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A novel strategy for the preparation of liposomes: rapid solvent exchange

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Abstract

During the preparation of multi-component model membranes, a primary consideration is that compositional homogeneity should prevail throughout the suspension. Some conventional sample preparation methods pass the lipid mixture through an intermediary, solvent-free state. This is an ordered, solid state and may favor the demixing of membrane components. A new preparative method has been developed which is specifically designed to avoid this intermediary state. This novel strategy is called rapid solvent exchange (RSE) and entails the direct transfer of lipid mixtures between organic solvent and aqueous buffer. RSE liposomes require no more than a minute to prepare and manifest considerable entrapment volumes with a high fraction of external surface area. In phospholipid/cholesterol mixtures of high cholesterol content, suspensions prepared by more conventional methods reveal evidence of artifactual demixing, whereas samples prepared by rapid solvent exchange do not. The principles which may lead to artifactual demixing during conventional sample preparation are discussed.

Keywords: Cholesterol; Demixing; Homogeneity; Lipid mixture; Liposome preparation; Phase behavior

1. Introduction

Biological membranes are chemically complex mixtures, and any given biomembrane may contain more than a hundred different lipid species [1]. By studying these chemically well-defined models of biomembranes, researchers have sought physical-chemical insight into membrane biology.

Our laboratory has been studying phospholipid/cholesterol membranes in the regime of high cholesterol content. Specifically, we have sought to determine the maximum solubility of cholesterol in bilayers composed of various phospholipids. Above this limit, excess cholesterol precipitates as crystals of pure cholesterol monohydrate.

Initially, we expected that this solubility limit would be straightforward to determine: simply prepare suspensions of increasing cholesterol content and examine each sample for the presence of crystals. We found that several methods (e.g. 90° light scattering, X-ray diffraction, ultrafiltration) could be used to detect cholesterol crystals, each with its own advantages and disadvantages. But regardless of detection method, one conclusion eventually became

Abbreviations: FEP, fluorinated ethylene propylene; LUV, large unilamellar vesicle; NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; PIPES, piperazine-1,4-bis(2-ethanesulfonic acid); SUV, small unilamellar vesicle

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clear: the reproducibility of these 'straightforward' solubility limits was surprisingly poor. We were led to conclude that the source of this variability was inhomogeneity in the samples themselves and we found that other workers had reported difficulty in preparing homogeneous samples of high cholesterol content [2]. Over the course of our research, we have learned that artifactual crystal formation can occur during conventional sample preparation [3]. This may help to explain the widely ranging values reported in the literature [3,4] for cholesterol solubility limits.

When preparing any model membrane, it is generally important that the lipids remain well mixed throughout the sample preparation procedure. This is especially true for physical-chemical studies of lipid mixtures, such as those of phase behavior. But the preparation methods which are most commonly used in such studies entail an incubation of the lipid mixture in the form of a solvent-free film or powder, which are solids. It has long been known that solid-state mixtures are, in general, far more likely to phase separate (i.e. demix) than non-solid mixtures [5]. Therefore, we became concerned that the spurious cholesterol crystal formation in our samples could be traced to phase behavior in the solid-state film or powder.

Here we describe the development of a novel method for model membrane preparation which we call rapid solvent exchange (RSE). This method is specifically designed to form compositionally homogeneous suspensions by the sudden precipitation of a lipid mixture in aqueous buffer. This is achieved by rapidly exchanging the lipids between an organic solvent environment and an aqueous environment. Accordingly, membrane structures are formed de novo in water without passing the lipid mixture through an intermediary solid state. Phospholipid/cholesterol suspensions have proven to be free of artifactual crystals when prepared by rapid solvent exchange.

2. Materials and methods

2.1. Chemicals and materials

Phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL) and cholesterol was purchased from Nu Chek Prep (Elysian, MN). Purity (>99%) was confirmed by thin layer chromatography on washed, activated silica gel plates (Alltech Associates, Deerfield, IL), developing with chloroform/methanol/water = 65:25:4 for phospholipid analysis or with petroleum ether/ethyl ether/chloroform = 7:3:3 for cholesterol analysis. All solvents used were of HPLC grade. TLC plates were quantitated by charring and densitometry. (16:0,18:1)-NBD-N-PE was synthesized and purified according to a procedure already described [6,7]. Phospholipid stock solutions, as well as some suspensions, were quantitated by phosphate assay [8]. Aqueous buffer (pH 7.0, 5 mM piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES), 200 mM KCl) was prepared from purified water (Milli-Q system, Millipore) and filtered through a 0.1 μm filter before use. Fluorinated ethylene propylene (FEP) Teflon Oak Ridge centrifuge tubes were purchased from Nalge. Anhydrous $MgSO_4$ was of analytical reagent grade (Mallinckrodt). Deuterated benzene (99.6% ^2H) and chloroform (99.8% ^2H) were purchased from Cambridge Isotope Laboratories (Andover, MA). Dialysis membrane was Spectra/Por brand (Spectrum Medical Industries). 5(6)-Carboxyfluorescein (Eastman Laboratory Chemicals, Rochester, NY) was used without further purification. Sodium hydrosulfite was purified grade (Fisher Sci.). 1 mm, special glass X-ray capillaries were purchased from Charles Supper (Natick, MA).

2.2. Rapid solvent exchange

The apparatus employed was developed through testing performed on a prototype. These devices were fabricated with the use of the Graduate Research Machine Shop facility supported by the Cornell Laboratory of Applied and Solid State Physics. The vacuum was maintained by a 1/3 h.p. mechanical pump (Gast, Benton Harbor, MI) with an adjustable inlet valve for pressure regulation, and the pump outlet was vented to a laboratory hood. Either 10 ml or 50 ml FEP Teflon Oak Ridge tubes served as vessels for solvent exchange. During RSE, precipitating lipids manifest transiently high surface activity and FEP tubes were judged least likely to adsorb lipid. A 100 μl gas-tight, blunt-tipped syringe (point-style #3, Hamilton, Reno, NV) was used to intro-

duce the lipid solution (10–100 μl of 10–100 mM lipid) to the injection tubing; see Section 3 for a description of the procedure. Standard vacuum pressure was 23 torr for sample preparation at room temperature (23°C). Samples containing di16:0-PC were prepared by RSE into buffer which was maintained at 50°C. For RSE at this temperature, the vacuum pressure was adjusted to 100 torr and the sample vessel was enclosed in a forced-air heating jacket which was coupled to a heat gun.

2.3. Conventional sample preparations

2.3.1. Film deposition

13×100 mm glass test tubes, each containing between 100 and 300 μl of a lipid solution in chloroform, were mounted on an analytical evaporator (Organomation Associates, South Berlin, MA) and bulk solvent was evaporated under a gentle stream of nitrogen gas. The test tubes were loaded into a vacuum desiccator which was then coupled to a vacuum line and the lipid films were incubated overnight under a measured pressure of approx. 30 millitorr. The films were hydrated with aqueous buffer at room temperature and vortexed for 1 min to disperse the lipid. Samples containing di16:0-PC were hydrated and vortexed at 50°C, before being allowed to cool to room temperature.

2.3.2. Lyophilization

13×100 mm glass test tubes, each containing between 100 and 300 μl of a lipid solution in cyclohexane/methanol (99:1), were plunged into a bath of liquid nitrogen. The frozen samples were loaded into a pre-cooled desiccator which was then coupled to a vacuum line. The desiccator was kept on water ice until the bulk solvent had been removed (2–3 h) after which the samples were allowed to warm to room temperature. The vacuum incubation continued overnight at approx. 30 millitorr. The voluminous, white lipid powders were hydrated and vortexed in the same manner as the films.

2.3.3. Freeze-thawing

Suspensions were subjected to five cycles of freeze-thawing, between a bath of liquid nitrogen and a room temperature water bath.

2.3.4. Extrusion

Large unilamellar vesicles (LUVs) were prepared by passing a suspension 10 times through a double stack of 0.1 μm Nucleopore polycarbonate membranes (Costar, Cambridge, MA), using a modified extruder apparatus (Lipex Biomembranes, Vancouver, B.C.).

2.4. Solvent residue analysis

Liposomes were prepared by rapid solvent exchange from 100 μl of organic solvent (25 mM lipid) into 5 ml of aqueous buffer. In order to quantitate solvent residue remaining at a given post-injection time-point, eight replicates were pooled and the suspension was centrifuged (20 000×g×5 min) to collect the vesicles in a pellet. The clear, lipid-free aqueous supernatant was removed and the pellet transferred, along with about 50 μl of associated buffer, to a 13×100 mm glass tube and dispersed in 1.0 ml of deuterated solvent. The resulting lipid-stabilized emulsion (buffer in deuterated solvent) was broken by chemical dehydration: 100 mg of anhydrous $MgSO_4$ was added, followed by vortexing and low-speed centrifugation. The desiccant pelleted, leaving a clear supernatant free of bulk water. This procedure quantitatively recovers both lipid and solvent residue from the original aqueous suspension, while leaving little water in the extract. Without dehydration, the extracts yielded NMR spectra containing large water peaks which interfered with the analysis.

When the injection solvent was dichloromethane, deuterated chloroform was used for sample extraction. In addition, samples for dichloromethane analysis were prepared from lipids with fully saturated acyl chains. This was to avoid interference from olefinic ^{1}H, whose resonance is near to that of dichloromethane ^{1}H. di12:0-PC was used to prepare samples at room temperature and di16:0-PC for sample preparations at 50°C.

When the injection solvent was chloroform, deuterated benzene was used for sample extraction. Since the chloroform ^{1}H resonance is well resolved from that of olefinic ^{1}H, 16:0,18:1-PC was used to prepare suspensions for chloroform analysis, at both room temperature and 50°C.

^{1}H NMR spectra were collected on a Varian XL-200 NMR spectrometer with a 3 s pulse delay. Typically, 160 transients were collected, though as many as 1000 transients were taken when high sensitivity was desired. When a solvent peak could be resolved (Fig. 1), it was integrated along with a nearby lipid proton peak, establishing a molar ratio between lipid and solvent residue. Control samples containing a known quantity of solvent residue (0.1–1.0 mole%) were extracted and analyzed to validate the procedure.

2.5. X-Ray diffraction

RSE liposomes (approx. 1 mg of lipid) were prepared and transferred to X-ray capillary tubes. These capillaries were then filled to the neck with buffer and placed in a buoyant support apparatus (Fig. 2) which allows the capillaries to withstand high speed centrifugation in a swinging bucket rotor. Vesicles were generally pelleted in the capillaries for 15 min at $20\,000 \times g$, after which they were sealed with paraffin wax under argon gas.

In order to observe the effects of centrifugation on the diffraction profile of RSE vesicles, replicate samples of 16:0,18:1-PC were prepared. Each of these suspensions was then exposed to centrifugation at a different level of centripetal force.

Diffraction data were collected at the Macromolecular Diffraction Facility of the Cornell High Energy Synchrotron Source (MacCHESS), on the F-1 beamline. Samples were exposed to an intense synchrotron beam of wavelength 0.908 Å, which was passed through a 0.2 mm collimator. Data were recorded either on Fuji imaging plates and then digitized by scanning, or with a Princeton 2K CCD detector containing 2048×2048 41-micron pixels [9,10]. When desired, circular integration was performed to transform the powder patterns into one-dimensional profiles of diffraction intensity.

2.6. External surface assay

Accessible external surface was assayed by a 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD)/dithionite assay [6]. In short, fluorescence is quantitated in samples containing a fluorescent surface marker (headgroup-labeled-NBD) both before and after chemical bleaching of the accessible external fraction by aqueous dithionite reagent. Subsequent addition of detergent micellizes the liposomes, resulting in complete bleaching of the remaining fluorophore.

For this assay, suspensions were prepared from 0.25 μmole of lipid (16:0,18:1-PC with 0.5 mole% 16:0,18:1-NBD-N-PE) in 5 ml of buffer. Five replicate samples were prepared by each of three methods: film deposition/freeze-thawing, film deposition/extrusion and rapid solvent exchange. Dithionite reagent (1 M) was prepared as described by Gruber and Schindler [6].

To estimate the accessible external marker fraction, 2 ml of sample was placed in a quartz cuvette with a masked stir bar, gently rotating at a set speed. After establishing the pre-dithionite signal, 5 μl of dithionite reagent was added to the cuvette. When the rapid bleaching phase was complete and the post-dithionite signal had been established, 50 μl of 20% (v/v) Triton X-100 was added to the cuvette to demonstrate complete bleaching.

Fluorescence time scans were collected on a Hitachi Fluorescence Spectrometer (model F-3010) with excitation at 470 nm (470 nm interference filter; 1.5 nm slit) and emission at 540 nm (480 nm long-pass filter; 10 nm slit). With our samples (0.5 mole% NBD, 50 μM suspension), this arrangement yielded negligible background signal ($< 0.05\%$), even with

Table 1
External surface fraction and entrapped volume of different liposome preparations

Preparative method	External surface fraction (%)	Entrapped volume (μl/μmole)
Film deposition	n.d.	0.25 (±0.04)
Lyophilization	n.d.	0.31 (±0.04)
Film deposition/extruded (LUV)	47.1 (±3.9)	n.d.
Film deposition/frozen and thawed (FT-MLV)	13.4 (±2.5)	4.07 (±0.06)
Rapid solvent exchange	33.1 (±1.6)	4.50 (±0.09)

Values presented are averaged from replicate samples (see Section 2); the range of values observed is shown in parentheses.

turbid suspensions. This was demonstrated by control samples prepared without fluorophore. For this reason, no corrections were necessary for turbid samples.

As observed by Gruber and Schindler, we found that turbid samples did yield higher fluorescence intensities (data not shown), but in our case this must have been due to a greater effective illuminated volume caused by scattering of light within the sample. Since this signal enhancement was significant (approx. 40%), we verified that the signal decrease upon dithionite addition was not due to changes in the light scattering properties of the suspension. Control samples were observed by 90° light scattering at 470 nm, and subjected to both dithionite and Triton addition as in the fluorescence assay. Dithionite injection had no effect on light scattering intensity (data not shown), so post-dithionite signal loss can safely be attributed to bleaching only. Triton addition decreased scattering intensity as expected, since this treatment micellizes the sample.

2.7. Entrapment assay

1 mM carboxyfluorescein (CF) was prepared in aqueous buffer and this was used to prepare three replicate samples by each of four methods: film deposition, lyophilization, film deposition/freeze-thawing and rapid solvent exchange. Samples contained 5 mg of 16:0,18:1-PC in 5 ml of buffer.

After preparation, suspensions were transferred to dialysis tubing (18 mm, 50 kDa MW cut-off) and dialyzed together against 600 ml CF-free buffer (changed every 3 h for 24 h) in order to remove unentrapped fluorophore. After dialysis, the CF remaining in each sample was quantified by fluorescence. In a quartz cuvette with a masked stir bar, 100 μl of the dialyzed sample was added to 3 ml of buffer containing 2% Triton X-100, emulsifying the liposomes and relieving the self-quenching of entrapped fluorophore. Each sample was also subjected to phosphate analysis to verify the phospholipid concentration in the suspension after dialysis, although no losses were ever observed. Control samples (CF buffer without lipid) were included in each experiment to quantitate the very low level of unentrapped, residual carboxyfluorescein remaining after dialysis.

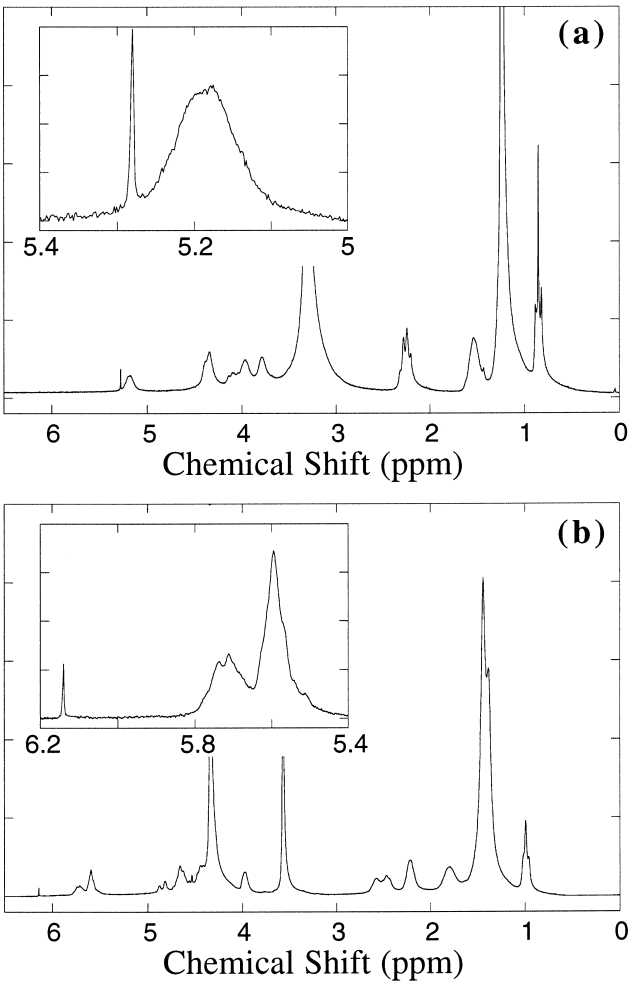

Fig. 1. Quantitation of solvent residue in aqueous lipid suspensions. ^{1}H NMR spectra of suspension extracts. Insets show expanded view of solvent peak together with adjacent lipid peak(s). (a) di12:0-PC with CH_2Cl_2 residue in $CDCl_3$. Integration of $C\underline{H}_2Cl_2$ peak (5.28 ppm) together with lipid glycerol backbone $\underline{H}$COCO peak (5.19 ppm broad) yield a solvent/lipid ratio = 0.036 for this sample. (b) 16:0,18:1-PC with $CHCl_3$ residue in C_6D_6. Integration of $C\underline{H}Cl_3$ peak (6.14 ppm) together with both nearby lipid peaks, $C\underline{H}=C\underline{H}$ (5.60 ppm broad) and $\underline{H}$COCO (5.72 ppm broad), yield a solvent/lipid ratio = 0.026.

2.8. Differential scanning calorimetry

Calorimetric measurements were made with a MicroCal MC-2 scanning calorimeter, at a scan rate of 0.18°C/min. di16:0-PC RSE liposomes were prepared at 50°C and then transferred to a 50°C water bath which was cooled to room temperature overnight. Samples were degassed for 5 min and were kept under nitrogen at 18 psi during data collection.

3. Results

Sample preparation by rapid solvent exchange is accomplished with the apparatus pictured in Fig. 3. This consists of a vacuum manifold, which couples an FEP Teflon sample tube to the vacuum line, and a laboratory sample vortexer. The vacuum pressure is adjusted to be slightly higher than the vapor pressure of water; we routinely set it to approx. 23 torr for sample preparation at room temperature. The FEP tube, containing aqueous buffer, is mounted on the vortexer. The vortexer is actuated, forming the buffer into a cylindrical shell, and the manifold is opened to the vacuum. Next, a gas-tight, blunt-tipped syringe containing the lipid solution in organic solvent is positioned at the injection port. Finally, the syringe needle is seated in the coupling sleeve and the syringe plunger is quickly depressed, forcing the solution down through the stainless steel injection needle and out through a narrow side-orifice in the tip (Fig. 4). As the organic solution passes through this orifice it experiences a sudden drop in pressure so that vaporization of the solvent begins, along with some evaporative cooling. The orifice is only about a centimeter from the tube wall, so the solution droplets quickly contact the vortexing buffer which serves as a heat reservoir, transferring heat to the droplets so that vaporization can proceed to completion. The cylindrical shell of buffer presents a large surface area, and the vortexing action aids in the transfer of solvent vapor to the vacuum. In this way, bulk organic solvent is very rapidly and very efficiently vaporized. Solvent residue analysis shows that $>99.99\%$ of dichloromethane is removed within a second of injection (Figs. 5 and 6). In order to remove trace, residual solvent the sample continues to vortex, maintaining maximal buffer surface area under reduced pressure for at least 20–30 s after injection. By this point no detectable solvent remains (Fig. 6), but our standard procedure is to leave each

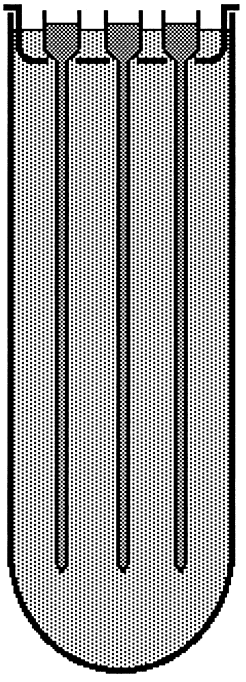

Fig. 2. Buoyant support for centrifugation of samples in X-ray capillaries. A 28.5×105 mm polycarbonate tube is filled with water and a friction-fit polypropylene closure, in which eight 2 mm diameter holes have been drilled, is pressed into place. The water level is adjusted to be approx. 3–5 mm above the bottom of the closure. X-Ray capillaries, containing liposome suspensions and filled with buffer up into the neck, are lowered into the holes. Supported in this way, the thin-walled capillaries can withstand centripetal forces in excess of $20\,000\times g$. The centrifuge must be started gently, so that capillaries are not broken against the tube wall by the sudden torque on the rotor.

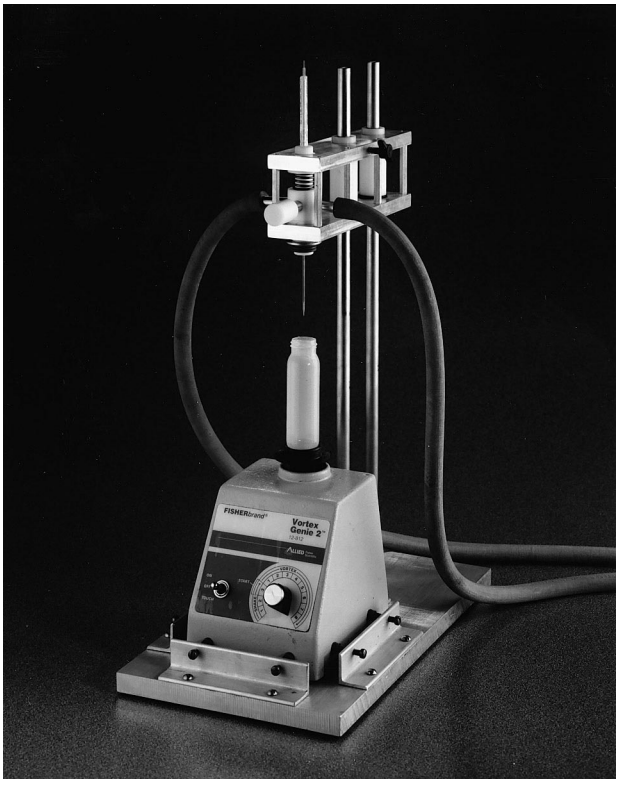

Fig. 3. Rapid solvent exchange apparatus.

sample vortexing under vacuum for 1 min after injection.

The X-ray diffraction pattern of 16:0,18:1-PC RSE liposomes is that of a fluid-phase bilayer with the expected lamellar repeat spacing of about 65 Å. The diffraction is rather weak, though, unless the sample is subjected to centrifugation, as illustrated by the diffraction profiles of samples exposed to increasing sedimentary force (Fig. 7). This is consistent with images we obtained by thin-section electron microscopy, which showed little multilamellar stacking in suspensions of RSE vesicles (data not shown). Likewise, the high fraction (approx. 33%) of accessible external surface (Table 1) is consistent with a low average lamellarity in RSE preparations.

RSE liposomes appear to be similar to frozen and thawed (FT) vesicles in aqueous entrapment volume, according to our entrapment assay (Table 1). No

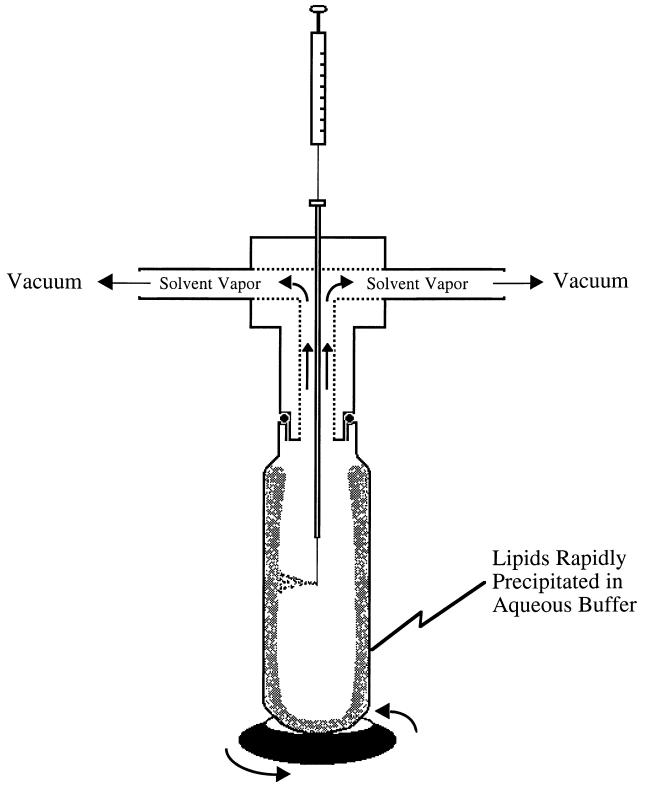

Fig. 4. Cross-sectional schematic of the process of rapid solvent exchange. A blunt-tipped, gas-tight syringe is seated in the injection sleeve and the plunger is depressed, forcing the lipid solution down through the injection needle and out the side-orifice. Bulk solvent vaporizes and is removed within about a second, while the lipid mixture precipitates in aqueous buffer.

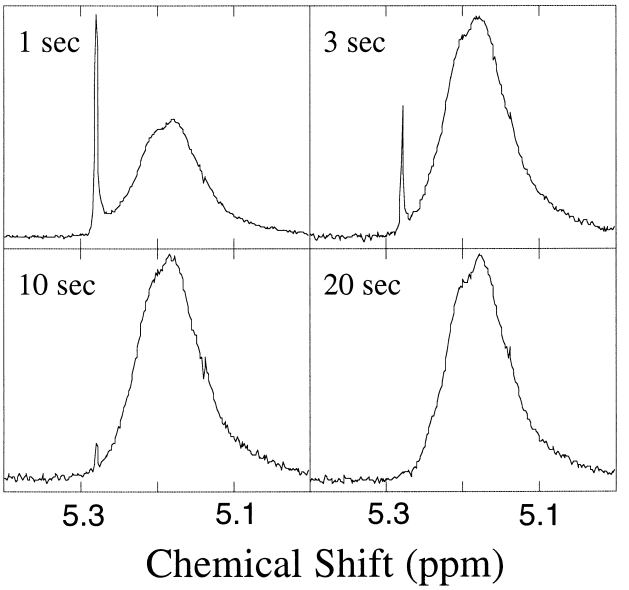

Fig. 5. Removal of dichloromethane residue at 23°C. Expanded ^{1}H NMR spectra (as in Fig. 1a inset) of extracted di12:0-PC samples prepared by rapid solvent exchange from a dichloromethane solution at room temperature. Shown are spectra for extracts at 1, 3, 10 and 20 s post injection. Bulk solvent is quickly vaporized; the solvent/lipid mole ratio (approx. 600 in the injection solution) has been reduced to approx. 0.05 one second after injection. Residual solvent is then removed by continued vortexing under vacuum so that by 20 s, no detectable solvent peak remains.

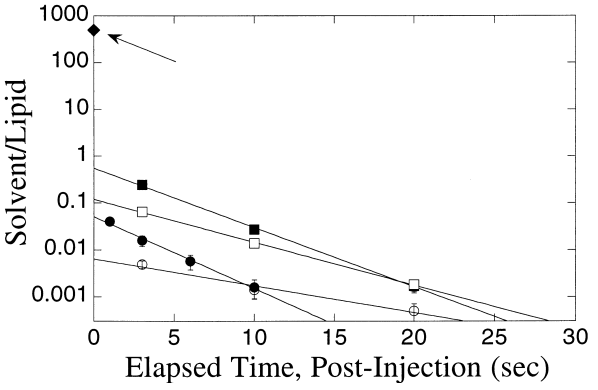

Fig. 6. Quantitative time courses of solvent residue removal. Zero time point (arrow) indicates solvent/lipid ratio in 25 mM injection solution. ●, dichloromethane removal at 23°C; each point represents the averaged result of five independent experiments. ○, dichloromethane removal at 50°C; results of three independent experiments. ■, chloroform removal at 23°C; three experiments. □, chloroform removal at 50°C; three experiments. The lines through the data represent exponential fits. Error bars span the range of solvent/lipid ratios obtained and the lower limit of the ordinate axis is the estimated detection limit of our assay.

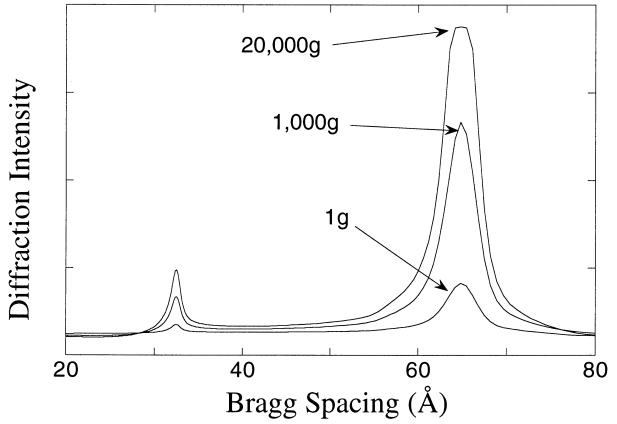

Fig. 7. X-Ray diffraction of liposomes prepared by rapid solvent exchange. X-Ray diffraction profiles for 16:0,18:1-PC suspensions subjected to increasing centripetal force. Both beam intensity and exposure time were identical. The first-order repeat is centered at about 65 Å, and the second-order repeat at about 32.5 Å. Centrifugation improves the diffraction intensity, though the positions and shape of the peaks are unaffected.

attempt was made to account for the possible slow leakage of carboxyfluorescein, and since the timescale of the dialysis assay was >24 h the estimated entrapment volumes should be regarded as lower limits.

Differential scanning calorimetry of di16:0-PC RSE liposomes yielded a thermogram typical of di16:0-PC (data not shown), revealing both a pretransition around 35°C and a sharp main transition centered at 41.3°C.

Fig. 8 shows X-ray diffraction powder patterns of a hydrated di16:0-PC/cholesterol mixture prepared in parallel by either film deposition or rapid solvent exchange. Cholesterol crystals, which here indicate artifactual demixing (see Section 4), are present in the sample prepared by film deposition (Fig. 8a). No crystals are evident in samples prepared by RSE (Fig. 8b).

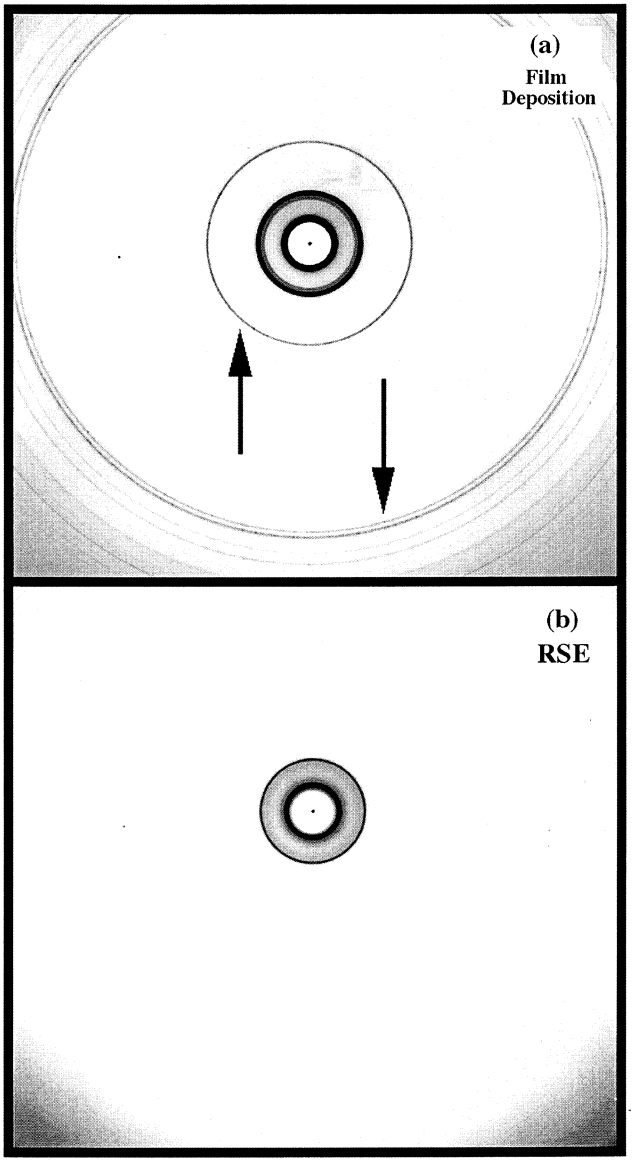

Fig. 8. X-Ray diffraction reveals artifactual cholesterol crystals in a sample prepared by film deposition, but not in a sample prepared by rapid solvent exchange. Powder patterns are shown for aqueous suspensions of di16:0-PC/cholesterol at 50 mole% cholesterol, prepared by either film deposition or rapid solvent exchange. This concentration of cholesterol is more than 15 mole% below the true solubility limit in a hydrated bilayer of di16:0-PC [3]. Both preparations began with an aliquot taken from the same solution of phospholipid and cholesterol codissolved in chloroform. (a) In the sample prepared by film deposition, the presence of crystalline cholesterol monohydrate is indicated by a characteristic pattern of lines at both low and wide angles (arrows). (b) No artifactual crystals appear in samples prepared by RSE; only lamellar-phase lipid is evident.

4. Discussion

4.1. Properties of RSE liposomes

Rapid solvent exchange is a simple, convenient method for the preparation of liposomes, requiring no more than 1 min for each sample. RSE membranes form de novo within an aqueous environ-

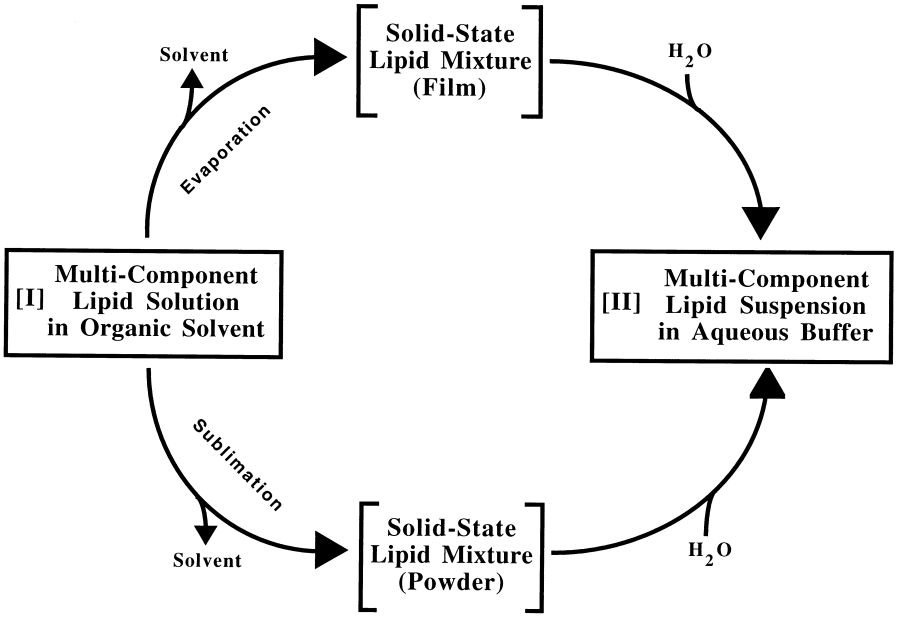

Fig. 9. Two conventional preparation methods which entail an intermediary solid-state lipid mixture. Film deposition (upper pathway): bulk solvent is removed by evaporation, depositing a solid-state lipid film on the wall of the sample vessel. After an extended vacuum incubation to remove residual solvent, the film is hydrated to form an aqueous lipid suspension. Lyophilization (lower pathway): the sample is frozen and bulk solvent is removed by sublimation, leaving a voluminous lipid powder. After vacuum incubation to remove residual solvent, the powder is hydrated to form an aqueous lipid suspension.

ment, so that larger entrapment volumes are achieved, and aqueous solutes should be uniformly distributed across lamellae. The suspensions are of low average lamellarity, with a high fraction of external surface area. More to the point, rapid solvent exchange does not pass the lipid mixture through an intermediary solid state.

4.2. RSE prevents artifactual cholesterol demixing

For some time, our laboratory has studied phospholipid/cholesterol bilayers in the regime of high cholesterol content. We have found [3] that cholesterol has a strong tendency to demix from such mixtures during sample preparation by either of the two methods most commonly used in physical-chemical studies of model membranes: film deposition and lyophilization. As discussed below, these preparative methods entail an intermediary, solid-state lipid mixture. Cholesterol tends to precipitate from solid-state lamellae at concentrations which are well below its true solubility limit in hydrated lamellae [3]. When a phospholipid/cholesterol suspension is prepared in this way, any demixed cholesterol can remain demixed after hydration, becoming crystals of pure cholesterol monohydrate. The presence of these crystals is clearly revealed by X-ray diffraction, as in Fig. 8.

The true solubility limit of cholesterol in a hydrated bilayer is an important parameter for the physical-chemical study of phospholipid-cholesterol interactions. Our studies of several phospholipid/cholesterol mixtures were frustrated by experiments which were based on either of the two more conventional preparative methods. These yielded apparent solubility limits whose reproducibility was poor, and which proved to be falsely low due to demixing artifacts. In contrast, experiments based on RSE preparations have proven to yield reproducible solubility limits. For every phospholipid/cholesterol mixture, these solubility limits have been higher than was indicated by the conventional preparations [3], consistent with the elimination of a demixing artifact.

4.3. Two conventional methods of preparation: film deposition and lyophilization

Liposome preparations are all aqueous lipid suspensions rather than solutions. On the other hand,

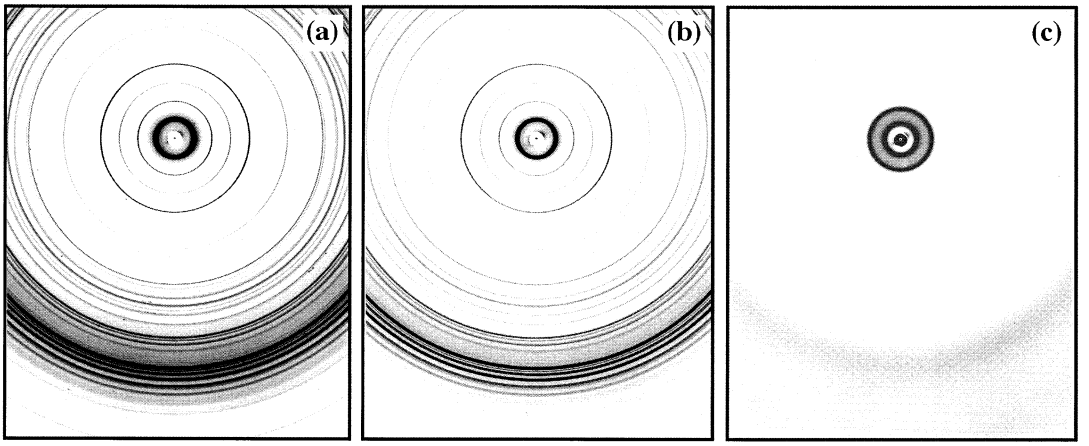

Fig. 10. X-Ray diffraction powder patterns contrasting the solid-state lamellar and hydrated, fluid lamellar states. Results are shown for 16:0,18:1-PC/cholesterol mixtures at 10 mole% cholesterol, but similar results were observed for other lipid mixtures. (a) Solvent-free solid-state mixture prepared by film deposition from chloroform. Note the multiple orders of low-angle rings, produced by intense diffraction from stacked lamellae formed in the solid state. Moreover, the dense array of wide-angle rings indicates that the solid-state lipid is highly organized within the lamellae, revealing many different, characteristic structural spacings all on the order of a few angstroms. (b) Solvent-free solid-state mixture prepared by lyophilization from cyclohexane/methanol 99:1. The diffraction pattern is similar to that in (a). (c) Lipid mixture film, as in (a), after hydration in aqueous buffer. The solid-state lamellae have swollen and fluidized; the many wide-angle rings have been replaced by a single, gentle undulation which is characteristic of a fluid-state bilayer.

lipids are purified, quantitated and stored as solutions in organic solvent. As a consequence, essentially all methods for liposome preparation begin with a lipid solution in organic solvent and end with a lipid suspension in water (Fig. 9). The various components are typically combined by codissolving the lipids in an organic solvent and the organic solvent is then most often removed by either of two methods: film deposition (Fig. 9 upper pathway) or lyophilization (Fig. 9 lower pathway). When all the solvent has been removed, the solid lipid mixture is hydrated with aqueous buffer. Upon exposure to water, the lipids spontaneously swell to form liposomes [11,12]. At this point, many methods diverge, processing the suspensions in various ways to affect their properties. Among these post-hydration treatments are vortexing, sonication [13], freeze-thawing [14], and high-pressure extrusion [15,16].

4.4. Intermediary solid-state lipid mixtures

Both film deposition and lyophilization entail a prolonged incubation in an intermediary state, neither organic solution nor aqueous suspension: the intermediary solid-state lipid mixture (Fig. 9, film/powder). It is well known that solvent-free lipids spontaneously form solid-state lamellae which exhibit an endothermic melting transition upon heating [12,17,18]. Likewise, X-ray diffraction reveals that these solid-state lamellae are highly structured [19], far in excess of the order observed in the hydrated, fluid-state bilayer (Fig. 10).[1] Our concern is that some apparent phase behavior observed in hydrated lipid mixtures may in fact be traced to the intermediary solid state. More than 20 years ago, Gershfeld argued that this sort of effect could explain disparate reports on phase behavior in phospholipid/cholesterol mixtures [21].

It is the hydrated bilayer which is the biologically relevant, physical-chemical state of interest, but both film deposition and lyophilization take the lipid mixture from solution to aqueous suspension via a long-

[1] It is interesting to note that the lamellae which we seek to study in water are formed *in a non-aqueous environment* by these preparation methods. The process of hydration is really then a process of swelling [11,12], or the penetration of water across these multilamellar structures. Of course, solutes permeate more slowly and mechanical treatments are employed, after hydration, to achieve uniform distribution of solutes across lamellae [14]. The lamellae themselves undergo transitions to new physical states associated with the shift to an aqueous environment [12,17,18,20,27], for example, the transition from an ordered, solid lamellar state to a fluid bilayer state upon hydration (compare Fig. 10a with c).

lived, intermediary solid state with its own phase diagram. The tendency of any mixture to remain stably mixed correlates with the phase state of the system: gas, liquid or solid. Gas-phase mixtures represent one extreme, being miscible in all proportions for all known cases. Solid-phase systems represent the other extreme. While some multi-component solids are known to be stably mixed, forming a single-phase 'solid solution', it is generally true that solid-phase mixtures can manifest complex phase behavior [5], resulting in the demixing of components.

4.5. Demixing in solid-state mixtures

For example, solvent-free mixtures of *n*-alkanes can have complicated phase behavior in the solid state. Binary mixtures of *n*-octane and *n*-decane are fully miscible in the fluid state but are largely immiscible in the solid state, phase separating between 2% and 90% *n*-octane [22]. Similarly, mixtures of *n*-eicosane (*n*-$C_{20}H_{42}$) and *n*-docosane (*n*-$C_{22}H_{46}$) are fully miscible in the fluid-state but manifest complex phase behavior in the solid state, with several different regimes of two-phase coexistence below the solidus temperature [23]. Recently, Dorset et al. have observed that while freshly solidified mixtures of *n*-$C_{30}H_{62}$ and *n*-$C_{36}H_{74}$ appear to be well-mixed, aged samples reveal extensive demixing between 10% and 90% *n*-$C_{36}H_{74}$ [24].

It has long been known that similar demixing can occur in solid-state mixtures of amphiphiles. Alkanols of differing chain lengths can be largely immiscible in the solid state, and the tendency to demix is enhanced when a primary alkanol is mixed with a secondary alkanol [25]. Binary mixtures of fatty acids will phase separate in the solid state, if one component is saturated and the other is unsaturated, or if the difference in chain length is significant [26]. Anhydrous soaps have shown similar tendencies in the solid state [27]. While studying a natural lipid extract by X-ray diffraction, Bear et al. concluded that the anhydrous lipid mixture showed evidence of three distinct lipid phases [28]. While studying the effects of drying on myelin, Elkes and Finean [29] observed the appearance of three new Bragg spacings which they attributed to lipid phase separation during the drying process. Finally, as discussed above, cholesterol can demix from solid-state mixtures with phospholipid, forming crystals of anhydrous cholesterol. Several groups have reported the presence of cholesterol crystals in solid-state phospholipid/cholesterol mixtures [3,28,30,31].

In all of these cases, components with significant structural differences seem to have a greater tendency to demix from each other. This is not surprising, considering that solids are characterized by a high propensity for organization and that space-filling, close-packing structures are favored in the solid state [32].

4.6. Possible manifestations of solid-state demixing in hydrated lipid mixtures

Of course, even if a solid-state mixture is unstable, demixing may be very slow to occur. On the timescale of sample preparation, many solid-state lipid mixtures are likely to remain well mixed, even as metastable solid solutions. However, in metastable solid solutions of hydrocarbons, the rate of demixing is known to be accelerated by differences in molecular structure [33], so it seems likely that some metastable solid-state lipid solutions could demix significantly during conventional sample preparation. If this occurs, then the resulting liposome suspension could be artifactually heterogeneous. This artifactual demixing could be either reproducible or variable in extent, depending on both the particular mixture and the timescale of sample preparation. In the case of reproducible solid-state demixing, the apparent phase behavior could be falsely assigned to the hydrated lipid mixture. On the other hand, variable (non-equilibrium) solid-state demixing might account for some lipid mixture formulations which are not well behaved, manifesting poorly reproducible properties.

4.7. Strategy of rapid solvent exchange

The strategy behind RSE is simple. Consider, for example, a chloroform solution of several lipid species. The interactions between chloroform and lipid molecules are favorable, so the lipids are solvated and can be considered to be well mixed. Now, imagine quickly exchanging the chloroform for water. Upon exposure to water, the lipids will begin to form lipidic colloids and the kinetics of this precipitation process should be collision-limited, so that

colloid formation is rapid and indiscriminate of species-specific interactions. During this collision-limited precipitation, the various lipid species ought to form compositionally homogeneous lipidic particles. These homogeneous particles can then relax to form stable structures de novo within the biologically relevant, aqueous environment.

4.8. Other methods compared with RSE

Of course, other sample preparation methods already exist which do not entail an intermediary solid-state lipid mixture. Like RSE, they should produce suspensions free of solid-state demixing artifacts. These other methods fall into two categories, depending on the nature of the organic solvent.

Preparation from a miscible organic solvent: liposomes can be formed by precipitation from an organic solvent which is miscible with water (see for example [34–36]). Lipid is dissolved in a suitable solvent (methanol, etc.) which is then mixed with water, causing lipids to precipitate. Unfortunately, poor lipid solubility is often an issue with such solvents. There is also the problem of residual solvent: since the solvent is miscible with water, removal requires exchange of the bulk aqueous buffer (e.g. by dialysis or gel-filtration chromatography) and this can be inconvenient and time-consuming. A final, important note to these procedures is that they can produce a high proportion of small unilamellar vesicles (SUVs) [34].

Preparation from an immiscible organic solvent: several publications have described the preparation of vesicles by evaporation of water-immiscible solvent from an organic solution of lipids which is in contact with aqueous buffer (see for example [37–41]). It is somewhat surprising that the publications describing these methods often do not provide the results of a quantitative assay for solvent residue. Moreover, we are not aware that any such publication has quantitated the time course of solvent removal, so we cannot assess the timescale of either bulk or residual solvent removal. It seems clear that the timescale of solvent removal has not been a controlling parameter in the design of the 'vaporization' methods published to date. Rather, the explicit goal has been to produce suspensions of low lamellarity and/or to entrap solutes. In contrast to these other methods, RSE has been specifically designed for the rapid, efficient removal of solvent and the timecourse of this process has been characterized quantitatively.

If solvent is not quickly and efficiently removed upon exposure of the lipid mixture to water, at least two concerns may be raised. First, some materials in the buffer may be sensitive to prolonged solvent exposure. For example, globular proteins targeted for entrapment may be irreversibly denatured by significant quantities of solvent [38], especially as the timescale of exposure increases. Second, lipid structures are likely to form in the presence of significant solvent, and this may create artifactual properties which remain even after solvent has been removed. Consider the 'ethanol injection' method, which tends to form a large proportion of SUV. The SUV structure, which is thermodynamically unstable, persists after the residual solvent has been removed or diluted [34].

We developed rapid solvent exchange in order to have a method which would better approximate the collision-limited precipitation strategy outlined above. RSE transfers the lipid mixture quickly between an essentially pure solvent environment and an essentially pure aqueous environment. When prepared by RSE, lipid structures form de novo in water, in the presence of insignificant solvent residue. Our explicit goal was to preserve compositional homogeneity throughout sample preparation, so that any apparent phase behavior observed in the aqueous suspension could be attributed only to the physical chemistry of the hydrated lipid mixture. Because the method is specifically designed for the fast, efficient removal of organic solvent, it does not require a highly volatile solvent. Rapid solvent exchange works with the most common, effective lipid solvents (dichloromethane, chloroform) and allows the preparation of lipid mixtures which incorporate components of widely ranging solubility properties.

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