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Interdigitation-fusion: a new method for producing lipid vesicles of high internal volume

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Abstract

Previously we demonstrated that fused phospholipid sheets can be formed from small unilamellar vesicles (SUVs) comprised of saturated symmetric chain lipids by exposing them to concentrations of ethanol sufficient to cause bilayer interdigitation (Boni et al. (1993) Biochim. Biophys. Acta 1146, 247–257). Here we report that these sheets spontaneously form large, predominately unilamellar vesicles, when exposed to temperatures above their main phase transition temperature (T_m). These vesicles, termed interdigitation-fusion vesicles (IFVs), have mean diameters between 1 and 6 μm, and, once produced, are stable both above and below the T_m of the lipid. The average captured volume of IFVs is dependent upon lipid chain length, the concentration of ethanol used to induce interdigitation-fusion, and size of the precursor liposomes. IFVs comprised of DPPC and DSPC had averaged captured volumes of 20–25 μl/μmol lipid. IFVs produced from SUVs containing only DPPG or DPPC/DPPG mixtures had captured volumes equivalent to those made from pure DPPC SUVs indicating that charge can be introduced without consequence to the IFV process. Inclusion of cholesterol in precursor vesicles reduced IFV captured volume in a concentration dependent fashion by interfering with interdigitation. Cholesterol could be incorporated, however, into IFVs through admixture with the already formed phospholipid sheets producing far less comprise to captured volume. IFVs are useful as model systems or drug carriers, since their large internal volume allows for efficient encapsulation particularly with regard to compounds such as iodinated radiocontrast agents which otherwise interfere with vesicularization.

Keywords: Interdigitation; Liposome; Captured volume; Drug delivery

1. Introduction

Since their discovery over a quarter of a century ago, liposomes have defined new approaches for rational drug design. Through encapsulation the pharmacodynamic properties of a wide variety of different drugs have been altered often resulting in dramatic reductions in toxicities and/or improvements in efficacy [1]. These applications have required various vesicle morphologies, and over the years numerous techniques have been developed for forming liposomes of different sizes, internal volumes, and lamellarities [2]. The simplest method involves hydrating dry lipids with an aqueous buffer. This procedure typically produces multilamellar vesicles, or MLVs, which are heterogeneous in their size distribution and have internal volumes of 0.5 to 1.0 μl/μmol lipid [3]. The internal volume of MLVs can be increased by an order of magnitude by freezing and thawing to produce 'frozen and thawed' MLVs or FATMLVs [4]. Other techniques for attaining liposomes of relatively high captured volume such as reverse phase evaporation [5] and the stable plurilamellar vesicle technique [6], rely on the addition and removal of organic solvents and generally produce systems with captured volumes below 10 μl/μmol lipid.

Abbreviations: DPPC, L-α-dipalmitoylphosphatidylcholine; DMPC, L-α-dimyristoylphosphatidylcholine; DSPC, L-α-distearoylphosphatidylcholine; DAPC, L-α-diarachidoylphosphatidylcholine; DOPC, L-α-dioleoylphosphatidylcholine; EPC, egg phosphatidylcholine; DPPG, L-α-dipalmitoylphosphatidylglycerol; CAT-1, 4-trimethylammonium 2,2,6,6-tretramethyl-1-piperidinyloxy free radical; SUV, small unilamellar vesicle; MLV, multilamellar vesicle; LUVET, large unilamellar vesicle by extrusion technique; IFV, interdigitation-fusion vesicle; IF, interdigitation-fusion; EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance; T_m, gel to liquid-crystalline phase transition temperature; P_i, phosphate

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In this report, we describe a novel and simple new method for preparing lipid vesicles from interdigitated phospholipid sheets. These liposomes, which we call interdigitation-fusion vesicles (IFVs), are predominately unilamellar, have mean diameters of 1–6 μm and have captured volumes of 20 μl/μmol lipid or above [7].

The production of IFVs takes advantage of the fact that the ethanol dependent induction to the interdigitated lipid phase is sensitive to bilayer radius of curvature [8]. Ethanol is one of several small amphiphilic molecules which produce interdigitation of symmetric saturated phosphatidylcholine bilayers [9–17]. Recently, we have described how small vesicles (less than 200 nm in diameter) comprised of saturated symmetric chain phospholipids coalesce to form a viscous suspension of interdigitated sheets at and above ethanol concentrations required to induce interdigitation [8]. In these studies, electron microscopy revealed that small (< 110 nm) liposomes underwent a collapse of their spherical geometry upon interdigitation. This created packing defects and hydrophobic exposure, driving vesicle coalescence and the formation of the extended interdigitated phospholipid sheets.

Here we describe how these sheets can be spontaneously transformed into liposomes (IFVs) by raising the solution temperature above the T_m of the lipid. In particular we describe the parameters by which SUVs can be transformed into IFVs. We examined the effects of the size of the precursor liposomes, ethanol and lipid concentration, and lipid species upon final IFV captured volume. IFVs are useful for a variety of different membrane modeling, biomedical, as well as pharmaceutical applications. Ethanol, the only non-aqueous solvent required, can be easily removed and is miscible with water. The large internal volume of IFVs makes them particularly attractive where high drug to lipid ratios are required [1].

2. Materials and methods

2.1. Materials

DPPC, DMPC, DSPC, and EPC were purchased from Princeton Lipids (Princeton, NJ). Cholesterol, was obtained from Sigma (St. Louis, MO), while DAPC and DOPC were obtained from Avanti Polar Lipids (Alabaster, AL). CAT-1 and TEMPONE were purchased from Molecular Probes (Eugene, OR). Ethanol (dehydrated 200 proof) was obtained from Pharmco Products (Bayonne, NJ).

2.2. Preparations of MLVs, SUVs, and LUVETs

MLVs were prepared by hydrating lipid at a concentration of 20 mg/ml in 150 mM NaCl, 10 mM Tris-HCl at pH 7.4. Lyophilized lipid was hydrated directly, but lipid supplied in chloroform was roto-evaporated to a thin film in a round bottom flask and vacuum dried overnight to remove residual chloroform prior to hydration. Binary lipid MLVs were prepared by mixing chloroform stock solutions of each lipid. In all cases the hydrated lipid samples were allowed to incubate for 10 min in buffer above their T_m in order to insure formation of intact vesicles. SUVs were made by sonicating (Branson Sonifier 450, Danbury, CN) MLV samples above the lipid T_m until optically clear. Titanium dust produced by the sonicator tip was removed from the sample by centrifugation. DPPC LUVETs were produced from the MLVs following the procedure described by Mayer et al. [18]. Briefly, the MLVs were forced ten times through two stacked polycarbonate filters (Nuclepore, Pleasanton, CA) of the required pore size using an Extruder (Lipex Biomembranes, Vancouver, BC) operating at 50–55°C. The average diameter of the LUVETs was determined by quasi-elastic light scattering using a NICOMP 270/370 (NICOMP Particle Sizing Systems, Santa Barbara, CA) operating at 50°C.

DPPC LUVETs which were prepared by extrusion through 50, 80, 100, and 200 nm polycarbonate filters could be fitted to a Gaussian distribution with average diameters of 41, 57, 79, and 143 nm. DPPC MLVs extruded through 400 and 800 nm pore size filters could not be fitted to Gaussian distributions unless they were disaggregated after which they sized at 219 and 451 nm, respectively.

2.3. Preparation of IFVs

Interdigitation-fusion vesicles (IFVs) were typically prepared in 4 ml batches using a lipid concentration of 20 mg/ml. We prepared IFVs in closed containers, such as scintillation vials or tubes with caps. An SUV suspension of the desired phospholipid was transferred into the vial or tube. DPPC, DSPC, and DAPC samples were equilibrated to room temperature, while DMPC, DOPC, and EPC samples were cooled to approx. 5°C. An isothermal volume of absolute ethanol was added to bring the final ethanol concentration in the sample to 4.0 M. The samples were immediately vortexed. This procedure generally quickly transformed the transparent SUV suspension into an extremely viscous opaque white suspension of interdigitated phospholipid sheets. We modified the ethanol addition step when the final ethanol concentration used to induce interdigitation was 1.5 M or below. For these experiments equal volumes of the SUV sample at 40 mg/ml and a buffer/ethanol solution at twice the desired final ethanol concentration were mixed. This procedure was required in order to avoid locally high ethanol concentrations in the SUV sample before the sample could be completely mixed. No difference in the internal volume of the DPPC IFVs formed at 2.0 M ethanol concentrations and above was observed regardless of whether the ethanol was added directly or prediluted.

After the ethanol addition, the samples were sealed and incubated for 15 min at room temperature, except for the

DMPC, DOPC, and EPC samples which were incubated at approx. 5°C. The samples were then incubated for 15 min at temperatures above the T_m of the lipid. The caps on the samples were then loosened to allow ethanol evaporation and the incubations continued for another 30 min at the same temperature. DMPC, DOPC, and EPC were incubated between 40 and 45°C. DPPC was incubated at 50–55°C, while DSPC and DAPC were incubated at 70–75°C. After this time the samples were 'sparged' above the T_m by the bubbling of a gentle stream of N_2 to remove ethanol. Next they were washed three times with 150 mM NaCl, 10 mM Tris-HCl (pH 7.4) at room temperature by 15-min centrifugations at 12000 × g using a Sorvall RE5B centrifuge (Dupont Instruments, Wilmington, DE) equipped with a Sorvall SA-600 rotor. Typically, 90 to 100% of the initial phospholipid was recovered. The IFV pellet was subsequently resuspended in NaCl-Tris buffer at a concentration near 20 mg/ml and stored at room temperature. IFV phospholipid concentrations were determined by a modified version of the procedure of Bartlett [19]. To prepare IFVs from SUVs comprised of two different lipids (at molar ratios of 0.2, 0.5, 0.67, or 0.8), the same general procedures as described above were followed. Care was taken to insure that the addition of ethanol was performed below the T_m of the mixture, that the high temperature incubation was performed above the T_m of the mixture, and that only miscible lipids were used. Cholesterol inhibited IF formulation (see Results and discussion)

Incorporation of cholesterol into DPPC IFVs was accomplished by first preparing interdigitated DPPC sheets from DPPC SUVs at a concentration of 20 mg/ml lipid using 4.0 M ethanol according to the procedures described above. DPPC/cholesterol SUVs (1:1, mol/mol) in 4.0 M ethanol were then mixed into the DPPC interdigitated sheets at room temperature in such a manner as to give the desired cholesterol to DPPC molar ratio. The mixtures were then incubated for 45 min at 50 to 55°C. Samples were then sparged above the lipid T_m with N_2 for 5 min to remove the ethanol. Since the size of the product DPPC/cholesterol IFVs decreased with cholesterol concentration, it was difficult to achieve good recovery following the centrifugation wash step at the high cholesterol concentration. Therefore, we omitted this step for this particular experiment. Control experiments with DPPC IFVs showed that leaving out the centrifugation wash steps did not change the final internal volume as determined by our standard method. Thus, residual ethanol was not present at sufficient quantities to change the permeability of the EPR probe used in the internal volume measurements.

2.4. Internal volume determination

Generally, the internal volumes of IFVs were determined using the ViVo method of Perkins et al. [3,20]. This method involves the addition to the liposome suspension of a membrane impermeant probe such as the EPR spin probe CAT-1. The concentration of the spin probe in the supernate or filtrate over that which would be expected in the absence of the liposomes is then determined using a standard curve. This allows an accurate determination of the external volume thereby permitting the internal volume of the liposomes to be calculated. In the experiments reported here, internal volumes were normalized to the inorganic phosphate content of the sample. In most cases, the product IFVs easily pelleted. In some situations, however, the product IFVs were too small to be efficiently pelleted and in these cases buffer was removed from the liposome suspension by filtration through a 0.02 μm Anotop 25 filter (Whatman, Clifton, NJ). For negatively charged lipid systems we used the distribution of the permeable spin probe TEMPONE to determine volume distribution [21]. A more complete discussion of internal volume in general, and measurements with CAT-1 in particular, can be found in Perkins et al. [20].

2.5. Optical microscopy

Phase contrast microscopy was accomplished using an Olympus BH-2 microscope in combination with an Olympus PM-CBSP exposure meter (New York/New Jersey Scientific, Middlebush, NJ). Kodak TMX-135 black and white print film was used to record the images. The samples were observed typically at room temperature.

2.6. Freeze-fracture electron microscopy

A 0.1–0.3-μl aliquot of sample was sandwiched between a pair of Balzer copper support plates (Nashua, NH) and rapidly plunged from room temperature into liquid propane. Samples were fractured and replicated on a Balzers BAF 400 freeze-fracture unit at −115°C with a vacuum of $4 \cdot 10^{-7}$ mbar. Platinum replicas were floated off in distilled water and transferred to a solution of commercial bleach (Chlorox) for overnight cleaning. The replicas were again washed in distilled water and mounted on 300 mesh copper grids (Polysciences, Warrington, PA) and viewed and photographed using a Philips 300 electron microscope.

2.7. DPPC IFV size determination

The mean diameter of DPPC IFVs was determined using a Malvern 3600 E laser diffraction particle sizer (Malvern Instruments, Malvern, UK). The measurements were made at room temperature with highly diluted samples. The number weighted particle diameter distribution was calculated by the instrument from the laser diffraction pattern.

2.8. Lamellarity determination

The lamellarity of the DPPC IFVs was determined by ^{31}P-NMR according to the method described by Hope et

al. [22]. This technique takes advantage of the fact that the addition of Mn^{2+} broadens only the ^{31}P signal arising from the lipid on the outer monolayer. The spectra were recorded with a Bruker 360 mHz spectrometer (Billerica, MA).

2.9. Differential scanning calorimetry

Differential scanning calorimetry was accomplished using a MC-2 Ultrasensitive Scanning Calorimeter (MicroCal, Northampton, MA). The sample concentrations were approximately 1 mg/ml. The samples were scanned from 20 to 55°C at a scan rate of 20°C/h.

3. Results and discussion

Previously we reported that the induction of the interdigitated gel phase in DPPC vesicles is sensitive to radius of curvature. We found that compared to large liposomes, smaller liposomes required higher concentrations of ethanol to induce bilayer interdigitation [8]. Most significantly, we

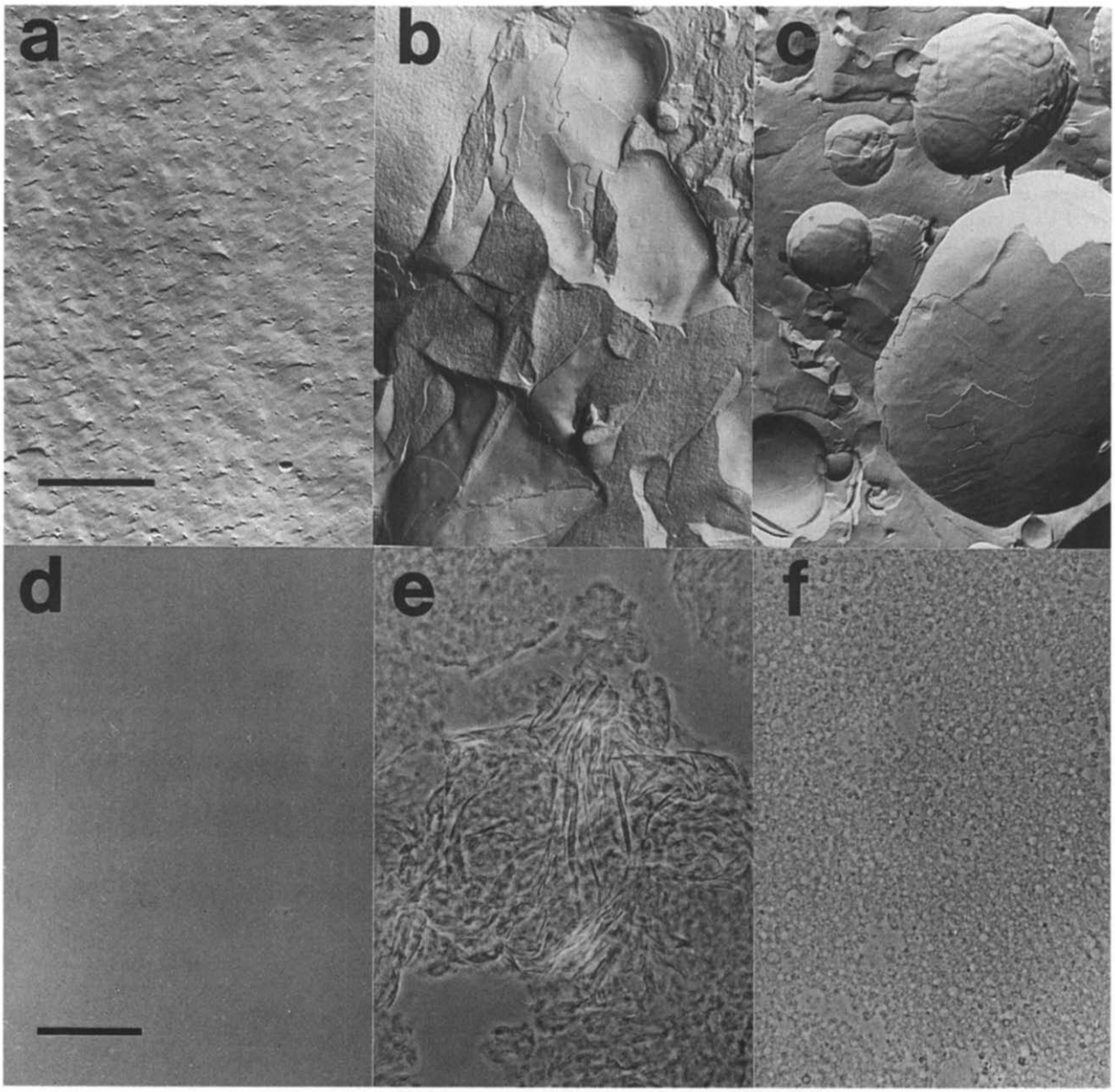

Fig. 1. Formation of interdigitation fusion vesicles (IFVs). The formation of DPPC IFVs was studied by freeze fracture (top) and phase contrast microscopy (bottom). DPPC SUVs (a and d), extended interdigitated sheets resulting from exposing DPPC SUVs at 20 mg/ml to 4.0 M ethanol at room temperature (b and e), IFVs annealed from the extended interdigitated sheets by transient incubation at 55°C for 45 min (c and f). All photomicrographs were obtained at room temperature. Removal of ethanol following the high temperature incubation step did not affect the morphology of vesicles. The bars represent 1 μm (freeze fracture) and 50 μm (phase contrast).

found that at concentrations of ethanol sufficient to induce interdigitation, DPPC vesicles below 200 nm in diameter changed in morphology and coalesced into an extremely viscous suspension of extended interdigitated phospholipid sheets. Here we show that this suspension of interdigitated phospholipid sheets can be annealed to form liposomes of high captured volume. We call these liposomes interdigitation-fusion vesicles (IFVs). IFVs can be formed from phospholipids or combinations of phospholipids which undergo ethanol-induced interdigitation, such as DMPC, DPPC, DSPC and DAPC. Phospholipids such as EPC or DOPC which do not form an interdigitated phase, do not form IFVs. Lipids which inhibit interdigitation such as cholesterol can be incorporated into IFVs by admixture into the intermediate interdigitated sheets.

The coalescence of DPPC SUVs into interdigitated sheets and the transformation of these sheets into IFVs is shown in Fig. 1. In these experiments we held the lipid concentration at 20 mg/ml and the ethanol concentration at 4.0 M. Formation of sheets from SUVs occurred immediately upon the addition of ethanol and could be detected visually because the optically clear lipid suspension was transformed into an opaque viscous material. The interdigitated sheets that comprised this material were measured by phase contrast microscopy to be on the order of tens of μm in length. Phase contrast microscopy allowed us to detect a decrease in viscosity and the curling off of predominately unilamellar liposomes (IFVs) from these sheets when the temperature passed through the T_m. The size distribution of these vesicles measured by light scattering (sensitive predominately to vesicles > 1 μm) is shown in Fig. 2. The distribution was relatively homogenous with a significant proportion of the liposomes in the 2 to 6 μm range. The average internal volume of these liposome samples was calculated to be 20.2 ± 0.2 μl/μmol by the technique of Perkins et al. [3]. ^{31}P-NMR measurements indicated that $44.3 \pm 12.5\%$ of the lipid was in the outer monolayer in these samples. This corresponds to a statistical lamellarity of 1.13 ± 0.32 and suggests that DPPC IFVs are predominately unilamellar [22]. As discussed in detail by Perkins et al. [20] statistical lamellarity together with captured volume provides an excellent means for gauging the size and lamellarity profile of a population of liposomes and prevents sampling errors which are common if only electron microscopic techniques are used.

The effects of ethanol concentration, vesicle size and lipid concentration upon the formation of IFVs as judged by captured volume are detailed in Fig. 3. We were already aware that induction of interdigitation in DPPC vesicles is dependent on both size and ethanol concentration [8] but did not know the role these variables might play in IFV formation. Because fusion efficiency is concentration dependent we also examined IFV formation as a function of lipid concentration. Fig. 3a shows the effect of varying the concentration of ethanol used to produce interdigitated sheets from DPPC SUVs. These sheets were

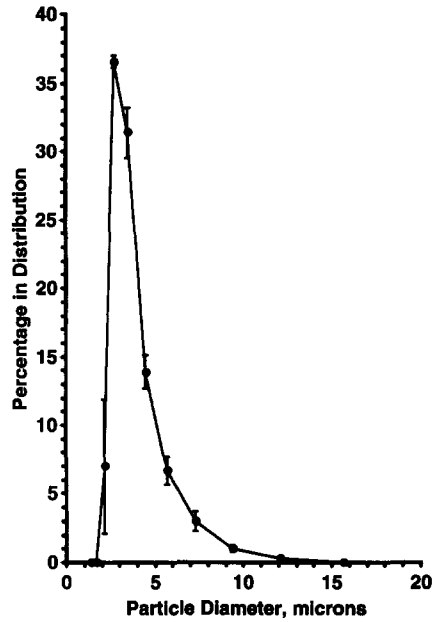

Fig. 2. Size distribution of DPPC IFVs. The normalized diameter distributions for three DPPC IFV samples were measured at room temperature using a Malvern 3600E laser diffraction particle sizer. The distributions were based on particle number and were calculated by the instrument. The average values and standard deviations for these preparations are shown in the figure. The number averaged diameter for the samples was 3.54 ± 0.12 μm. The IFVs were formed from DPPC SUVs at 20 mg/ml using 4.0 M ethanol. This technique is not sensitive to particles under 1 μm.

subsequently incubated at 55°C for 45 min, cooled to room temperature and the resultant IFVs measured for internal volume. Concentrations of ethanol below 2.0 M did not result in the efficient production of IFVs. In fact, at ethanol concentrations below 1.0 M, SUVs do not interdigitate at all [8] and as expected did not produce large vesicles. At ethanol concentrations between 2.0 and 4.0 M, however, internal volumes of the resulting IFVs increased in a roughly linear manner. IFVs were not formed efficiently at ethanol concentrations above 5.0 M, presumably due to disruption of bilayer integrity. Ethanol at this concentration constitutes 30% (v/v) of the solution.

Fig. 3b shows how the interdigitation-fusion (IF) process depends on the diameter of the precursor DPPC liposomes. In these experiments, the ethanol concentration was held at 4.0 M, which appeared to be near the optimum concentration for obtaining high internal volumes with DPPC SUVs. The diameter of precursor DPPC LUVETs was determined by quasi-elastic light scattering (see Materials and methods). Precursor vesicles below 100 nm yielded DPPC IFVs of the greatest internal volumes. Large IFVs were not observed unless the diameter of the precursor vesicles was 150 nm or less. Vesicles of 200 nm or above were relatively ineffective as precursor liposomes. This steep size dependence was consistent with our earlier work which indicated that only liposomes below approx. 110 nm in diameter were altered in geometry upon induc-

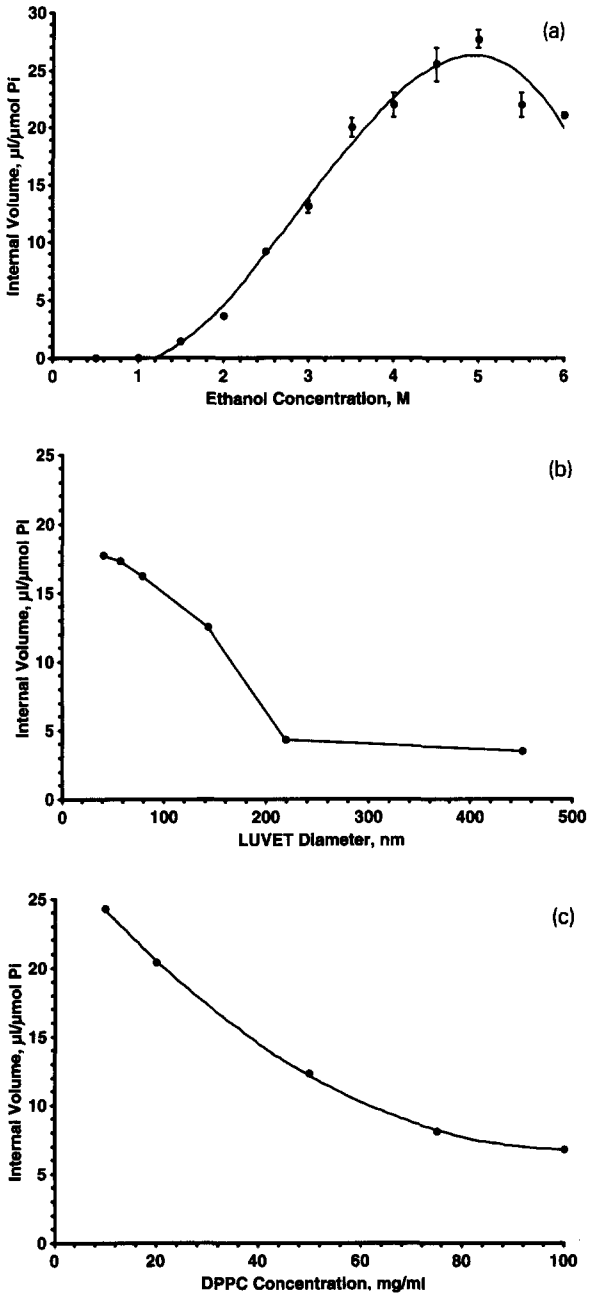

Fig. 3. Effect of formation parameters on the internal volume of DPPC IFVs. (a) Internal volume of DPPC IFVs as a function of ethanol concentration. For ethanol concentrations of 2.0 M and above the DPPC IFVs were formed by direct addition of ethanol to precursor DPPC SUVs which were at 20 mg/ml. For ethanol concentrations of 1.5 M and below the ethanol was prediluted before addition in order to avoid mixing artifacts (see Materials and methods). The internal volume was measured for three separate preparations at each ethanol concentration using the method of Perkins et al. [3]. The error bars indicate the standard deviations of the measurements. (b) Internal volume of DPPC IFVs as a function of precursor DPPC LUVET diameters. DPPC IFVs were formed using 4.0 M ethanol at 20 mg/ml DPPC. The DPPC LUVETs were prepared by extrusion of MLVs through two polycarbonate filters. Diameters of the DPPC LUVETs were determined by quasi-elastic light-scatting (see Materials and methods). (c) Internal volume of precursor DPPC IFVs as a function of DPPC SUV concentration. The interdigitation-fusion was induced with 4.0 M ethanol.

tion of interdigitation. The cutoff is not as abrupt as one might expect here because the population of extruded liposomes is distributed over a range of diameters which includes liposomes above and below the size at which deformation and fusion occurs.

Because fusion processes are generally greatly affected by concentration of reagents, we examined the effect of lipid concentration upon the final captured volume of IFVs. The effect of DPPC SUV concentration on the production of IFVs at 4.0 M ethanol is shown in Fig. 3c. As indicated, internal volumes were maximal when the initial DPPC concentration was 20 mg/ml or less and decreased as the lipid concentration was increased above this value. This is not surprising since at higher lipid concentrations, the amount of aqueous volume not associated with lipids would be expected to become limiting resulting in a tighter packaging of the interdigitated sheets. In fact, while the size distributions for DPPC IFVs formed at 100 mg/ml and 20 mg/ml lipid were similar, their statistical lamellarities determined by ^{31}P-NMR were 2.9 bilayers/vesicle versus 1.1 bilayers/vesicle, respectively. The simplest interpretation for this increased lamellarity is that the sheets, now more oriented because of crowding at high lipid concentrations, curled off as stacked bilayers (concentric stacking) rather than single bilayers to form liposomes. As we have described elsewhere multilamellar liposome systems can exclude solute when formed via certain procedures [6]. Importantly in all the experiments depicted in Fig. 3 the internal volumes measured by the CAT-1 solvent dependent spin label technique did not significantly differ from those determined by labeling internal volumes with [^{14}C]sucrose and assuming ideal distribution of this solute (data not shown). This demonstrates that under the range of conditions described, exclusion of solute did not occur and the resultant IFVs existed in osmotic equilibrium with the formation buffer [3,6]. The fact that solutes are ideally distributed in IFVs is not surprising given the means by which these vesicles form and that the T_m, where permeability is greatest, is traversed twice.

Using parameters optimized for DPPC, we were able to produce IFVs from a variety of different saturated phospholipids. These vesicles were prepared by fusing SUVs at 20 mg/ml in 4.0 M ethanol at temperatures which allowed for the formation of interdigitated sheets. Vesicles were produced by transiently heating the resulting interdigitated sheets above the gel to liquid-crystalline temperature of the phospholipid chosen. Table 1 shows the internal volumes of IFVs produced from DMPC, DPPC, DSPC and DAPC. EPC and DOPC, lipids that do not interdigitate in the presence of ethanol, were included as controls. The internal volumes of vesicles formed from these phospholipids via the same protocol were less than 1 μl/μmol P_i.

We found, however, that IFVs could be produced from binary combinations of lipids if both were capable of

interdigitation. High internal volume IFVs reflective of their constituent lipids were produced from mixtures of DPPC/DMPC, DPPC/DSPC and DPPC/DPPG only if ethanol additions were performed at temperatures favoring the L_β phase for both lipid constituents. In fact, DMPC inhibited the formation IFVs when the addition of ethanol was performed at room temperature, a temperature at which DPPC is in the L_β phase, but DMPC is in the L_α phase. In a typical experiment DMPC/DPPC SUVs (1:1, molar ratio) yielded IFVs with captured volumes of 16.6 μl/μmol lipid, if ethanol was added at 5°C but IFVs with captured volumes of 2.3 μl/μmol lipid if ethanol was added at room temperature. Similarly, cholesterol quite effectively inhibited the formation of DPPC IFVs. This is consistent with the findings of Komatsu and Rowe [23] who found that cholesterol inhibits bilayer interdigitation.

Our findings that the IF process is sensitive to the inclusion of cholesterol (or lipids that do not interdigitate) posed a potential problem regarding the limitation of the technique. Accordingly we searched for a way to avoid such limitations. We found that if a non-interdigitating lipid component was added after ethanol had been allowed to induce interdigitated sheets from an interdigitating component, both components could be induced to fuse into mixed component IFVs. An example of this manipulation is illustrated in Fig. 4. The internal volume of IFVs formed from DPPC/cholesterol SUVs rapidly decreased as the cholesterol content was increased. In contrast, adding cholesterol after the formation of DPPC interdigitated sheets significantly increased the internal volume of the IFVs for a given mol percent cholesterol. Differential calorimetry scans of 35 mol percent DPPC/cholesterol IFVs prepared by either method showed that the DPPC gel to liquid-crystalline phase transition at 41°C was eliminated in both samples suggesting complete mixing of the cholesterol into the phospholipid component.

In summary we conclude that interdigitation-fusion provides a new method for preparing predominately unilamellar liposomes with exceptionally high internal volumes. IFVs are particularly well suited for drug delivery applications which require high drug to lipid ratios. One application is delivery of contrast agents to the liver and spleen in order to improve detection of lesions by X-ray computed tomography [24–26]. In this application high iodine to lipid ratios are required so that the diagnostic concentrations of iodine can be achieved before reticuloendothelial blocking concentrations of lipid accumulate in Kupffer cells. In fact, the IF procedure allows unprecedented iodine to lipid ratios [20]. Moreover, using IFVs carrying the contrast agent iotrolan, we have been able to produce diagnostic images in the livers of rats and dogs of a quality heretofore unobtainable [27]. IFVs will also be useful as model membranes since they are essentially unilamellar and their size is amendable to electrochemical probing.

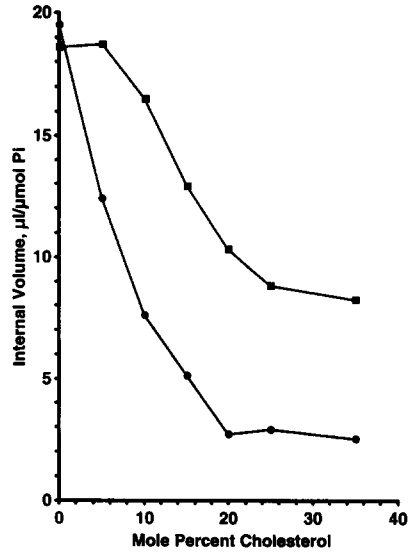

Fig. 4. Incorporation of cholesterol into DPPC IFVs. The final internal volume of DPPC/cholesterol IFVs is compared for two methods of cholesterol incorporation. In one method, DPPC/cholesterol IFVs were formed directly from precursor DPPC/cholesterol SUVs of various mol percents cholesterol (–●–). With this method, the internal volume of the product IFVs decreased rapidly with increased cholesterol content. In the second method, the cholesterol was introduced in the form of DPPC/cholesterol SUVs (1:1, molar ratio) in 4.0 M ethanol after ethanol induced DPPC interdigitated sheets were formed (–■–). This latter procedure produced a significantly higher IFV internal volume at each mol percent cholesterol. Differential scanning calorimetry was used to demonstrate that the cholesterol from the SUVs was incorporated directly into the bilayer of the IFVs. At 35 mol percent cholesterol, the DPPC gel to liquid-crystalline phase transition was eliminated in DPPC/cholesterol IFVs formed by either method. Moreover, the incorporation of ^{14}C-labelled cholesterol into the final IFVs was shown to be equivalent in both methods (data not shown).

Table 1
Internal volume of IFVs formed from saturated phosphatidylcholines

	Phospholipid			
	DMPC	DPPC	DSPC	DAPC
Internal volume (μl/μmol P_i)	6.63 ± 0.06	20.23 ± 0.21	23.67 ± 0.06	11.80 ± 0.96

The average internal volumes are shown for DMPC (di-14:0), DPPC (di-16:0), DSPC (di-18:0), and DAPC (di-20:0). The IFVs were prepared from precursor SUVs at 20 mg/ml using 4.0 M ethanol to induce interdigitation-fusion (see Materials and methods). The internal volumes were measured using the ViVo method of Perkins et al. [20]. The error bars indicate the standard deviation for $n = 3$ measurements. Internal volumes for DAPC IFVs were diminished presumably because of the difficulty of vesicularization from the rigid interdigitated sheets of this highly saturated lipid.

4. Conclusions

We have developed a simple new method for preparing unilamellar liposomes with internal volumes in the 20 to 25 μl/μmol P_i range. We call these liposomes interdigitation-fusion vesicles or IFVs. The method as summarized in Fig. 5 requires the ethanol-induced interdigitation-fusion of

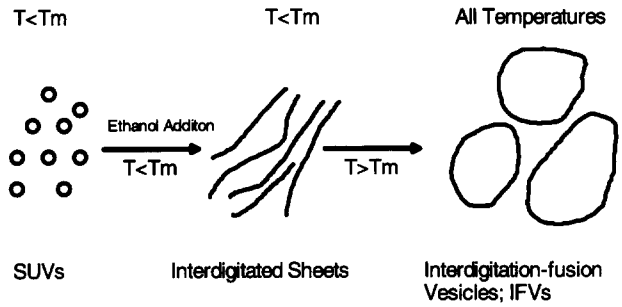

Fig. 5. Liposome formation by interdigitation fusion. See text for details. Vesicles, once formed are stable both above and below the T_m in the presence or absence of ethanol. Ethanol can be removed after vesicle formulation is complete resulting in a noninterdigitated bilayer. If ethanol is not removed, depending on its concentration, the bilayer has the potential to exist in an interdigitated packing motif below the T_m of the lipid.

SUVs into large interdigitated lipid sheets which are subsequently annealed into vesicles. Precursor SUVs composed of saturated phospholipids such as DPPC and DSPC form high internal volume IFVs. Non-interdigitating lipids such as cholesterol, can be introduced into IFVs by admixture with the interdigitated lipid sheets. We have also used high pressure to prepare high internal volume liposomes from SUVs using the interdigitation-fusion process [28]. This technique is particularly attractive because no organic solvents are involved in the process and it produces qualitatively similar vesicles.

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