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Magnetically induced orientation of phosphatidylcholine membranes

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Lipid bilayers prepared from natural phospholipids orient in magnetic fields with the long axis of the lipid molecules perpendicular to the magnetic field. This magnetically induced orientation was studied at high (11.7 Tesla (T)), mid (9.36 T), and low (4.68 T) magnetic field strengths using lipid aggregates prepared from natural and synthetic phosphatidylcholine analogs. Phosphatidycholine analogs containing saturated diacylated chains (12 to 16 carbons/chain) exhibited extensive orientation of the lipid when bilayer formation occurred by gentle hydration conditions. Gentle hydration involved incubating dried phosphatidylcholine lipid films in excess water at temperatures that were ~ 5–10°C above the main phase transition (T_m); brief shaking or swirling by hand was occasionally needed to completely disperse the lipids. The method of bilayer formation significantly influenced the amount of lipid that orients in magnetic fields. Thus the supramolecular structures (and % orientation) above T_m in an 11.7 T field of dimyristoylphosphatidylcholine (DMPC) bilayers are SUV (0%), LUV (~ 15%), SPLV (~ 40%), vortexed-MLV (~ 60%) and non-vortexted MLV (~ 90%). Single layered vesicles prepared by the REV method exhibited orientation at 11.7 T similar to LUV prepared by freeze thaw cycles. Aqueous dispersions of eggPC prepared by gentle hydration exhibit ~ 40% orientation at 11.7 T which decreased to ~ 30% orientation if 30% cholesterol is added to the membrane. Magnetic orientation of bilayers thus appears to be a general phenomenon for both saturated and unsaturated natural phospholipids either with or without cholesterol in the membrane.

Introduction

Artificial membranes prepared from natural and synthetic phospholipids are routinely studied by ^{31}P-NMR to obtain the conformation and dynamic properties of phospholipids [1–4]. Although many studies utilize pure phospholipid membranes [5–7], ^{31}P-NMR is also used to probe the effect of membrane guest molecules on bilayer phospholipids. For instance, ^{31}P-NMR studies of phospholipids in membranes containing fatty acids [8], signal peptides [9] and proteins [10–12] have been reported.

With the exception of small single layered vesicles, the ^{31}P-NMR spectra of liposomes is an axially symmetric powder pattern (Scheme I). Although most phospholipid dispersions exhibit axially symmetric powder patterns, several examples of distorted lineshapes have been reported (Table I). Distortion of the axially symmetric powder pattern has been correctly attributed to the orientation of bilayers such that the bilayer surface is parallel to the magnetic field. In other words phospholipid molecules in the bilayer tend to orient with their long axis perpendicular to the magnetic field. Generally, the magnetic orientation of aggregated lipids is very small, and observable only by more sensitive methods such as magnetic birefringence [13,14]. Consequently, most ^{31}P-NMR spectra show axial symmetric powder patterns with no evidence of magnetically induced orientation because only a small

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Abbreviations: DMPC L-α-dimyristoylphosphatidylcholine; T_m, main phase transition temperature; SUV, small unilamellar vesicles; MLV, multilamellar vesicles; MLV-REV, multilayered vesicles prepared by the REV method; SPLV, stable plurilamellar vesicles; REV, reverse phase evaporation vesicles; LUV, large unilamellar vesicles; DLPC, L-α-dilaurylphosphatidylcholine; DPPC, L-α-dipalmitoylphosphatidylcholine; CH, cholesterol; AC2, 1,2-di(12-methoxydodecanoyl)-*sn*-glycero-3-phosphocholine; NPSM, *N*-palmitoylsphingomyelin; NMR, nuclear magnetic resonance; DMPS, L-α-dimyristoylphosphatidylserine; T, Tesla; CHAPSO, 3-(cholamidopropyl)dimethylammonio-2-hydroxy-1-propane sulfonate; DEPE, L-α-dielaidoylphosphatidylethanolamine; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; DOPC, L-α-dioleoylphosphatidylcholine; DOPA, L-α-dioleoylphosphatidic acid; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol; bis-sorPC, 1,2-bis[10-(2′,4′-hexadienoyloxy)decanoyl]-*sn*-gly cero-3-phosphocholine; T_2, transverse relaxation time; ppm, part per million.

fraction of the lipid molecules in the membrane preparation are oriented when NMR measurements are made with low and mid-field NMR spectrometers. However, the orientation of lipid bilayers in magnetic fields is becoming increasing common due to the availability of modern high field spectrometers for membrane studies.

The first report of quantitative bilayer orientation induced by the magnetic field (7.05 T) of a modern NMR spectrometer was the study of phospholipids extracted from *Escherichia coli* membranes [15]. Complete orientation of phospholipids occurred only if the 'lipid mixture' extracted from *E. coli* was not vortexed during dispersion of the lipids. Most interesting was the observation that liposomes prepared from pure lipids in the *E. coli* 'lipid-extract' would not orient suggesting that lipid mixtures were required for magnetic orientation. Several other studies have also suggested that magnetic orientation of phospholipid bilayers required particular lipid 'mixtures' such as DMPC/NPSM [16], DMPC/DMPS [17], and DMPC (or DPPC) liposomes containing detergents [18–19]. These last reports also indicated that pure DMPC lipid dispersion at 9.36 T [16] or DPPC lipid dispersions at 11.7 T [18] could not be oriented. More recent studies utilizing either ^{31}P-or ^{2}H-NMR spectroscopy clearly showed that the magnetic orientation of bilayers strongly depends on the T_m of the phospholipids, the phospholipid composition, and the method of liposome preparation [16–21].

Most observations to date indicate that lipid mixtures are required for orientation of membranes in magnetic fields. Recently, we synthesized a phosphatidycholine analog, denoted as AC2, that exhibits potent anti-HIV activity in infected T-cells and macrophages [22]. During our preliminary ^{31}P-NMR studies of AC2, we observed that AC2 liposome membranes partially oriented at 11.7 T (i.e., the field strength of an ^{1}H 500 NMR spectrometer). The magnetically induced orientation of AC2 liposomes encouraged us to study the phenomenon with both natural and synthetic lipids in magnetic fields common to modern NMR spectrometers. Phosphatidylcholine lipids were used to prepare SUV, REV, LUV, SPLV, non-vortexed-MLV, and vortexed-MLV liposomes. Our results show that magnetic orientation of liposome membranes is in fact a general phenomenon for pure lipid systems but the extent of orientation depends on magnetic field strength, size of the liposomes, and T_m of the lipid dispersion.

Materials and Methods

Chemicals

DLPC, DMPC, DPPC and egg PC were obtained from Avanti Polar Lipids, Birmingham, AL and used without further purification. Cholesterol (CH) was obtained from Sigma, St. Louis, MO. 1,2-Di(12-methoxydodecanoyl)-*sn*-glycer-3-ophosphocholine (AC2) was synthesized in our lab by reacting 12-methoxydodecanoyl anhydride with glycerolphosphocholine in dry $CHCl_3$ using dimethylaminopyridine as catalyst [22]. Dimethylaminopyridine was obtained from Aldrich. D_2O (99.99% D) was also purchased from Aldrich. Tris and Sephadex G-50–80 were purchased from Sigma. EDTA was purchased from Fisher Scientific. Diethyl ether from fresh unopened containers, obtained from Fisher Scientific, was used as received to form SPLV, REV and MLV-REV liposomes.

Liposome preparation

Liposomes made from natural phospholipids were prepared from either H_2O/D_2O (80:20, v/v) or Tris buffer which contained 10 mM Tris-HCl (pH 7.0), 1 mM EDTA, and 20% D_2O. However, H_2O/D_2O (80:20, v/v) was used to prepare liposomes from the synthetic phospholipid AC2. All aqueous media for liposomes contained 10–20% (v/v) D_2O in the final liposome population which was used for field-locking during NMR measurements. The mobile phase for Sephadex chromatography was the same aqueous solution used to prepared the liposomes except D_2O was omitted. Liposomes prepared in D_2O aqueous media that were eluted from the Sephadex columns in non-D_2O mobile phases contained residual D_2O that was used for field-locking during NMR measurements; however, some liposome samples were concentrated and resuspended in aqueous media containing D_2O for the same purpose. Lipid mixtures corresponding to each type of liposome are given in the figure legends.

Both single layered and multilayered liposomes were prepared from predried lipid films. Lipids (60–100 mg) in chloroform were rotary evaporated in a 100 ml round-bottom flask at 30–45°C to form a thin dry film. This film was then vacuum evaporated using a pump for 4–12 h to completely remove trace organic solvents. These predried lipid films were used to prepare the liposome preparations described below.

Multilayered liposomes

Multilayered liposomes were not concentrated prior to NMR measurements. For preparing non-vortexed-MLV liposomes, predried phospholipid films were suspended in 0.8 ml of H_2O/D_2O (80:20, v/v) or Tris buffer by slow hydration with gentle swirling above the lipid T_m [23]. Converting non-vortexed MLV liposomes into vortexed-MLV liposomes merely required vigorously vortexing for 10 minutes. SPLV liposomes were prepared as described [24,25]. The SPLV emulsion contained 0.3 ml Tris buffer in 5 ml of ethyl ether. MLV-REV vesicles were prepared as described [25,26] using 0.3 ml buffer emulsified with 10 ml of diethyl

ether. The structural differences between SPLV and MLV-REV liposomes is predominantly the size of the liposomes' core; MLV-REV have larger cores than SPLVs because during vesicle formation, the SPLV liposomes are continuously sonicated. Structural differences between SPLV and MLV-REV liposomes have been extensively discussed [25,26].

Single layered liposomes

SUV liposomes were prepared by sonication of vortexed-MLVs. The vortexed-MLV suspension (~ 50 mg lipid/1.5 ml buffer) was sonicated to near optical clarity at room temperature for 30–40 min under nitrogen with a Sonic Materials VC40 ultrasonic processor equipped with a Titanium probe. To remove Titanium particles from the SUV suspension, SUV liposomes were chromatographied on a Sephadex G-50–80 column. SUV liposomes were not concentrated prior to NMR measurements. LUV liposomes were prepared from dilute SUV liposomes eluting in the void volume of the Sephadex G-50–80 column. Thus SUV liposomes were subjected to 10 freeze-thaw cycles using an acetone/dry ice bath (−95°C) to freeze the lipid dispersions between cycles. Thawing was performed at room temperature. LUV liposomes prepared by the freeze thaw method were concentrated by pelleting ($14\,000 \times g$) in an Eppendorf tube. The pellet was resuspended in 0.4 ml Tris buffer for NMR measurements. REV vesicles were prepared from 80 mg lipid emulsified in 9 ml diethyl ether and 4 ml of Tris buffer [27]. REV liposomes were concentrated by floatation using D_2O buffer [25,26].

^{31}P-NMR spectroscopy

The field strength used for each NMR measurement is given in each Figure legend. Most ^{31}P-NMR spectra were obtained at 11.7 T on a Varian VXR 500 spectrometer using a completely phase cycled Hahn echo pulse sequence [28] with WALTZ proton decoupling which was gated on during acquisition. The $\pi/2$ pulse width was 17 μs, the pulse spacings were 40 μs and 35 μs, and the recycle time was 3 s. Data were acquired over 100 kHz spectral width and a 0.05 s acquisition time. Spectra were signal-averaged from 200–1000 transients; the actual transients for each spectra are given in each figure legend. The free induction decay (FID) was left-shifted to the maximum of the first echo and then processed with an exponential line broadening of 100 Hz before Fourier transformation. It is critical to left-shift the FID exactly on the top of the first echo; overshifting the FID distorts the transformed spectrum by reducing low field spectral intensities [16]. The temperature was controlled to an accuracy of ±0.5°C, and the time allowed for temperature equilibration after a 5°C increment was 15–30 min.

^{31}P-NMR spectra were also obtained at 9.36 T and 4.68 T. ^{31}P-NMR spectra at 9.36 T were obtained on a Varian Unity 400 NMR spectrometer. A single 75° pulse of 5 μs was used with a 4 s recycle time. Gated proton decoupling was applied during data acquisition. Data were acquired with a 50 kHz spectral width, a 0.08 s acquisition time, and were signal-averaged for 1000 transients and an exponential line broadening of 50 Hz was applied before Fourier transformation. The ^{31}P-NMR spectrum at 4.68 T was obtained on Varian XL-200 spectrometer using a single 45° pulse of 5 μs, gated WALTZ proton decoupling and 3 s recycle time. Data were acquired with a 50 kHz spectral width using a 0.1 s acquisition time. The spectrum was signal-averaged from 970 transients and processed with an exponential line broadening of 50 Hz. Chemical shifts were referenced to external 85% phosphoric acid.

Spectral simulation

It is rare that NMR spectrometers, even high field spectrometers, can quantitatively orient the lipid in membrane preparations. To compare the extent of lipid-orientation in liposome dispersions subjected to different magnetic field strengths and different temperatures, it was necessary to simulate the ^{31}P-NMR spectra. Based on the superposition principle inherent to NMR spectroscopy, partially oriented phospholipids exhibit ^{31}P-NMR spectra containing contributions from both the oriented lipid molecules and the nonoriented lipid molecules in the sample. Initially we simulated ^{31}P-NMR spectra corresponding to these two extreme conditions, i.e., a spectrum $F_0(\sigma)$ representing completely unoriented phospholipids and a spectrum $F_{100}(\sigma)$ representing phospholipids completely oriented at 90° relative to the applied field. Partially oriented lipid samples could then be conveniently simulated using a linear combination of $F_0(\sigma)$ and $F_{100}(\sigma)$ with appropriate weighting coefficients to reflect the amount of oriented lipid. The simulated spectra were considered a good fit of the experimental spectra when the overlaid spectra showed no apparent systematic deviations [7]; typically greater than 95% of the simulated spectra exactly matched the experimental spectra which indicates that we have at most a 5% error associated with the simulated data.

The $F_0(\sigma)$ spectrum was simulated by the theoretical equation for axially symmetric powder patterns [29]

$$F_0(\sigma) = \frac{1}{4} \times \left(\frac{\sigma_{\parallel} - \sigma_{\perp}}{\sigma - \sigma_{\perp}} \right)^{1/2} \quad (1)$$

where $\sigma_{\parallel}$ and $\sigma_{\perp}$ were obtained from each experimental spectra to be simulated. Eqn. 1 does not account for relaxation and consequently simulating the spectra required convoluting $F_0(\sigma)$ with a line broadening function. Thus, $F_0(\sigma)$, ~ 1 K data points, was transformed into the time domain using a Fourier transformation

algorithm and multiplied by the line broadening function $\exp^{-t/T_2}$, then Fourier transformed back into the frequency domain.

A Lorenzian function was used for simulating $F_{100}(\sigma)$

$$F_{100}(\sigma) = \frac{2T_2}{1+4\pi^2 T_2^2(\sigma-\sigma_\perp)^2} \quad (2)$$

which corresponds to a spectra centered at the perpendicular edge of the powder pattern. The area under the spectral curves generated by Eqns. 1 and 2 were normalized to have equal areas and the experimental spectra $F_E(\sigma)$ exhibiting partial lipid orientation was simulated by summing the normalized spectra

$$F_E(\sigma) = C_1 F_0(\sigma) + C_2 F_{100}(\sigma) \quad (3)$$

where the sum of the weighting coefficients C_1 and C_2 equals unity and $F_E(\sigma)$ is the simulated data. The fraction of lipid oriented in the experimental spectra was C_2 and the % lipid oriented was $100 \times C_2$.

Spectral simulations using Eqns. 1 and 2 required estimates of T_2 which is the transverse relaxation time. In principle, T_2 depends on the orientation of the lipid in the magnetic field [10,30,31]; therefore, anisotropic T_2 values should be used for the simulated data. However, since our main objective was to qualitatively compare magnetically induced orientation of lipids dispersed by common procedures, it was not necessary to use an anisotropic T_2 values for the spectral simulations. In addition, an orientation-independent 100 Hz line broadening function was applied to the experimentally measured FID prior to Fourier Transformation to obtain the experimental spectra. This strong weighting function averages the real anisotropic T_2 values that exist across the powder pattern and spectral intensities at high field (i.e., near the perpendicular edge of the spectrum) are spread towards the spectral regions corresponding to low field strengths (i.e., near the parallel edge of the spectrum). This strong weighting function thus causes an underestimation in the amount of lipid-orientation because the spectral intensity corresponding to the orientation is spread to regions of the powder pattern that do not correspond to lipid-orientation. Nevertheless, an isotropic T_2 value was used to simplify the calculations but all calculations of the % lipid oriented should be understood to be an approximation, albeit a very good approximation.

Measurement of T_m

The T_m of AC2 was measured using a Perkin Elmer Differential scanning calorimeter and also by infrared spectroscopy. IR measurements utilized the methylene symmetric and asymmetric stretch and were accurate to ± 1°C. Our infrared spectrometer has been described in detail [32,33] and methods for measuring T_m using changes in these band positions are well established [34,35]. Infrared spectroscopy measurements were difficult because of the changes in background absorbance of the AC2 suspension when heated above the T_m. Above T_m the background absorbance significantly decreased. AC2 liposomes were collected on the filter of a 0.2 μ microfilterfuge tube as described earlier for DLPC liposomes [32]. However, great care had to be taken to assure that the AC2 liposomes did not pass through the filter. Unlike DLPC liposomes, AC2 liposomes are very easily extruded through 0.2 m filters even below T_m.

Results

Complete orientation of lipid bilayers is rarely observed in NMR spectrometers. However, nonvortexed MLV liposomes prepared from DMPC ($T_m = 23$°C) incubated above T_m have nearly all the spectral intensity at the perpendicular edge (−16 ppm) indicating near complete orientation (Fig. 1). Although most studies report that magnetically induced lipid orientation requires lipid mixtures (Table I), Fig. 1 clearly demonstrates that quantitative orientation at high magnetic field strengths (11.7 T) occurs for pure lipid dispersions. Fig. 1 also demonstrates that non-vortexed MLV liposomes of pure DMPC are not oriented below T_m at 22°C but when heated above T_m followed by cooling back down to 22°C, approximately 10% lipid orienta-

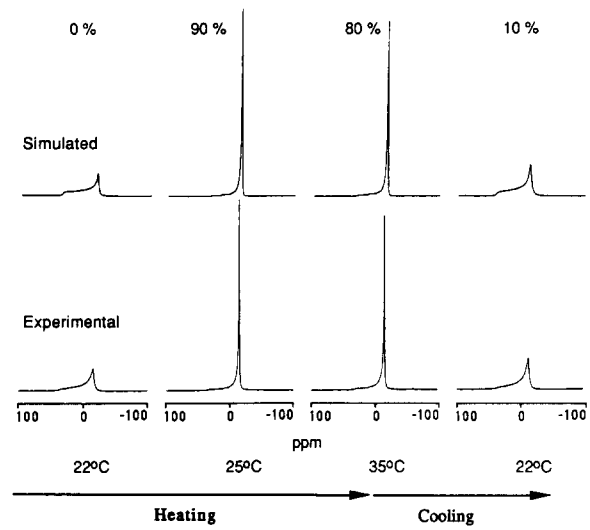


Fig. 1. Experimental ^{31}P-NMR spectra (bottom) and simulated spectra (top) of non-vortexed MLV liposomes prepared from DMPC (13 wt% suspension). The temperature dependent spectra were obtained on the same lipid dispersion at a field strength of 11.7 T and the spectra were signal-averaged from 300 transients and plotted with absolute intensity. The amount of phospholipid oriented by the magnetic filed (expressed as% of total lipid) is given at the top of the simulated spectra. Spectral measurements were made at several temperatures. The non-vortexed MLV lipid dispersion was initially heated above T_m (23°C) and then cooled down below T_m.

TABLE I

Orientation of lipid dispersions in magnetic fields

Phospholipids [c]	Lipid dispersion	Magnetic field strength	Temperature for orientation	Ref.
POPE/POPG (80:20)	88 wt% buffer nonvortexed MLV	7.05 T	45°C (T_m = 37) [a]	15
NPSM/DMPC (60:40)	50 wt% H_2O nonvortexed MLV	9.36 T	44°C (T_m = 32)	16
DMPC/DMPS (50:50)	90 wt% buffer nonvortexed MLV	8.42 T	35°C (T_m = 23)	17
DMPC/CHAPSO (83:17)	25 wt% buffer MLV	11.7 T	40°C (T_m = 23)	19
DPPC/MPPC (67:33)	50 wt% buffer MLV	11.7 T	55°C (T_m = 41)	18
DEPE/melittin (80:20)	94 wt% buffer nonvortexed MLV	7.05 T	50°C (T_m = 37)	48
DPPC/phytanic acid (80:20)	85 wt% buffer MLV	5.9 T	45°C (T_m = 41)	49
DOPA + Na^+	95 wt% D_2O MLV	5.9 T	25°C	21
DOPE/SorbPC (75:25)	50 wt% buffer MLV	11.7 T	25°C	20
DOPC	97 wt% buffer nonvortexed MLV	7.05 T	25°C	50

[a] The T_m is for the highest melting lipid in the membrane.

[b] Lipid dispersions were subjected to several freeze-thaw cycles prior to NMR studies.

[c] All of the lipid dispersions exhibited ~90% lipid orientation under the conditions given in the table except for DOPE/SorbPC which exhibited approx. 50% lipid orientation. Lipid orientation was observed by either ^{31}P-NMR and/or ^{2}H-NMR.

tion persists as long as the lipid dispersion was not removed from the field (Compare the spectra labeled 0% and 10% in Fig. 1). Spectral simulations were needed to identify this small amount of lipid-orientation.

Nonvortexed MLV liposomes prepared from DPPC behaved similar to nonvortex liposomes prepared from DMPC regarding the magnetically induced orientation

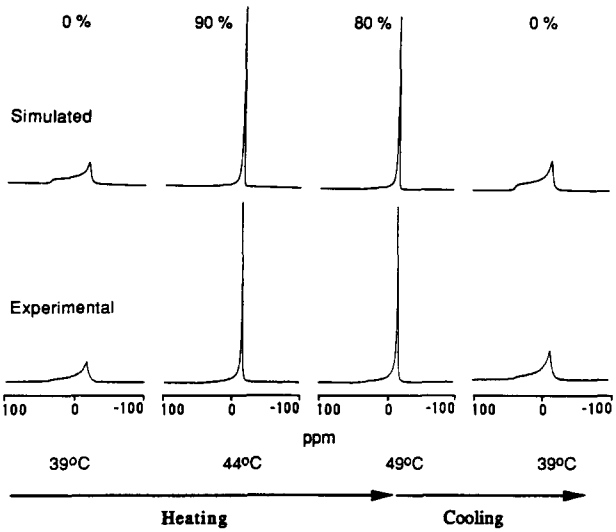


Fig. 2. Experimental ^{31}P-NMR spectra (bottom) and simulated spectra (top) of non-vortexed MLV liposomes prepared from DPPC (10 wt% suspension). The temperature dependent spectra were obtained on the same sample at 11.7 T and spectra were signal-averaged from 250 transients and plotted with absolute intensity. The amount of lipid oriented by the magnetic filed (expressed as % of total lipid) is given at the top of the simulated spectra. Spectral measurements were made at several temperatures. The non-vortexed MLV lipid dispersion was initially heated above T_m (42°C) and then cooled down below T_m.

above T_m; however, unlike DMPC dispersions, residual lipid orientation did not occur for DPPC after the lipid dispersion was cooled below T_m in the magnetic field (Fig. 2). Thus, below the T_m of DPPC, no lipid orientation occurred and an axially symmetric powder pattern was obtained but this spectrum collapsed to a single resonance near $\sigma_\perp$ when the lipid dispersion was heated above T_m. Upon cooling, the spectrum returned to the typical powder pattern with no change in $\sigma_\perp$ (−16 ppm), $\sigma_\parallel$ (35 ppm) and $\Delta\sigma$ (51 ppm); $\Delta\sigma$ is the the breadth of the powder pattern $\Delta\sigma = \sigma_\perp - \sigma_\parallel$. Similar to the residual lipid orientation below T_m for the DMPC dispersion (Fig. 1, lower right spectrum), others have observed partial phospholipid orientation below T_m if the incubation above T_m occurred in the magnetic field and this was attributed to a super cooling effect of the lipid dispersion [15,16].

Phospholipid bilayers exist in many phases which have been defined as L_c, P_β, $P_{\beta'}$ and L_α and the lipid-phase adopted by the lipid depends on both the temperature and lipid [33,36]. The main phase transition temperature T_m corresponds to the phase change from the gel ($P_{\beta'}$) to liquid crystalline (L_α). The molecular changes that occur at T_m have been well studied in the absence of high magnetic fields. Magnetically induced orientation of lipids may require incubating the lipids at T_m in the magnetic field (Fig. 1). In other words, molecular events associated with T_m, like hydrocarbon chain melting, expansion of the bilayer, etc., may be modified by the magnetic field and consequently lipid orientation may require incubating the lipids at T_m in the magnetic field. To test this possibility, nonvortexed MLV made from DMPC in a 5-mm NMR tube at 30°C (above T_m outside the magnetic

field) was inserted into the NMR spectrometer which was preequilibrated to 30°C. The ^{31}P-NMR spectrum of this lipid dispersion was identical to that in Fig. 1 indicating that the lipid orientation was also the same (not shown). This indicates phospholipid dispersions do not need to be incubated in the magnetic field at T_m for lipid orientation to occur as shown in Figs. 1 and 2.

The effect of magnetic field strength (H) on lipid orientation was evaluated using nonvortexed DMPC lipid dispersions. Aliquots of the same DMPC dispersion were used to obtain ^{31}P-NMR spectra at 30°C in either a 4.68 T, 9.36 T or 11.7 T magnetic field (Fig. 3). Theses results show that spectral line shapes are field dependent. At low field (4.68 T) the ^{31}P-NMR spectrum has a lineshape of a typical axially symmetric powder pattern. However, as the field strength increases the intensity at $\sigma_{\|}$ decreases and the intensity at $\sigma_{\perp}$ increases and dominates the spectrum. From spectral simulations, the lipid orientation was ~15%, ~55% and ~90% at field strengths of 4.68 T, 9.36 T and 11.7 T, respectively. This indicates that the magnetically induced lipid orientation is approximately proportional to H^2 as shown in Fig. 3 (inset). This H^2 dependence of lipid orientation is the main reason that observing lipid orientation was uncommon until high field (11.7 T) spectrometers became more available. Low and mid field spectrometers do not have sufficient energy to orient the lipid dispersions.

Although lipid orientation strongly depends on the field strength of the NMR spectrometer it also significantly depends on the liposome structure. Liposomes can be ranked ordered by size (or approximate diameters) as

SUV (~250 Å) < LUV (<0.5 μ) < SPLV (0.4–2 μ) < vortexed MLV (1–5 μ) < nonvortexed MLV (≫10 μ)

and we used DMPC to prepare each of these liposomes and evaluated magnetically induced lipid orientation. The ^{31}P-NMR spectra clearly demonstrate that the amount of lipid orientation increases from 0 to 90% as the average liposome size increased (Fig. 4). Similar to the ^{31}P-NMR spectra of DMPC (Figs. 1 and 3), spectra were obtained at 11.7 T at 30°C. The amount of lipid orientation increases from ~50% to >90% if the lipid dispersion is not vortexed. Although large single layered LUV and REV liposomes showed little lipid orientation (<15%), the small single layered vesicles, SUVs, do not exhibit any lipid orientation. In fact the ^{31}P spectral intensity of SUVs do not correspond to the perpendicular edge of the spectrum because SUVs exhibit isotropic motion on the NMR time scale causing the ^{31}P-NMR spectrum to be a Lorenzian line shape centered at 0 ppm (Fig. 4).

Although both DMPC and DPPC liposomes orient most of their bilayers above T_m in 11.7 T fields (Figs. 1 and 2), DLPC liposomes, egg PC liposomes and egg PC/CH 70:30 liposomes (all non vortexed dispersions) are only partially orientated (Fig. 5). DLPC contains hydrocarbon chains with 12 carbons which is only two

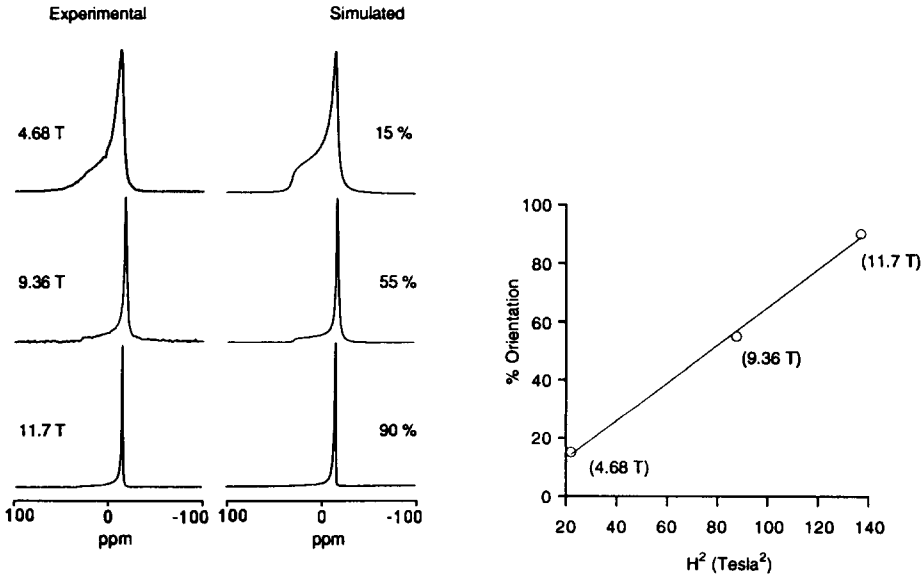


Fig. 3. Experimental ^{31}P-NMR spectra (left) and simulated spectra (right) of non-vortexed MLVs prepared from DMPC (13 wt% suspension) at spectrometers operating at low-field (4.68 T, 970 transients), mid-field (9.36 T, 1000 transients), and high-field (H = 1.7 T, 300 transients). All spectra were obtained at 30°C and plotted with normalized intensity. The amount of lipid oriented in each magnetic field (expressed as % of total lipid) is given on the right side of the simulated spectra. The smoothed parallel edge of the spectrum $\sigma_{\|}$ obtained at 4.68 T was due to residual dipolar broadening. The graph shows that the extent of orientation is proportional to H^2.

methlyene groups shorter than DMPC. However, the spectrum of DLPC indicates that only ~45% of the lipid would orient at 11.7 T (Fig. 5) compared to ~90% orientation of DMPC under the same conditions (Fig. 1). In contrast to liposomes prepared from saturated lipids, liposomes prepared from unsaturated lipids exhibit significantly less lipid orientation. Non-vortexed egg PC and nonvortexed egg PC/CH 70/30 lipid dispersions exhibit ~40% and ~30% orientation, respectively. Although lipid orientation is incomplete above T_m for DLPC, all non-vortexed MLV liposome populations prepared from saturated longer alkyl chain phosphocholines (DMPC and DPPC) exhibit significant orientation at 11.7 T (Figs. 1, 2 and 4).

The above results indicate that bilayers prepared from natural phospholipids will orient to an extent >50%, and usually ~80–90%, if the bilayers are above the T_m in a high magnetic field (11.7 T), and

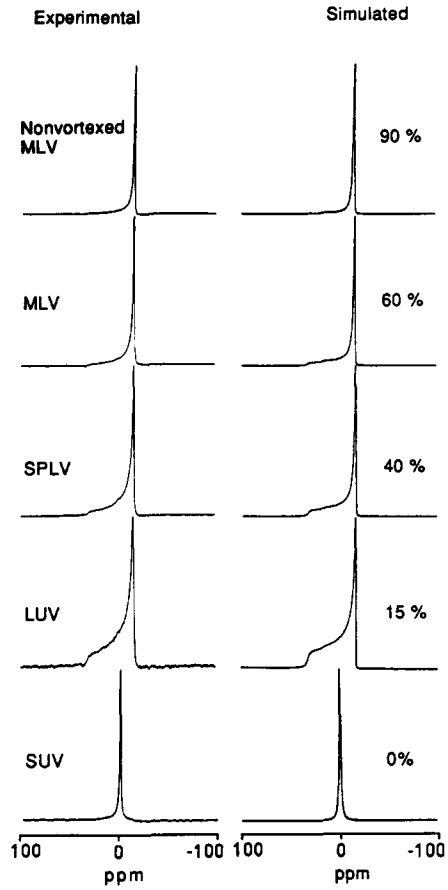


Fig. 4. Experimental ^{31}P-NMR spectra (left) and simulated spectra (right) of DMPC liposomes prepared by several methods (~10 wt% suspensions except for SUVs which was approximately a 2 wt% suspension). All spectra were obtained at 11.7 T at 30°C from 300 transients except for the SUV (spectrum which required 1000 transients. The spectra were plotted with normalized intensity. The amount of oriented-lipid for each type of liposome (expressed as % of total lipid) is given on the right side of the simulated spectra. The ^{31}P-NMR spectrum of MLV-REV vesicles was similar to the ^{31}P-NMR spectrum of SPLVs and is therefore not shown.

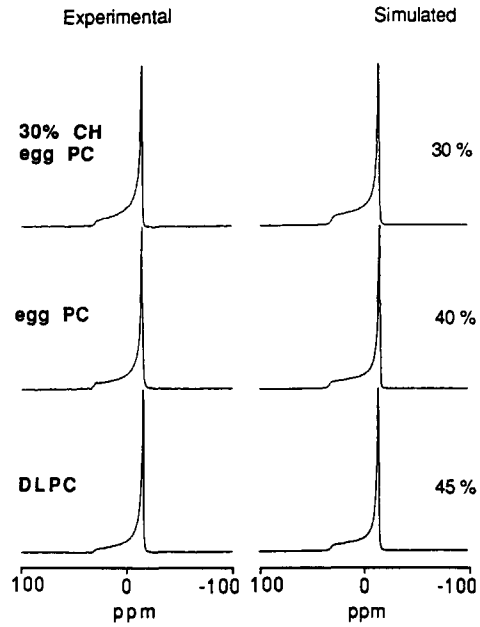


Fig. 5. Experimental ^{31}P-NMR spectra (left) and simulated spectra (right) of non-vortexed MLV liposomes prepared from egg PC (10 wt% suspension), egg PC/CH 70:30 w/w (10 wt% suspension) and DLPC (7 wt% suspension). The spectra were obtained at 11.7 T at 30°C from 500 transients and plotted with normalized intensity. The amount of oriented-lipid in each sample (expressed as % of total lipid) is given on the right side of the simulated spectra.

most importantly lipid orientation is maximum if the the dispersion is not vortexed. Thus, magnetic orientation appears to be a general phenomena. Since novel synthetic phospholipids that self assemble into membranes are routinely prepared by several laboratories (e.g., Ref. 37), it is important to test lipid orientation on bilayers prepared from synthetic lipids. Thus, we evaluated the magnetic orientation of lipid dispersions prepared from the synthetic lipid AC2 which is a chemical analog of DMPC. In the AC2 molecule the 13th atom in each alkyl chain is an oxygen instead of a methylene. Fig. 6 shows the ^{31}P-NMR spectra of AC2 nonvortexed dispersions above and below T_m. Spectral simulations show that the ^{31}P-NMR spectrum of AC2 liposomes obtained below T_m at 30°C corresponds to ~5% lipid orientation compared to DMPC dispersions that did not exhibit any lipid orientation below T_m unless the DMPC lipid dispersion was initially incubated above T_m in the magnetic field then cooled below T_m in the field (Fig. 1). As the temperature increases toward T_m, the fraction of AC2 bilayers that orient increases to 20%. Increasing the temperature above the T_m of AC2, caused 'decreased' lipid orientation from the maximum value of ~20% to ~10%. This is in contrast to all other lipid dispersions (Figs. 1–5) which exhibited increased lipid orientation as the temperature increased further beyond T_m. When cooling from above the T_m to below the T_m of AC2 the spectrum was similar to the initial spectrum at 30°C

with the same amount of lipid orientation (~ 5%) and the same spectral width $\Delta\sigma$ (~ 47 ppm) was obtained (top spectrum, Fig. 6).

Most interesting about the AC2 dispersion was that when heated above T_m, $\sigma_\perp$ and $\sigma_\parallel$ suddenly decreased and $\Delta\sigma$ was reduced from 47 to 36 ppm. These changes in $\sigma_\perp$, $\sigma_\parallel$ and $\Delta\sigma$ were reversible when the AC2 lipid dispersion was cycled through T_m (41.4°C). The singularities at 35°C are observable at $\sigma_\perp = -15.7$ and $\sigma_\parallel = 33$ ppm; whereas, at 45°C $\sigma_\perp = -11.5$ and $\sigma_\parallel = 24$ ppm (Fig. 6). However, the changes in $\sigma_\parallel$, $\sigma_\perp$ and $\Delta\sigma$ that occurred above T_m actually began at T_m and the experimental spectrum obtained at T_m is thus a combination of these two states. This is shown more clearly in Fig. 7 which is the ^{31}P-NMR spectrum obtained on an AC2 lipid dispersion that was not equilibrated for 15 min prior to data acquisition; two distinct $\sigma_\perp$ peaks are observed. However, the two states do not contribute equally to the spectra obtained at T_m (compare the intensities of $\sigma_\parallel$, $\sigma_\perp$ above and below T_m in Fig. 6). Since we would expect that at the true T_m the two states should give equal contributions to the experimental spectra, the true T_m may actually be slight higher than 41.4°C. This reversible change in the breadth of the powder pattern for the AC2 lipid dispersion cycled through the main phase transition temperature indicates increased lipid headgroup motion above T_m which reduces the chemical shift anisotropy of the headgroup phosphate.

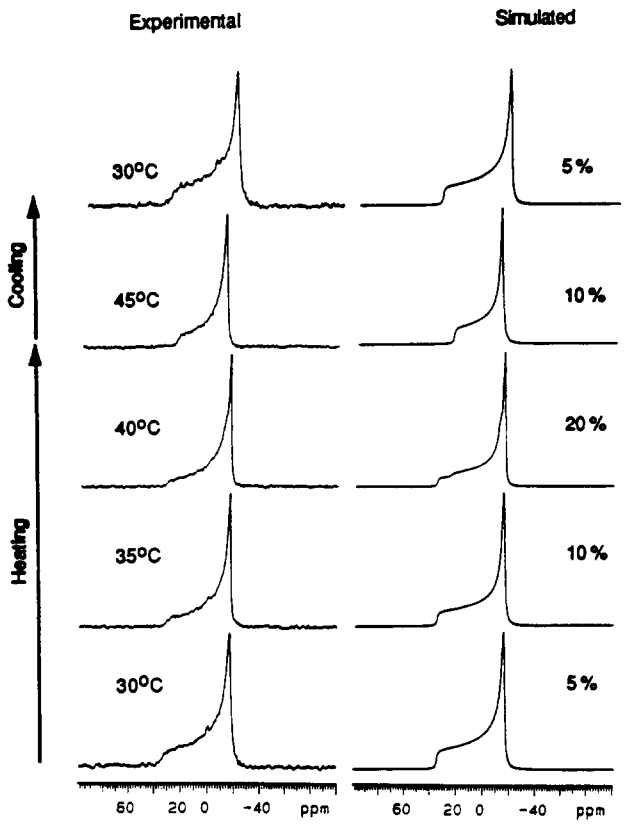


Fig. 6. Experimental ^{31}P-NMR spectra (left) and simulated spectra (right) of non-vortexed MLV liposomes prepared from the synthetic phospholipid AC2 (7 wt% suspension). The spectra were obtained at 11.7 T field at several temperatures from 800 transients. The spectra were plotted with normalized intensity and the amount of oriented-lipid (expressed as % of total lipid) in each spectrum is given next to the simulated spectra. Similar to Figs. 1 and 2, ^{31}P-NMR spectra were initially obtained below T_m (~ 40°C) followed spectra obtained at several temperatures near and higher than T_m before cooling the sample for obtaining the final spectrum.

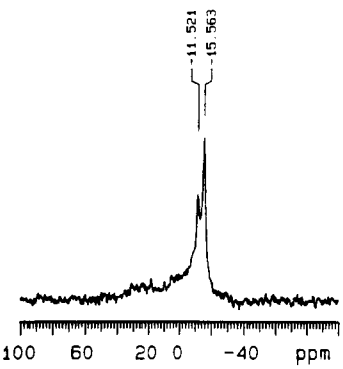
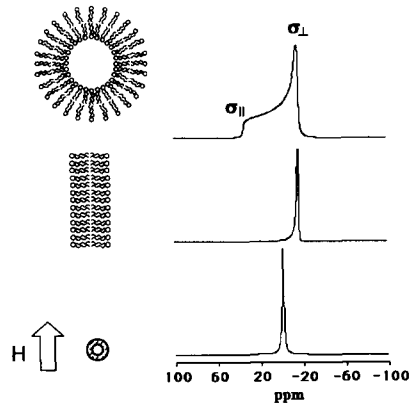


Fig. 7. ^{31}P-NMR spectrum of nonvortexed AC2 lipid dispersions at T_m without temperature equilibration prior to data acquisition. In contrast to Fig. 6, data acquisition began within 5 min of raising the temperature to 40°C. See Fig. 6 legend for data acquisition parameters.

In summary, unlike any other lipid dispersion, AC2 did not exhibit significant lipid orientation under any experimental conditions and the breadth of the powder pattern decreased above T_m and returned to the same value below T_m before heating. These reversible changes in the powder pattern did not occur for any other lipid dispersion and most importantly, the distinct resonance of $\sigma_\parallel$, $\sigma_\perp$ above and below T_m indicate that the AC2 lipid dispersion exists in two states depending on whether the sample is above or below T_m.

Discussion

When a circular liposome containing a closed membrane is placed in a magnetic field, the confluent phospholipid molecules are oriented at all possible angles relative to the field. If sliced at different latitudes, liposome slices contain lipid molecules at many orientations relative to the field and different amounts of lipid are contained in each slice. Assuming liposome tumbling is slow, the 'latitude' of each slice determines the chemical shift (i.e., lipid orientation in the field) but it is the number of lipid molecules in each slice that determines the spectral intensity. The large number of phospholipid molecules forming the liposomes' equator (the largest slice), are oriented at 90° relative to the static field and contribute to the largest spectral intensity ($\sigma_\perp$). As one moves away from the liposomes equator smaller amounts of lipid molecules are in each hypothetical slice and the spectral intensity decreases.



Scheme I. Comparison of the expected ^{31}P-NMR spectra of liposomes exhibiting no lipid orientation to liposomes exhibiting complete lipid orientation. Unoriented liposomes exhibit axially symmetric powder patterns (upper spectrum). The resonance at $\sigma_\perp$ is from phospholipid molecules oriented perpendicular to the magnetic field whereas $\sigma_\parallel$ is from phospholipid molecules oriented parallel to the magnetic field. Spherical liposomes in magnetic fields contain phospholipids at all angles relative to the field, not just perpendicular and parallel, and the resonances between $\sigma_\perp$ and $\sigma_\parallel$ reflect these states because chemical shifts depend on the molecular orientation relative to the magnetic field. Large liposomes themselves exhibit slow tumbling, but the phospholipids in the bilayer exhibit rapid axial rotation. Rapid axial rotation of lipids in the slow tumbling liposome results in chemical shift anisotropy among the population of lipid molecules comprising the membrane causes an axially symmetric powder pattern (upper spectrum). As the liposome size decreases, vesicle tumbling increases which causes a reduction in the chemical shift anisotropy, i.e.the breadth of the powder pattern decreases. For very small liposomes, vesicle tumbling is rapid on the NMR time scale causing the chemical shift anisotropy to completely average and the expected spectra contains a single resonance at 0 ppm (lower spectrum). For lipids completely oriented at 90° relative to the applied field, the chemical shift of all other orientations other than 90° are eliminated; consequently the axially symmetric powder pattern collapses to a single resonance centered at $\sigma_\perp$ (middle spectrum). For lipid dispersions that contain only a fraction of the lipid molecules oriented in the magnetic field, the observed spectrum will contain contributions from the nonoriented axially symmetric powder patter (upper spectrum) and oriented spectrum (middle spectrum) and Eqn. 3 can be used to estimate the fraction of oriented lipid.

Two particular chemical shifts correspond to specific lipid-orientations relative to the magnetic field. The chemical shift at high field (Scheme I) exhibits the highest spectral intensity and corresponds to lipids oriented at 90° relative to the applied field and hence the notation 'perpendicular edge' ($\sigma_\perp$) denotes this spectral resonance. As one moves toward lower field, chemical shifts correspond to lipid orientations less than 90°, until the chemical shift at $\sigma_\parallel$ is reached which corresponds to a lipid orientation of 0° relative to the applied field. The chemical shift corresponding to molecules oriented at 0° is denoted as the 'parallel edge' ($\sigma_\parallel$) of the spectrum (Scheme I). The spectral 'intensities' in the range between $\sigma_\perp$ to $\sigma_\parallel$ determine the NMR lineshape and are proportional to the population of phospholipid molecules at orientation angles between 0° and 90°. Both the residual chemical shift anisotropy ($\Delta\sigma = \sigma_\parallel - \sigma_\perp$) and the spectral lineshape between $\sigma_\parallel$ and $\sigma_\perp$ are important for characterizing the magnetically induced orientation of bilayers.

Magnetic orientation of bilayers is usually observed when liposomes are made from mixed lipids (Table I). However, our studies have shown that almost complete orientation of bilayers could be obtained on pure DMPC and DPPC liposomes at 11.7 T field (Figs. 1 and 2). In addition, our results demonstrate for the first time that liposomes prepared from DLPC, egg PC, and egg PC/CH 70:30 orient in high magnetic fields. Collectively these observations suggest that magnetic orientation of bilayers is a general phenomenon of phospholipid membranes. Spherical, or approximately spherical liposomes contain lipid molecules distributed at all orientations relative to the magnetic field and magnetically induced bilayer orientation requires distortion of the liposome structure by the magnetic field. In other words, when $> 90\%$ of the lipid bilayers in DMPC and DPPC liposomes are oriented (Figs. 1 and 2), the spherical liposome must become either partially cylindrical or cigar shaped; a short fat cigar has a shape similar to an elliptical vesicle and this reflects our use of the term cigar shape.

The origin of the magnetic orientation force is the inherent orientation-dependent diamagnetic susceptibility χ of molecules. The term 'susceptibility' denotes that when a molecule is placed in a magnetic field (H), the electrons that bond each pair of atoms are 'susceptible' to magnetically induced currents. The induced current of each molecular bond generates a magnetic moment (μ) antiparallel to the direction of H. The magnitude of μ is proportional to H which merely indicates that as the field strength increases, the induced currents increase and the magnetic moment μ increases. Diamagnetic susceptibility χ of the molecule is the proportionality constant between the small magnetically induced moments μ and H

$$\mu = \chi \cdot H \tag{4}$$

In other words, it is χ (controlled by contributions from each bond) that determines the magnitude of μ for a particular magnetic field strength. Induced currents in the bonding electrons actually depends on the orientation of each bond relative to the magnetic field. Since all bonds do not have the same orientation, the induced local moments from each electron-pair are randomly distributed over the entire molecule. Consequently, the diamagnetic susceptibility of the entire molecule has contributions from the induced currents in each bond and diamagnetic anisotropy indicates that

the diamagnetic susceptibility at each point on the molecular surface is not uniform. Bond diamagnetic susceptibility should thus not be confused with diamagnetic anisotropy of the molecule.

Changing the orientation of a molecule in a magnetic field changes the orientation of the bonds relative to the field. Because bond diamagnetic susceptibilities depend on the orientation of the bond relative to the field, the molecular diamagnetic anisotropy is significantly effected by the orientation of the molecule itself relative to the magnetic field. However, there is some particular molecular 'orientation' that generates a maximum diamagnetic susceptibility. For this 'orientation', a maximum induced moment μ is created according to Eqn. 4, and the static field H thus torques $(H \times \mu)$ the molecule. When μ is at a maximum, the torque is maximum and consequently, the molecular orientation corresponding to the maximum diamagnetism (induced magnetism) corresponds to the position of maximum torque on the molecule (i.e., this is an energetically unfavorable molecular orientation in the field). For phospholipids, χ is maximum when the long axis of the lipid molecule is parallel to H and minimum when the long axis is perpendicular to H. The molecular diamagnetic anisotropy $\Delta\chi$ of individual phospholipid molecules is expressed as

$$\Delta\chi = \chi_{\parallel} - \chi_{\perp} \tag{5}$$

$\chi_{\parallel}$ is the diamagnetic susceptibility of molecules oriented parallel to the field, and $\chi_{\perp}$ is the diamagnetic susceptibility when molecules are oriented perpendicular to the field. When the values of $\chi_{\perp}$ and $\chi_{\parallel}$ are significantly different, a large diamagnetic anisotropy $\Delta\chi$ exists for the molecule. The 'sign' of $\Delta\chi$ may be positive or negative and determines the direction of the orientation force. If $\Delta\chi$ is positive the molecules tend to orient parallel to the field, but if $\Delta\chi$ is negative the molecules tend to orient perpendicular to the field.

The 'magnitude' of $\Delta\chi$ is determined by the contributions from individual functional groups in the lipid molecule: hydrocarbon chains, glycerol esters, and the phospholipid headgroup. For DPPC crystals, $\Delta\chi$ is $-68 \cdot 10^{-6}$ EMU/mol [38] yet $\Delta\chi$ is only $-2 \cdot 10^{-6}$ EMU/mol for egg PC in the L_{α} phase [39]. This decrease in $\Delta\chi$ for phosphocholine molecules containing all trans alkyl chains (i.e., DPPC) vs. unsaturated and fluid alkyl chains (i.e., eggPC) indicates that the magnitude and sign of $\Delta\chi$ is determined by the lipid alkyl chains instead of the lipid headgroup. In a field H, individual functional groups within the molecule will tend to adopt the orientation that corresponds to the lowest energy level. When lipid ester carbonyls are oriented parallel to the static field, they attain their lowest energy level; whereas, hydrocarbon chains attain their lowest energy level when oriented perpendicular to the static field. Since lipid ester carbonyls are approximately perpendicular to lipid hydrocarbon chains, both of these functional groups tend to orient the lipid molecule in the same direction to attain the lowest energy state of the molecule [38].

Individual molecules can not orient because the magnetic orientation energy E_m calculated by

$$E_m = \Delta\chi H^2 \tag{6}$$

is much less than thermal energy responsible for Brownian motion which occurs both in and out of magnetic fields. However, when aggregated lipid molecules are aligned approximately parallel as found in bilayers, $\Delta\chi$ is additive and the magnetic orientation energy becomes proportional to the the aggregation number N. Thus, the orientation force of lipid aggregates becomes cooperative and the bilayer magnetic orientation energy E_M is

$$E_M = N\Delta\chi H^2 \tag{7}$$

In a magnetic field, liposome structure will be determined by the balance of all forces, i.e., membrane curvature energy, thermal energy, and magnetic energy and if E_M exceeds both the membrane curvature energy and thermal energy, then liposome bilayers will orient. Based on the earlier work of Helfrich [40], the distortion of spherical liposomes by magnetic fields has been modeled as

$$\frac{\Delta R}{R} = \frac{R^2 d \Delta\chi H^2}{12\kappa} \tag{8}$$

where R is the radius of the nondeformed liposome, ΔR corresponds to the deformed liposome and is the difference between the half-axes parallel and perpendicular to the field, d is the membrane thickness, H is magnetic field strength, and κ is the membrane curvature energy which is linearly related to the bending elastic modulus of the membrane K_m (i.e., $\kappa = K_m d$) [40,41]. The bending elastic modulus characterizes the membrane rigidity which is the energy needed to bend the membrane. Although Helfrich's model was derived for single layered liposomes, it also applies to multilayered liposomes if each individual liposomes lamellae is considered as an individual liposome; in other words multilayered liposome structures can be considered as liposomes within liposomes. Eqn. 8 contains parameters for liposome structure (R, d), magnetic energy (H), thermal energy (κ significantly depends on temperature), and membrane energy (κ). Because liposome deformation is necessary for lipid orientation the proportion of lipid oriented in any lipid dispersion is proportional to Eqn. 8 and therefore the % orientation

can be written as

$$\% \text{ Orientation} \propto \frac{R^2 d \Delta\chi H^2}{12\kappa} \quad (9)$$

From Eqn. 9, H (homogeneous field strength) and R (initial liposome radius) are the most important factors determining if a given membrane preparation will orient during NMR measurements at high field because these parameters are raised to the second power. The H^2 dependence was verified (Fig. 3) because H can be easily controlled; however, since it is very difficult to obtain completely homogeneous populations of liposomes with a narrow size distribution, the effect of R on the % orientation could only be qualitatively confirmed as shown in Fig. 4.

The importance of temperature and lipid mixtures on membrane orientation is not obvious from Eqn. 9. However, both the strong temperature-dependent (Figs. 1, 2) and lipid-mixture dependent orientation (Table I) can be attributed to changes in κ (the bending elastic energy of the membrane). κ decreases dramatically during the phase transition from gel to liquid crystal [36] and based on Eqn. 9, as κ decreases the % orientation will increase. The decrease in κ above T_m indicates that the fluid membrane is more elastic and can be deformed by the magnetic field. Guest molecules in the membrane (particularly lysolipids) affect κ in a manner similar to raising the temperature. Lysolipids significantly decrease the bending elastic modulus of membrane bilayers making them more easily deformed [18]. Eqn. 9 thus demonstrates that the bending elastic modulus is an important parameter for membrane orientation but lipid diamagnetic anisotropy, magnetic field strength, liposome radius, and membrane thickness all contribute to the phenomenon and merely being above the T_m is not sufficient to orient the bilayers.

Our experimental results (Figs. 1–6) and the results of others (Table I) are consistent with Eqn. 9. For a given field strength, the liposomes' structure (Fig. 4) and lipids forming the membrane (Figs. 1, 2 and 5) determine if orientation occurs. If the liposomes are of sufficient size (Fig. 4) then temperatures above T_m are sufficient for the magnetic energy to induce bilayer orientation. Spherical liposomes have isotropic membrane curvature energy which does not depend on liposome size [42]. In contrast to membrane curvature energy, the anisotropic magnetic energy is additive (according to Eqn. 7) and increases as the liposomes size increases, i.e., as the aggregation number increases.

For a given number of lipid molecules per unit area of membrane, the average magnetic force on the bilayer depends on the aggregation number (based on Eqn. 7) in the unit area of membrane. However, diamagnetic susceptibility depends on the orientation of lipid molecules relative to the static field. Therefore the membrane curvature gradient per unit area of membrane, may influence the total orientation force experienced by a particular bilayer if the bilayer has high curvature. Stated differently, the molecules forming the liposome equator (perpendicular to the field) are at right angles to the lipid molecules at 0° latitude (liposomes north or south pole relative to the static field) and the lipid orientation gradient from the equator, to 0° latitude, can be steep (for very small liposomes) or very small (for large liposomes). Membrane curvature thus determines the orientation gradient of lipid molecules in the liposomes and can affect the amount of magnetic energy available to deform the membrane. The key concept is that membrane curvature determines the lipid orientation gradient relative to the static field and hence membrane curvature can affect E_M for liposomes.

For small liposomes (SUVs) with high membrane curvature, the rapid change in lipid orientation as one moves away from the liposomes' equator results in minimal magnetic energy per unit area to deform the liposome structure. For large liposomes, with a significant surface area near the equator, the magnetic deformation force can be substantial. However, when the curvature of closed liposome membranes approaches the (lack of) curvature common to planar membranes, the magnetic force per unit area of membrane will remain approximately constant. We have previously demonstrated that on a molecular level phospholipid bilayers are approximately planar, i.e., have no curvature, when the liposomes' radius exceeds approx. 0.1 μ [25]. Most methods for preparing liposomes generate liposomes with diameters much greater than 1 μ and consequently most of the 'lipid' in liposomal preparations contributes to the formation of planar membranes; thus the magnetic energy per unit area of membrane (at a particular orientation relative to the static magnetic field) is similar for all liposomes that exceed 0.1 μ in radius.

For most membrane systems, magnetically induced bilayer orientation is reversible such that cooling below T_m causes the liposome structure to return to a 'nondeformed' state which eliminates bilayer orientation in the field. Thus, when the temperature drops below T_m, κ increases to its original value and the liposome returns to a spherical nondeformed shape and the typical ^{31}P-NMR powder pattern is obtained (Figs. 1 and 2). One report is available demonstrating that cooling below T_m did not destroy lipid orientation in this mixed lipid system [1]. Because κ decreases for mixed lipids systems [43], they are more easily oriented compared to pure lipid bilayers. The persistent orientation of lipid after cooling below T_m does not appear to be a general phenomenon and more experiments are

needed to understand lipid-dispersion regarding the persistent lipid orientation below T_m when the orientation initially occurred above T_m.

The effect of bulk viscosity on lipid orientation is not defined in Eqn. 9. Bulk viscosity of suspensions is determined by the ratio water/lipid in the dispersion, and lipid orientation is hindered at high viscosity. Typically lipid dispersions require > 65 wt% water to have sufficient bulk water to form closed membrane structures [25]. At < 50 wt% water in the lipid dispersion there is insufficient bulk water to suspend the lipid mixture into closed liposome membranes [25] and consequently the aqueous swelling of lipids under limiting hydration conditions results in predominantly stacks of lamellae; the lipid dispersion has a very high viscosity similar to a paste. High viscosity of membrane preparations are expected to interfere with magnetically induce bilayer orientation. For instance ^{31}P-NMR studies by Jansson et al. [18] have demonstrated that an aqueous 50 wt% dispersion of DPPC will not orient in an 11.7 T field; whereas, our ^{31}P-NMR spectra (Fig. 2) obtained on ~ 10–20 wt% DPPC clearly demonstrates almost quantitative lipid orientation. Thus, bulk viscosity and/or > 65 wt% water can be significant factors effecting lipid orientation in magnetic fields.

Lipid dispersions can exist in complex phases and the structure of the self assembled lipids depends on the lipids, buffers, counter ions etc. Lipid structures can be micelles, hexagonal phases, and bilayer phases and even within the bilayer phases several stable phases have been studied (L_c, P_β, $P_{\beta'}$, L_α etc.). The lipid bilayers of AC2 dispersions did not significantly orient in high magnetic fields either above or below T_m(40°C). AC2 is a chemical analog of DMPC that contains an oxygen atom substituted for each C13 methylene group in the lipid alkyl chains. Thus, AC2 contains terminal methyl groups tethered to the lipid alkyl chains by ether bonds. For free fatty acids, this oxygen for methylene substitution causes a decrease in the hydrophobicity equivalent to removing ~ 2–4 carbon atoms from the fatty acid [44]. Thus, AC2 with two fatty acid chains is expected to be significantly less hydrophobic than DMPC. The $\Delta\chi$ for a C-O bond is slightly greater than the $\Delta\chi$ for a single C-C bond [38] and therefore the $\Delta\chi$ for AC2 should be similar or slightly greater than the $\Delta\chi$ of DMPC. Therefore, E_m for AC2 should be greater than E_m for DMPC; however, bilayer orientation does not occur in spite of the higher magnetic deformation force on AC2 bilayers compared to DMPC bilayers. Because differences in the $\Delta\chi$ of DMPC and AC2 do not account for the inability of AC2 dispersion to orient in magnetic fields, temperature dependent membrane properties must be responsible as described below.

AC2 is a novel phosphatidylcholine lipid analog with unusual physical chemical properties when compared to DMPC which is an equal chain length phosphatidylcholine. AC2 has a T_m ~ 41.4°C which is ~ 18°C higher than the T_m of DMPC and unlike DMPC, AC2 liposomes do not aggregate during prolonged storage at 4°C. This suggests that the molecular packing of AC2 phosphocholine molecules is significantly more compact in AC2 bilayers compared to the molecular packing of DMPC phosphocholine molecules in DMPC bilayers. Nonvortexed AC2 lipid dispersions heated above T_m without vortexing, form typical spherical liposome structures 1–5 μ in radius which are smaller in size to nonvortexed DMPC lipid dispersions. However, unlike DMPC suspensions when heated above T_m, the bright white AC2 suspension is converted into a suspension that appears similar to dilute liposomes, i.e., a significant reduction in light scattering occurred above T_m. This decrease in optical density above T_m suggests that AC2 liposomes spontaneously convert from large structures to smaller liposomes. The decreased size of AC2 liposomes above T_m can explain the lack of magnetically induced lipid orientation (Fig. 6) and is consistent with the R^2 dependence in Eqn. 9. Liposome fission above T_m has recently been well studied [45].

If the average size of AC2 liposomes reversibly decreases above T_m, then AC2 membranes have unusual thermaoelastic properties (κ) compared to DLPC, DMPC, DPPC and eggPC lipids which do not undergo fission above T_m. This suggests that κ for AC2 must be much less than κ for endogenous lipids. Liposome fission above T_m has been observed for large single layered DMPC liposomes but only when the incubation temperature was much greater than T_m, i.e., for temperatures more than 25°C above T_m [45].

The possibility of a size transformation in AC2 liposomes is consistent with the sudden 10 ppm reduction in $\Delta\sigma$ of the powder pattern when the spectrum was obtained above T_m. The reduced $\Delta\sigma$ occurs because smaller AC2 liposomes undergo more rapid Brownian tumbling which further averages the ^{31}P principle chemical shielding tensors. In addition, the reversible change in $\Delta\sigma$ above and below T_m indicates that cooling AC2 liposomes below T_m causes the smaller liposomes to fuse into larger liposomes, i.e., the size transformations of AC2 are reversible. In addition to changes in liposome size, increased wobbling of the phospholipid headgroup in the plane of the bilayer can also explain the decrease in $\Delta\sigma$. If the lipid molecule can wobble in the plane of the membrane, the principle shielding tensors begin to average causing a reduction in the spectral width. Alterations in the breadth of the powder pattern are currently being evaluated and the contribution of molecular wobbling at the liposome-surface vs. size reduction in the liposome-structure of AC2 liposomes is under investigation.

In the absence of a magnetic field, liposomes are approximately spherical because this shape has minimum curvature energy. Thus, when liposomes are initially put into a magnetic field, the spherical liposome contains minimum membrane curvature energy and maximum magnetic orientation energy. The liposome structure will change from this high energy state to a lower energy state. Liposome rotation in the *xy* plane does not affect the magnetic orientation force; whereas liposome tumbling in the *xz* plane (or *yz* plane) competes with the orientation force. Liposomes that exhibit significant orientation tend to aggregate; aggregation either eliminates or significantly reduces liposome tumbling (and rotation) allowing the magnetic force to deform the liposome.

Lipid orientation requires deformation of the liposome structure from approximately circular to most likely a cigar shape (i.e., prolate ellipsoid). Spherical liposome membranes are thus under 'strain' in magnetic fields; strain-1 involves lipid molecules at the equator which resist the magnetically induced reorientation force, and strain-2 involves the lipid molecules at the apex that experience the highest magnetically induced reorientation force which torques lipid molecules at the liposome-north (or south) pole towards the liposome equator. These two counter-acting forces cause strain on each hemisphere of the liposome and the strain is relieved when the liposome is distorted into a cigar shape; the cigar shape minimizes only strain-2 or the torque on the liposome hemispheres. Strain-2 is minimized because only a small fraction of the lipid molecules are needed to form the apex whereas the long sides of the cigar continue to resist magnetically induced motion. Cigar shaped liposomes will exhibit extremely slow (or no) tumbling but all other lipid motions remain. This includes rapid liposome rotation around the long axis of the distorted liposome (this axis is parallel with H) and lateral diffusion of lipid molecules over the entire distorted surface.

Very recently [46,47], the magnetically induced distortion of liposomes caused by high magnetic field strengths was observed by freezing DPPC/cholesterol liposomes preequilibrated in a 9.4 T magnetic field [46]. Electron micrographs of the frozen-liposomes clearly showed that MLV liposomes can be distorted into elliptical shapes by the magnetic field.

Conclusions

High field NMR spectrometers (11.7 T) have sufficient magnetic energy to cause either complete or partial orientation of lipids that form the liposome membrane. The extent of orientation is greatest above the main transition temperature of the lipid. Frequently the fraction of oriented lipid is small relative to the total lipid content of the lipid dispersion and spectral simulations are needed to quantify the orientation.

Acknowledgments

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