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Phase behaviour of amphotericin B multilamellar vesicles

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Because side effect profiles and key physical properties of liposomal amphotericin B reflect the molecular nature of the hydrated preparations, effort has been directed toward understanding this nature. We describe here an examination by differential scanning calorimetry in the region of the main transition of the phase behaviour of amphotericin B multilamellar liposomes used investigationally for patient treatment. Liposomes were composed of 7:3 dimyristoylphosphatidylcholine/dimyristoylphosphatidylglycerol (7:3 DMPC/DMPG) containing up to 33 mol% drug. Preparations in which pure DMPC or pure 1-oleoyl-2-stearoylphosphatidylcholine (OSPC) was substituted for 7:3 DMPC/DMPG were subjected to the same measurements for comparison. The DSC-derived partial phase diagrams were similar to those previously recorded using EPR spectroscopy for unsonicated liposomes of 7:3 DMPC/DMPG containing amphotericin B, and for mixtures with different pure saturated and unsaturated phosphatidylcholines (Grant, C.W.M., et al. (1989) Biochim. Biophys. Acta 984, 11–20). Fluidization onset temperatures for liposome host matrices were relatively unaffected by drug compared to the temperatures of completion. This effect was particularly marked for the unsaturated phospholipid matrix. Partial phase diagrams were interpreted as demonstrating that amphotericin B has a tendency to separate into a rigid phase within the membrane. This is consistent with molecular modelling considerations which suggest that amphotericin B may exist as oligomers in a phospholipid matrix. Drug-induced alterations of DSC melting profiles for the phospholipid bilayers studied were less extensive than those reported for partially sonicated preparations of 7:3 DMPC/DMPG (Janoff, A.S., et al. (1989) Proc. Natl. Acad. Sci. USA 85, 6122–6126). Melting profiles obtained did not change upon further sample incubation, suggesting that the hydrated preparation represented a thermodynamically stable form.

Introduction

As liposomal drugs begin to see regular use in certain centres, considerable attention is being focussed on the complex nature of the preparations employed. Liposomal amphotericin B (AmB) is a striking example. The non-liposomal drug must be solubilized for intravenous use by adding a 1:1 weight ratio of the bile salt detergent, deoxycholate, and is quite toxic. Nevertheless it is an extremely effective anti-mycotic; and has long been the agent of choice for systemic fungal infections [1,2]. AmB is well suited to liposomal format since it does not dissolve beyond 10^{-7} M in water at physiological pH and associates strongly with lipid bilayers [2]. The clinical attraction of liposomal AmB is that it appears not to cause the well known severe side effects of the conventional material at normal dosage (Refs. 2–4 and our unpublished observation). There are, however, two factors which greatly complicate the picture. Firstly, it has become clear that subtle details of AmB molecular arrangement, and liposome composition, size and history very significantly affect *in vitro* and *in vivo* toxicity [2,3,5–8]. Secondly, it appears that molecular arrangement of AmB relative to any given bilayer is a sensitive function of temperature, lipid choice, method of liposome preparation, and even drug concentration [2,8–16].

Abbreviations: DSC, differential scanning calorimetry; EPR, electron paramagnetic resonance; DMPC, dimyristoylphosphatidylcholine; DMPG, dimyristoylphosphatidylglycerol; OSPC, 1-oleoyl-2-stearoyl phosphatidylcholine; DEPC, dielaidoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; AmB, amphotericin B; MLV, multilamellar vesicles; SUV, small unilamellar vesicles.

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Probably the most influential work on AmB arrangement in membranes has been that of De Kruijff and Demel, which was based on a detailed consideration of molecular models [17] made possible by the structural analyses and considerations of groups such as Ganis et al. [18], Finkelstein and Holz [19] and Andreoli [20]. AmB has a cyclic hydrocarbon backbone which likely forms an elongated structure roughly comparable in size and shape to a phospholipid in a membrane. One end, bearing an amino sugar, is highly polar and would not be expected to be capable of membrane insertion. However, AmB also has polar groups down one 'side', that should impose thermodynamic constraints on any form of membrane insertion. This feature led workers to suggest that AmB molecules might aggregate with sterols to form hydrophilic pores in cell membranes (e.g., as a mechanism of fungal cell killing and host toxicity). Optical spectroscopy has proven to be an excellent technique for experimentally measuring the interaction of AmB with bilayer membranes, since the conjugated double bond system provides a chromophore whose spectrum is exquisitely sensitive to arrangement and environment [8,10–14,16,21]. Such experiments have been done with SUV since light scattering prevents good measurement on structures larger than 100 nm (but see Ref. 12). This approach has permitted detailed analysis of the kinetics and thermodynamics of AmB association with SUV. Unfortunately, in spite of the extreme sensitivity of optical spectroscopy to *changes* in molecular environment, the technique has not provided information as to absolute molecular arrangement in the membrane – which remains the subject of speculation.

Above 10^{-7} M concentrations, AmB exists as aggregates in aqueous solution [2,22]. Aggregates of unknown size have also been claimed to be the predominant form for polyene antibiotics in phospholipid SUV without sterols, based on optical spectroscopy measurements [12,23,24]. There is evidence of alteration in such clusters below the phase transition temperature [11,12]; but whether the alteration seen is related to reduced aggregation or to some other change such as partial extrusion of AmB is unknown (personal communication with Bolard, J. and Petersen, N.O.). Much of the work with SUV was done by adding drug to preformed bilayers in aqueous suspension. In such studies binding of AmB by saturated phospholipid SUV was seen to be more extensive (and unsaturable) compared to that by similar SUV composed of unsaturated lipid (egg phosphatidylcholine) [12]. Liposome size was seen to exert considerable control over the experimental results. It is also very important to note that absolute and relative concentrations of drug and lipid determined the extent of association and type of association [8,11–14,16]. These optical spectroscopy experiments were performed with dilute suspensions (typically up to 0.1–1 mg/ml lipid and 0.01 mg/ml drug) – as appropriate to many aspects of liposomal drug investigation.

Our work has centered around very different preparations of liposomal AmB. Firstly, instead of adding drug to preformed liposomes, drug and lipid were dissolved together in organic solvent, evaporated to a dry film, and subsequently hydrated. Secondly, preparations of liposomes were not sonicated: we have dealt only with hand shaken multilamellar vesicles (MLV). Thirdly, we have studied much higher concentrations of lipid and drug (15 mg/ml and 1 mg/ml, respectively). MLV of this type are typical of many preparations that have been used *in vitro*, in animal studies, and in several clinical trials including our own [3]. The high concentration range is representative of conditions existing at preparation, storage, and delivery of liposomal AmB for clinical use. The presence of multilamellar structures may be expected to greatly influence any equilibrium between membrane-bound and aqueous AmB. Questions originally asked by us, in collaboration with the groups of Juliano and Lopez-Berestein, were quite basic: (i) was drug actually mixed in with lipid as conventionally prepared for patient treatment? (ii) if so, were the resultant structures liposomal in nature? The answers to both questions as addressed by freeze-etch electron microscopy and EPR spectroscopy were 'yes' [5,15]. However, more subtle biophysical details were also apparent. Liposomes prepared by simple hydration (i.e., without sonication) of drug/lipid films dried down from $CHCl_3/CH_3OH$ were heterogeneous in size and drug concentration. In addition, there appeared to be a tendency for drug/lipid phase separation. These findings and others were similar for pure saturated dipalmitoylphosphatidylcholine (DPPC) and for the unsaturated dielaidoylphosphatidylcholine (DEPC), as well as for the 7 : 3 mixture of dimyristoylphosphatidylcholine and phosphatidylglycerol (7 : 3 DMPC/DMPG) used clinically. This was somewhat unexpected since some workers reported greater toxicity for liposomal AmB when unsaturated phospholipids were used [3,5,6] (but see Ref. 4). However, DEPC is unusual in being a diunsaturated species with *trans* double bonds.

A study by deuterium NMR of unsonicated DMPC bilayers bearing AmB found evidence of phase separation into drug-poor and drug-rich domains (the latter having an apparent composition of 1 : 1 AmB/ DMPC) [25]. A report by Janoff et al. using differential scanning calorimetry (DSC) on 7 : 3 DMPC/DMPG bearing AmB also noted results consistent with phase separation of drug [7]. However, it seemed to indicate greater dispersion of drug than we found with EPR spectroscopy of a spin label probe [5,15]. A significant difference in the approaches is that the above DSC work was done on MLV which had been partially sonicated.

We have continued to focus our efforts on liposomes

produced by manual agitation ('hand shaking') of dry lipid films hydrated with aqueous salt solutions above the phase transition temperature. Our interest in the present work was to test by DSC our earlier observations. In addition to 7 : 3 DMPC/DMPG, we examined pure DMPC and the *cis*-unsaturated species, OSPC, with amphotericin B.

Materials and Methods

Pure amphotericin B was obtained from Lyphomed, Inc., Melrose Park, IL. The phospholipids, L-α-dimyristoylphosphatidylcholine (DMPC), L-α-dimyristoylphosphatidylglycerol (DMPG), and L-α-1-oleoyl-2-stearoylphosphatidylcholine (OSPC), were purchased from Avanti Polar Lipids, Birmingham, AL. Organic solvents were reagent grade. The spin label, TEMPO, was from Aldrich, Milwaukee, WI.

Liposome preparation

Pure amphotericin B was dissolved in methanol at 0.5 mg/ml to produce a slightly cloudy solution which was filtered through a glass wool plug to give a clear solution. The lipids were dissolved in chloroform at a 25 mg/ml concentration and combined with the appropriate amount of amphotericin B in methanol. The solvent was removed using a rotary evaporator and a desiccator under vacuum. Samples were hydrated with 10 mM Hepes buffer containing 5 mM EDTA using mild vortexing and manual agitation at a temperature 10 C° above the lipid phase transition. All samples containing amphotericin B were protected from light exposure during preparation and storage.

Phase transition studies

Differential scanning calorimetry (DSC) was performed on samples containing 8–10 mg lipid. Once prepared, samples were transferred to DSC sample cells, and scans were taken with a Microcal (Microcal Inc., Amherst, MA) MC-1 high sensitivity differential scanning calorimeter at a scan rate of 12–14 C°/h. Onset, completion, and main transition temperatures were determined manually from the plots, and by a computer program that permitted baseline corrections. Once scans were run samples were stored at 4°C in a methanol solution. Phospholipid concentration was quantitated using a phosphate assay method [26] with amphotericin B content being determined by absorbance vs. standards in methanol at 405 nm [27].

Electron paramagnetic resonance (EPR) spectroscopy was performed on liposome suspensions containing 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 [28]. Sample temperature was monitored with a copper/constantan thermocouple in the Dewar insert. Data were treated as recommended by Shimshick and McConnell [29].

Electron microscopy

Liposome samples studied by freeze-etch electron microscopy were rapidly quenched from 33°C by plunging suspension droplets on gold discs into liquid freon cooled in liquid nitrogen. Frozen samples were fractured at −105°C in a Balzers BAF 300 high-vacuum coating unit equipped with electron beam guns. After fracturing samples were 'etched' for 2 min prior to platinum shadowing. Replicas were cleaned in bleach, rinsed in distilled water, and picked up from 1 : 1 acetone/ethanol. Replicas were examined using a Phillips 300 electron microscope.

Results and Discussion

Bilamellar lipid membranes with relatively homogeneous phospholipid composition are well known to undergo temperature induced fluid gel transitions analogous to melting, while retaining their basic bilayer structure. Details of such 'melting' behaviour reflect underlying molecular arrangement. A number of the most important liposome compositions which have been investigated for experimental and clinical use in the case of amphotericin B are particularly simple in composition and manifest striking phase behaviour. Thus DMPC has only 14-carbon, fully saturated fatty acid chains and undergoes a sharp main transition at 23°C [26]. Its phosphatidylglycerol analogue, DMPG, has the same fatty acid composition and is known to also have a phase transition temperature of 23°C in the absence of Ca^{2+} [27,28]. Interestingly, hydrated combinations of these two phospholipids 'melt' sharply at 23°C and they are considered to mix homogeneously [27,28]. Such phospholipids (with saturated as opposed to unsaturated fatty acids) have sometimes been observed to minimize amphotericin B toxicity when used to produce liposomal drug [3,5,6]. OSPC is a phospholipid possessing a *cis*-unsaturated 18-carbon fatty acid and displaying a main transition at 8.6°C that can be accurately monitored over a similar temperature range to the saturated species [32].

Fig. 1 illustrates DSC melting profiles for the abovementioned fully hydrated phosphatidylcholines with amphotericin B and for the 7 : 3 DMPC/DMPG mixture used clinically by us and by the groups of Lopez-Berestein and Juliano [3]. All samples were prepared identically and data shown were obtained under identical conditions of slow heating. The only difference in sample history relative to liposomes used clinically was that they were warmed slowly (12–14 C°/h) through

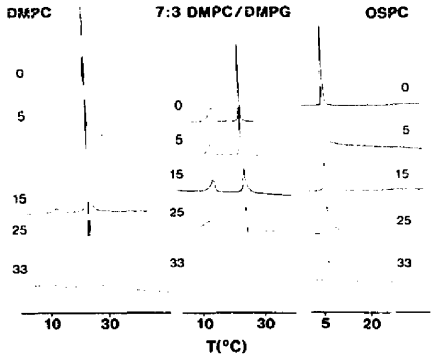

Fig. 1. Differential scanning calorimetry traces for fully hydrated multilamellar liposomes of DMPC, 7:3 DMPC/DMPG, and OSPC containing amphotericin B. Traces are shown for drug concentrations of 0, 5, 15, 25 and 33 mol%. Each sample contained approximately 8–10 mg dry lipid hydrated with 10 mM Hepes buffer (pH 7.4) containing 5 mM EDTA. Scan rates were 12–14 C°/h. Traces shown have not been normalized or baseline-corrected.

the phase transition and then cooled, prior to recording DSC curves (i.e., first DSC runs were not used). First runs tended to produce sporadically irregular baselines as is typical of liposomes without drug, but otherwise were comparable to subsequent runs. Results remained unchanged between DSC experiments over 24 h apart, and repeated cycles of freezing and thawing were not seen to alter the melting profiles.

The presence of impurities in a phospholipid bilayer alters melting behaviour in ways that depend upon interactions among the molecules involved – in this work the 'impurity' was amphotericin B. There are several aspects of the data in Fig. 1 that deserve comment. Firstly, the lipid main transition was preserved in each case up to the highest drug concentrations. Even pretransitions remained detectable. This is an extension of the observation made originally on the 5 mol% liposomes used clinically, that the drug had remarkably little effect on liposome melting profiles [5]. These observations are consistent with (a) a tendency for drug molecules to self-associate (reducing their area of contact with phospholipids) and/or (b) the presence of a phase from which drug is relatively excluded. Secondly, drug effect was relatively small on the onset of melting, while significantly raising and broadening the completion of melting. We originally recorded this phenomenon for the 7:3 mixture, DPPC, and DEPC using EPR spectroscopy [15]. A typical such EPR result, as determined for DMPC, is shown in Fig. 2. Based upon our early EPR results, we considered that amphotericin B in the MLV studied showed significant solid phase immiscibility (as described for binary phospholipid systems by McConnell, H.M. and co-workers [26]). Thirdly, the

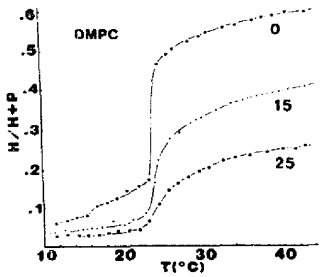

Fig. 2. Typical membrane melting profiles, comparable to differential scanning calorimetry traces in Fig. 1, derived from EPR data of TEMPO spin label partitioning between buffer and membrane. Curves shown are for multilamellar liposomes of DMPC prepared as in the caption to Fig. 1 and containing 0, 15 and 25 mol% amphotericin B. Samples were scanned from low to high temperature at 0.4 C°/min.

overall melting curve appearance was basically similar for AmB MLV formed from the various lipid types.

Partial phase diagrams derived from the drug liposome DSC curves are shown in Fig. 3. They reflect the features described in the DSC curves of Fig. 1. The minimal drug-induced change in onset of membrane melting is most marked for DMPC and OSPC as evidenced by a horizontal solidus in each case. There is a significant upward slope to the solidus for the 7:3 mixture. An upward sloping solidus was also seen for similarly-prepared liposomes of the 7:3 mixture as

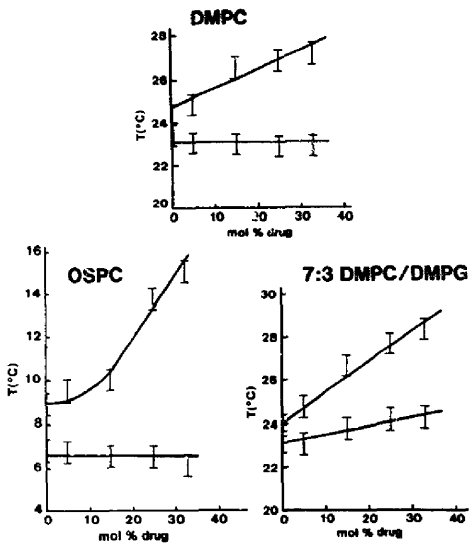

Fig. 3. Partial phase diagrams for multilamellar vesicles of DMPC, 7:3 DMPC/DMPG, and OSPC containing amphotericin B. Temperatures of onset and completion of melting were obtained from differential scanning calorimetry traces, averaging two or three traces per point.

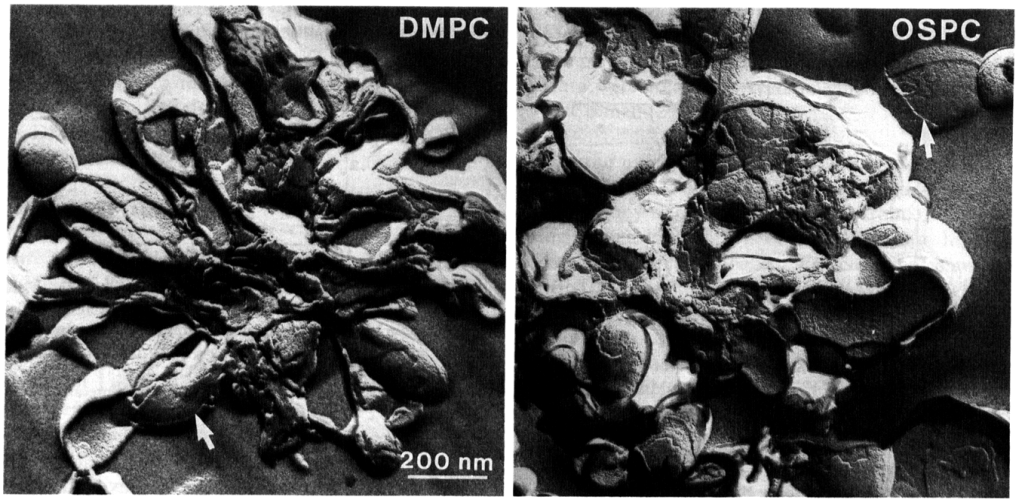

Fig. 4. Freeze-etch electron micrographs of samples used to derive the DSC and EPR information reported here. Samples shown are DMPC and OSPC containing 15 mol% amphotericin B. Fracture face/etch face junction is indicated by an arrow. Magnification: ×65000. Shadow is from bottom to top.

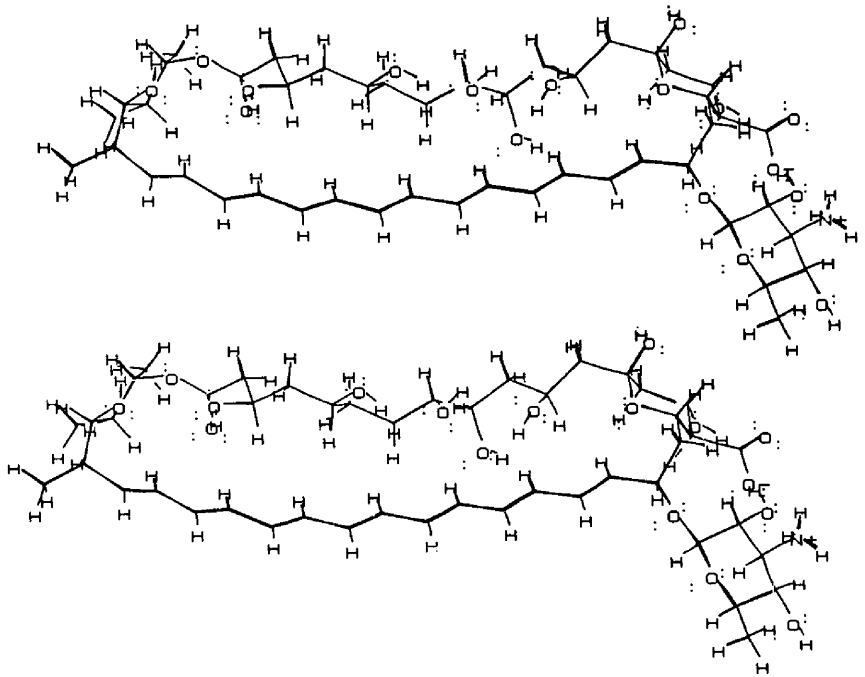

Fig. 5. Structure of zwitterionic amphotericin B obtained by molecular modeling in the absence of intermolecular constraints. Software used: PCMODEL (Serena software, Bloomington, IN) running default parameters, with intramolecular H-bonding permitted for acidic hydrogens to the left of the ketal ring and for the $-COO^-$ function (upper), or toggled off and the structure re-minimized (lower). PCMODEL utilizes an MMX force field.

studied via EPR spectroscopy, although in the latter experiments it was within experimental error [15]. The extent of phospholipid/drug phase separation was most evident for the liposomes composed of OSPC. The physical appearance of DMPC/AmB and OSPC/AmB drug liposomes used for these measurements is illustrated in Fig. 4. The micrographs show the same features described previously in freeze-etch electron micrographs of DPPC, 7 : 3 DMPC/DMPG and DEPC/drug MLV [15].

An explanation of the DSC results obtained in this work is suggested by the structure of amphotericin B [17–20] (Fig. 5). AmB is water insoluble beyond 10^{-7} molar at neutral pH, and strongly lipophilic [2]. Like a phospholipid, it has one very polar end and a lengthy hydrocarbon portion. However, a number of polar substituents that would be expected to make membrane insertion energetically unfavourable occur down one side of the cyclic hydrocarbon chain (and these are not readily shielded by rotation about bonds [33] or by internal H-bonding) (Fig. 5). Conceptually, the amphotericin B molecule could meet both constraints (hydrophobicity and restricted lipophilicity) by forming aggregates such that the polar groups of one AmB molecule interacted with those of one or more others within the bilayer. The size of such aggregates is not known, however, De Kruijff and Demel have used similar logic to propose amphotericin B octamers in tight 1 : 1 association with sterols as the mechanism of drug-induced pore formation in cell membranes [17] and related structures have been suggested in sterol-free phospholipid bilayers [34]. Phase separation of binary phospholipid mixtures has been typically reported in combinations of lipids whose structures are dissimilar. Certainly an aggregate of AmB molecules would have structural characteristics very dissimilar to those of the surrounding phospholipids of the bilayer. The observed drug-induced extension of the temperature range over which completion of membrane melting occurs would be consistent with the presence of a coexisting phase relatively enriched in AmB oligomers. It was mentioned earlier that partially sonicated MLV of 7 : 3 DMPC/DMPG show greater suppression of the DSC phase transition [7] upon incorporation of AmB than is apparent in our otherwise identical unsonicated systems. The same partially sonicated 7 : 3 DMPC/DMPG liposomes containing 5 mol% AmB appear to have been more toxic in a mouse model than their unsonicated counterparts [3,6,7]. It would seem reasonable to suggest that these observations are associated with greater physical dispersion of the phase-separated drug into the lipid membrane.

The observation that AmB shows a greater tendency to phase separate in (unsaturated) OSPC than in (saturated) DMPC is the first direct evidence of a different physical drug arrangement in unsaturated vs. saturated phospholipid MLV. It recalls the observation that binding of exogenously-added AmB to SUV prepared from phospholipids having unsaturated fatty acids was markedly less than was its binding to analogous SUV prepared from phospholipids with saturated fatty acids [12]. Unfortunately, while selecting OSPC and DMPC for comparison on the basis of their having sharp phase transitions well above 0°C and fairly close together, we have not been able to control for acyl chain length (18 for OSPC and 14 for DMPC). Chain length is known to influence AmB/phospholipid binding [13,21].

Conclusions

An important aspect of liposomal amphotericin B (and probably of liposomal drugs in general) is the complexity of the preparation itself. In this article we have focussed on unsonicated MLV at the high drug and lipid concentrations used clinically, without sterols. Our results are consistent with the existence of AmB oligomers that phase separate into domains of relative enrichment. This tendency was greatest in the unsaturated host matrix, OSPC, however, it was not possible to isolate the effect of unsaturation from the effect of acyl chain length.

We would emphasize that the MLV described here appear to be thermodynamically stable. It is interesting to note that even partial sonication of 7 : 3 DMPC/DMPG with amphotericin B (i.e., bath sonication for 30 min to reduce the size of the multilamellar structures) gave very different results: Janoff et al. found that the melting peak almost disappeared in sonicated dispersions of 7 : 3 DMPC/DMPG containing 25 mol% AmB [7]. Upon incubation at 65°C the above preparation returned to a DSC behaviour similar to our own – consistent with phase separation of drug being thermodynamically favoured. Interestingly the toxicity of the partially sonicated preparations [7] also appears to have been very different from that of its unsonicated counterpart [3,6]. The physical characteristics of amphotericin B MLV recorded here may be involved in the sensitivity of the material to method of preparation and history: domain size and molecular arrangement would be expected to be sensitive to such factors.

Acknowledgements

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