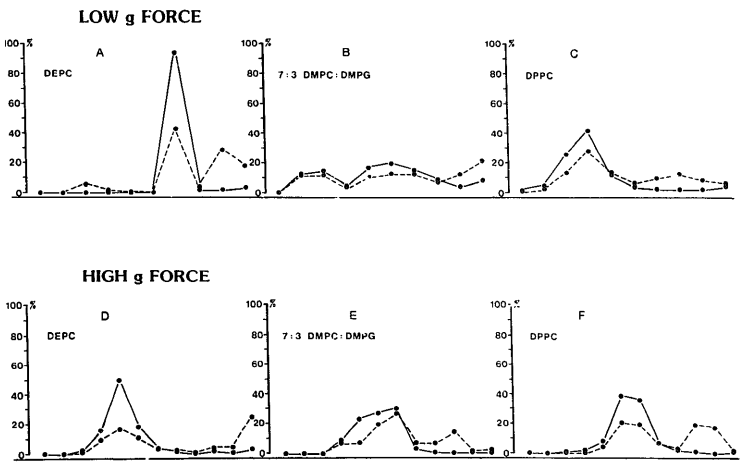

Fig. 5. Typical density gradient dispersion of hand-shaken liposomal amphotericin B preparations: (A)–(C), at low g-force for 15 min; and (D)–(F), at high g-force for 24 h. Data are shown for 20 mol% amphotericin B in the unsaturated DEPC, the 7:3 saturated mixture DMPC/DMPG, and the saturated DPPC. In each case, 750 μl of liposome suspension (6 mg lipid) was dispersed in the uppermost layer of a 0–45 wt% 12 ml linear sucrose gradient in 16×76 mm or 14×95 mm Beckman ultra-centrifuge tube. Liposomes had been doped at preparation with a tracer of [^{14}C]PC. After centrifugation at 1700×g for 15 min (A)–(C) or 100000×g for 24 h (D)–(F) in swinging bucket rotors, tubes were punctured at their bases and 1 ml fractions collected. All runs were performed at 22° C. Amphotericin B was quantitated by absorbance at 405 nm after dilution into CH_3OH, and lipid determination was done by scintillation counting. Results are displayed as % of total amphotericin B (solid line) and lipid (broken line), plotted from left to right in order of extrusion from the bottom of the tube.

by hydrating lipid films dried down from solvent. As organic solvent is removed prior to the hydration step, the rate of deposition of lipid vs. drug depends on their relative solubilities. Subsequent hydration will then lead to production of liposomes, whose composition reflects local conditions within the flask in addition to the ratio of components originally added. Thus, careful scrutiny will generally reveal all features in all preparations. It is the proportions that vary depending upon overall stoichiometry as would be expected: i.e., at higher drug concentrations there are relatively fewer liposomes resembling those in Figs. 3A–C, and more showing features of high drug content. It is interesting that, even at 25 mol% overall amphotericin B, bilayer features are by far the predominant finding, although focal evidence of extreme bilayer disruption may be found amongst the platelike lamellae (Figs. 4H, I). We consider that the 'distorted liposomal' structures recorded here are closely related to the 'non-liposomal' structures reported recently [12] in freeze-fracture preparations of sonicated 7:3 DMPC/DMPG bearing amphotericin B. We have chosen not to draw a clear distinction between 'liposome' and 'non-liposome' since the structures in our hands generally are lamellar, have an identifiable etch face/fracture face junction, and appear not to have unsealed edges; however, the argument is probably somewhat semantic. It seems likely that the 30 min sonication step used by Janoff et al. [12] leads to greater dispersion of drug and also greater sample homogeneity than is seen in our unsonicated preparations. Rapid freezing of samples ('slam freezing' using a Heuser-type Cryopress – see Materials and Methods) from 22 and 35° C did not alter our results.

Sucrose density gradients of liposomal amphotericin B

Given the appreciable visual heterogeneity of liposomal amphotericin B, we have experimented with centrifugation of our preparations, as have others [11,12]. The approach we describe here involved centrifugation of samples after dispersion in the uppermost layers of sucrose density gradients, since liposome density is a function of drug content in the bilayer. Experiments

were quantitated by doping lipid with a tracer of [^{14}C]PC and measuring drug concentration spectroscopically at 405 nm in methanol. Two approaches were used: (i) brief (15 min) centrifugation at 1700 × g in which the bulk of the drug and lipid sedimented to leave behind liposomes with lesser amounts of drug, or (ii) 24 h centrifugation at 100 000 × g (isopycnic density gradient centrifugation) in the same medium. Typical results of both methods at 22°C are shown in Fig. 5 for the three lipid types studied in this work bearing 20 mol% drug. In each case, amphotericin B was strongly associated with lipid. However, as in the freeze-etch electron microscopy data, there is evidence of some liposome heterogeneity. Similar results were obtained over the range of drug concentration explored here. We have isolated and dialysed selected density gradient fractions from each lipid class at several drug concentrations for study by EPR spectroscopy and freeze-etch electron microscopy. For any given sample the densest fractions showed selective enrichment in liposomes having the melting curve and electron micrograph features associated with greater drug content as described in the previous two sections. Less dense fractions of each sample displayed the narrower melting profiles and reduced visible disruption expected of lower drug content.

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