Polymerization of the Inverted Hexagonal Phase

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Abstract: The hydration of polar natural and synthetic lipids yields a variety of lipid phases including various inverted cubic phases and the inverted hexagonal (H_{II}) phase. The H_{II} phase can be considered as aqueous columns encased with a monolayer of lipids and arranged in a hexagonal pattern. The polar head groups are well-ordered at the water interface, whereas the lipid tails are disordered to fill the volume between the tubes of water. A particularly interesting characteristic of the H_{II} phase is the large temperature effect on the basis vector length *d* of the hexagonal lattice. Previous studies indicate that polymerization of the lipid region of the H_{II} phase might reduce the sensitivity of the basis vector to temperature. A phosphoethanolamine (PE) was designed and synthesized with dienoyl groups in each lipid tail in an attempt to cross-link the lipids around and along the water core of the H_{II} phase. The synthesis of the the PE was accomplished by acylation of 3-(4-methoxybenzyl)-*sn*-glycerol with 2,4,13-(*E*,*E*,*Z*)-docosatrienoic acid, followed by deprotection, then phosphorylation with dichloro-[[*N*-[(2,2,2-trichloroethoxy)carbonyl]-2-amino]-ethyl]phosphinic acid to give the Troc-PE, which was converted to the PE with activated zinc and acetic acid. The hydrated PE (1/1 weight lipid/water) formed the H_{II} phase over an extended temperature range. Polymerization to high conversion was accomplished at 60 °C with the aid of redox initiators. Polymerization was followed in-situ using X-ray diffraction over a period of 48 h. The scattering, which weakened over the course of the reaction, remained consistent with a hexagonal phase. Temperature cycling of the polymerized H_{II} phase showed an unaltered pattern on decreasing temperature while maintaining the same lattice parameter, unlike that of the unpolymerized phase where the value increased with decreasing temperature. Thus it is possible to fix the dimensions of the H_{II} phase by cross-linking polymerization of appropriately designed reactive lipids.

Introduction

The hydration of polar natural and synthetic lipids yields a variety of lipid phases depending on the concentration, temperature, and pressure. At high concentrations certain lipids form quite complex lyotropic liquid crystals. These include various inverted cubic phases and the inverted hexagonal (H_{II}) phase.[1−3] The most commonly studied of these phases is the H_{II}, which is readily formed from lipids that exhibit a large spontaneous curvature under certain conditions. Curvature is a consequence of an imbalance of forces across a lipid layer.[4] When the repulsive lateral pressure in the lipid tail region is greater than the repulsive forces acting at the head group, a lipid layer assumes a negative curvature as shown in Figure 1. Lipids with cis-double bonds or branching substitutents in the tails and relatively small, poorly hydrated head groups form the H_{II} phase at moderate to low temperatures.[5]

The H_{II} phase can be considered as aqueous columns arranged in a hexagonal pattern (Figure 1). Each water channel is encased within a monolayer of lipid. The polar head groups are well-ordered at the water interface, whereas the lipid tails are disordered to fill the volume between the columns of water. A particularly interesting characteristic of the H_{II} phase is the large temperature effect on the basis vector length *d* of the hexagonal lattice. Tate and Gruner showed that reduction in the radius of the water channel accounts for most of the change in this lattice vector.[5] It may therefore be possible to polymerize of the lipid region in a manner to lock-in the column diameter rendering the unit cell of the H_{II} phase insensitive to changes in temperature.

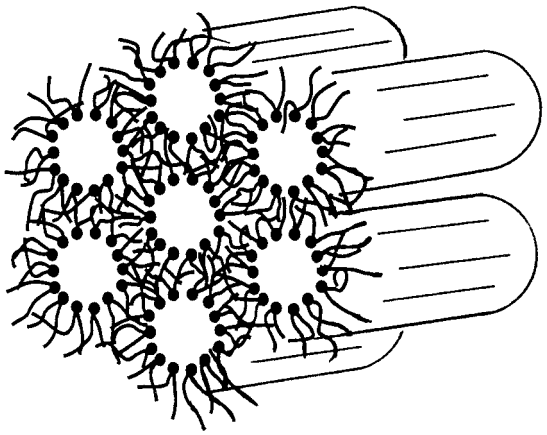

Figure 1. Schematic representation of the inverted hexagonal phase.

Previously reported methods to polymerize and stabilize lamellar assemblies, i.e. bilayer vesicles, offer approaches to the polymerization of nonlamellar assemblies. Since the first reports of the polymerization of hydrated bilayers in the early 1980s,[6−9] a wide variety of polymerizable groups have been successfully employed. The various strategies for the polym-

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erization of lamellar phases have been reviewed.[10−13] A particularly useful method relies on the design of suitable polymerizable amphiphiles, which upon hydration form assemblies that can then be polymerized in place. Several reactive lipids, e.g. acryloyl, dienoyl, and sorbyl, may be polymerized by radical chain processes in either the L_β or L_α phases, whereas the topotactic polymerization of diacetylenes limits this chemistry to the solid-analogous (L_β) phase.[9] Lipids in the L_α phase exhibit rapid lateral diffusion,[14] in a manner that facilitates the polymerization process.[15] A series of systematic studies have provided new insights into the effect of the two-dimensional nature of the lipid bilayer on the rate and degree of polymerization in the L_α phase.[16−19] In recent years the effects of polymerization on the partial phase diagram of some hydrated lipids have been reported.[20−23]

On the basis of the current understanding of the polymerization of the lamellar phase and our success in extending these methods to polymerization of a bicontinuous cubic phase,[22] we began an examination of the polymerization of the H_{II} phase. Various strategies for the design of polymerizable lipids were considered at the outset of these studies. First, the reactive group could be located on either or both lipid tail(s) near the lipid backbone, i.e. the glycerol unit in the case of phospholipids. Second, the reactive group could be placed at the end of the lipid tails, although this may be less desirable because polymerization of the disordered terminal end of the lipid tails may perturb the H_{II} phase. Third, the reactive group could be attached to the lipid head group as a hydrophilic substitutent. The first approach appeared to be the most promising because covalent linkage of lipids near the backbone would have less effect on the important forces that act at the head group and tails of the lipids. Furthermore the use of a diene group conjugated with the acyl chain carbonyl does not interfere with the biocompatibility of the lipid−water interface. Here we report the design and synthesis of a PE with dienoyl groups in each lipid tail in a manner that is expected to yield a cross-linked polymer around and along the water core of the H_{II} phase. Studies of the polymerization of bis-substituted dienoyl lipids, e.g. phosphatidylcholines (PC), in the lamellar phase show the formation of cross-linked bilayer membranes.[24] In addition the formation and polymerization of the H_{II} phase was accomplished in a manner that preserves the lipid assembly as well as leads to the formation of individually polymerized unit cells, i.e. tubes of lipid surrounding the water core.

Results and Discussion

[1,2-Bis[2,4,13-(*E*,*E*,*Z*)-docosatrienoyl]-*sn*-glycerol]-3-phosphoethanolamine (**1**) that contains a polymerizable dienoyl group in each hydrophobic chain was designed and synthesized. A cis-double bond was incorporated into each hydrophobic tail to facilitate the formation of H_{II} phase at suitable temperatures. Polymerization of the diene substituted lipids can be accomplished with the aid of either thermal or redox initiators or by direct photopolymerization.[24] In this study, redox-initiated radical polymerizations were employed, as they can be performed at reasonable rates over a wide range of temperatures.

Synthesis of Lipid 1. The synthesis of PE lipids has been studied extensively in the past decade, and several methods have been reported.[25−27] However, methods for the chemical synthesis of polymerizable PE lipids have only recently been described.[28] Our preparative approach started with the synthesis of a 1,2-bis[2,4,13-(*E*,*E*,*Z*)-docosatrienoyl]glycerol (**2**) followed by phosphorylation. Acylation of 3-(4-methoxybenzyl)-*sn*-glycerol[29,30] with 2,4,13-(*E*,*E*,*Z*)-docosatrienoic acid (**3a**) and then deprotection of the 4-methoxybenzyl ether group via Lewis acid catalyzed hydrolysis at low temperature afforded **2** (Scheme 1).

The polymerizable fatty acid **3** was accessible in three steps from commercially available oleoyl alcohol (Scheme 2). The alcohol was oxidized to the corresponding aldehyde **4** using pyridinium dichromate (PDC) in CH_2Cl_2.[31] The Wittig−Horner reaction of **4** and trimethyl 4-phosphonocrotonate gave methyl dienoate **5**, which upon base-catalyzed hydrolysis using 1.5 mol equiv of KOH in methanol afforded acid **3**. This acid was obtained as a mixture between (*E*,*E*)- and (*E*,*Z*)-isomers as determined by ^{1}H NMR spectroscopy. We utilized urea inclusion complexation to separate the (*E*,*E*)-acid **3a** from its (*E*,*Z*)-isomer.[28] This purification was undertaken to prepare a single lipid in order to minimize the complexity of the phase and polymerization studies. Urea is known to form a crystalline inclusion complex with certain compounds. The hydrogen bonded urea molecules in methanol orient in a helical crystal lattice in such a way as to leave a narrow cylindrical channel with a diameter of 5.3 Å, and compounds with small cross-section diameters can reside within these clathrate channels.[32,33] The optimum ratio of the host urea to the guest fatty acid was predetermined to be 18. The mixed-isomer fatty acid sample was added to a methanolic solution of urea. The more linear (*E*,*E*)-dienoyl acid **3a** preferentially formed an inclusion complex with the urea and precipitated. The bent (*E*,*Z*)-isomer apparently did not fit within the urea clathrate channel. Filtration followed by extraction of the inclusion complex with ether produced dienoic acid that was predominantly (*E*,*E*)-isomer based on the absence of the characteristic vinyl proton of (*E*,*Z*)-isomer at 7.55−7.68 ppm in the ^{1}H NMR spectrum (Figure 2).[34]

The selection of the protecting group for glycerol is crucial for a successful synthesis. The deprotection step cannot interfere with the dienoyl polymerizable group, and it must not facilitate

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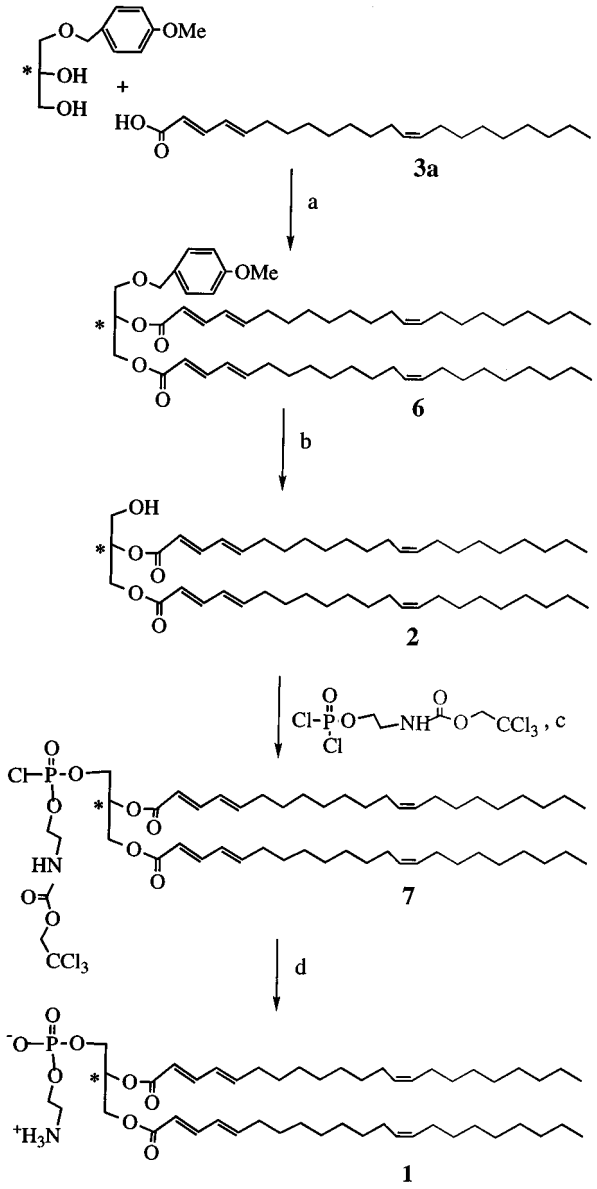
Scheme 1[a]

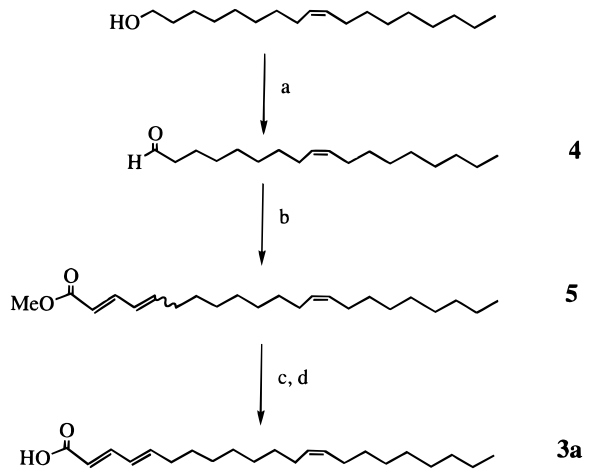


[a] (a) DCC, DMAP, $CHCl_3$. (b) Me_2BBr, CH_2Cl_2, −78 °C. (c) Pyridine. (d) Zn/HOAc.

the isomerization from 1,2-diacylglycerol to 1,3-diacylglycerol. The 4-methoxybenzyl ether meets these criteria.[29,30] Herbert et al. reported that its deprotection via Lewis acid Me_2BBr catalyzed hydrolysis at very low temperature (−78 °C) for a short period of time (10−15 min) proceeded in quantitative yield without any isomerization of 1,2-diacylglycerol.[30]

The acylation of 3-(4-methoxybenzyl)-*sn*-glycerol with 2,4,-13-(*E*,*E*,*Z*)-docosatrienoic acid (**3a**) using dicyclohexylcarbodiimide (DCC) and 4-(dimethylamino)pyridine (DMAP) in CH_2Cl_2 gave 1,2-bis(dienoyl)-3-(4-methoxybenzyl)-*sn*-glycerol **6**. When an excess of fatty acid was employed, **6** was obtained in less than 20% yield and accompanied by acid anhydride and acid−DCC complex side products. The yield of **6** was improved to 50% (based on fatty acid) when excess protected glycerol (1.5 mol equiv) was used. The deprotection of the 4-methoxybenzyl ether group was accomplished by treating it with excess dimethylboron bromide in dry CH_2Cl_2 at −78 °C for 15 min. The reaction was quenched by diluting with ether and washing with water until the aqueous extracts were neutral. The purity of the product was determined by thin layer chromatography and ^{1}H NMR spectroscopy, which showed that

Scheme 2[a]



[a] (a) PDC, CH_2Cl_2. (b) NaH, trimethyl phosphonocrotonate, THF. (c) KOH, MeOH. (d) Urea inclusion.

the deprotection proceeded without interfering with the polymerizable dienoyl group and isomerization of 1,2-diacylglycerol to its 1,3-isomer.[28,35]

The final step was phosphorylation of **2** with a N-protected phosphorylating agent. 2,2,2-Trichloroethoxylamide (Troc-amide) was employed as an amine protecting group since it could be readily removed via reduction with activated zinc under nonhydrolytic conditions thereby avoiding reduction of the carbon−carbon double bonds.[36] Treatment of **2** with phosphorylating agent dichloro-[[*N*-[(2,2,2-trichloroethoxy)carbonyl]-2-amino]ethyl]phosphinic acid[36] afforded Troc-PE **7** in 70% yield. The Troc-protecting group was removed by stirring **7** overnight with activated zinc in a mixture of glacial acetic acid and ether at room temperature (rt) to yield the desired polymerizable PE **1** as a white solid in 80% yield.

Unsaturated PEs such as those found in biological membranes are particularly sensitive to oxidation.[37] Lipid **1** suffers from a similar sensitivity. Samples of hydrated **1** in the presence of oxygen, without initiator, began to noticeably deteriorate at temperatures of 60 °C or higher as judged by an increase in yellow color. If the lipid samples were handled under argon they were significantly more stable. Consequently the experiments described here were performed under argon to minimize lipid degradation. The nature of the oxygen-dependent degradation of lipid **1** remains to be determined.

Phase Study of Lipid 1. Variable-temperature ^{31}P NMR was used to examine the phase of lipid **1** at high concentration. A 1:1 mixture of **1** and MilliQ water was incubated at 5 °C under Ar for 48 h after hydration yielding an opaque solid. The proton-decoupled ^{31}P-NMR spectrum of **1** at 5 °C showed the characteristic line shape of a well-ordered hexagonal assembly with a baseline width of 26 ppm (Figure 3a). The sample temperature of **1** was then increased rapidly to 60 °C and another spectrum acquired after the temperature stabilized for ca. 5 min. The spectrum at 60 °C had the same line shape as that acquired at 5 °C (Figure 3b). The apparent slight narrowing of the width (25 ppm) is probably due to increased rotational and translational motions of the lipids at the higher temperature.

X-ray diffraction characterization of the phase behavior of a 1:1 mixture of **1** and MilliQ water revealed the formation of

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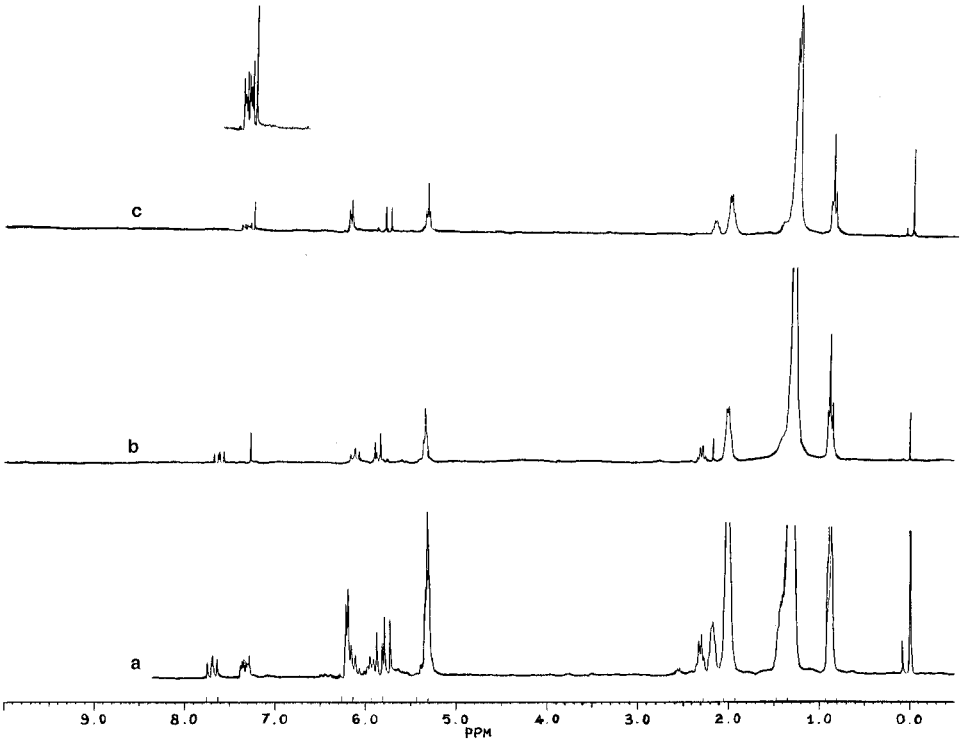

Figure 2. ^{1}H-NMR spectra of (a) mixture of (*E*,*E*)- and (*E*,*Z*)-dienoic acid **3**, (b) (*E*,*Z*)-dienoic acid, and (c) (*E*,*E*)-dienoic acid **3a**.

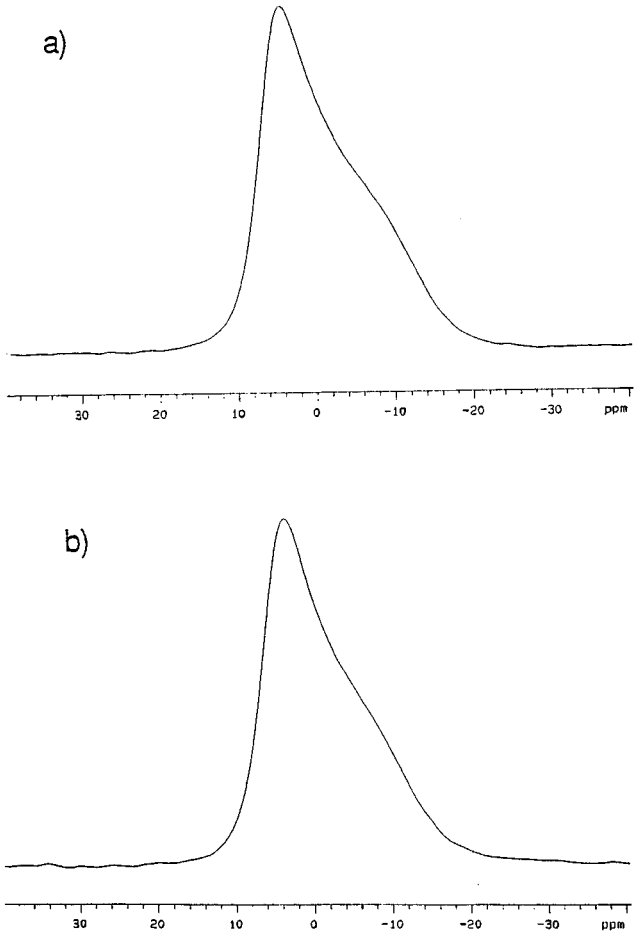

Figure 3. ^{31}P-NMR spectra of lipid **1** and water (1:1 by wt) at (a) 5 °C and (b) 60 °C.

two liquid crystalline phases, an inverse hexagonal and a bicontinuous cubic (consistent with the $Pn\bar{3}m$ space group) over the temperature range 0−80 °C. Figure 4 shows the variation with temperature in the unit cell parameters for each of these

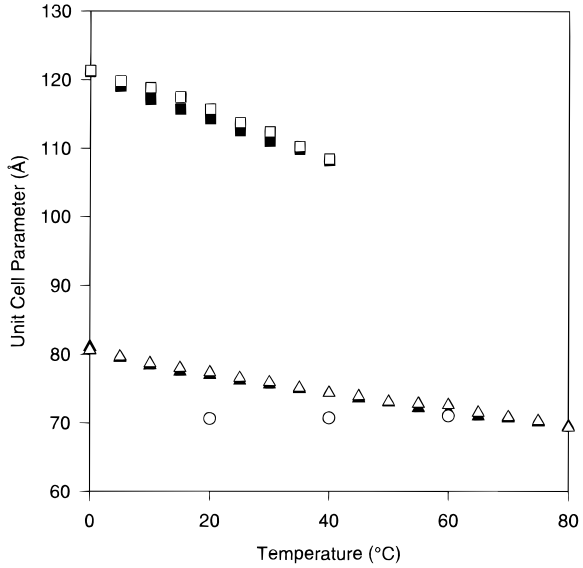

Figure 4. Variation in unit cell parameters measured via X-ray diffraction as a function of temperature along the isopleth 1/1 lipid to MilliQ water. Squares = double diamond bicontinuous cubic phase, triangles = H_{II} phase, and circles = polymerized H_{II} phase. Closed symbols indicate increasing temperature run and open decreasing temperature run.

phases. Wide-angle X-ray diffraction showed a single diffuse ring centered at 4.5 Å for all temperatures, consistent with the phases having fluid hydrocarbon chains. At 0 °C the two phases coexist. The dominant signal is due to the H_{II} phase, although it is difficult to quantitate the contribution of the bicontinuous cubic phase. The absence of an isotropic component in the ^{31}P-NMR spectrum of a sample of lipid **1**/water with a weight ratio of 1 at 5 °C indicates that the amount of the cubic phase is quite small. An attempt to produce a pure double diamond ($Pn\bar{3}m$) bicontinuous cubic phase by continued heat cycling and incubation about the temperature range of the transition from lamellar to nonlamellar (ca. −10 °C) was unsuccessful.[38] A pure lamellar phase with a lattice parameter of approximately

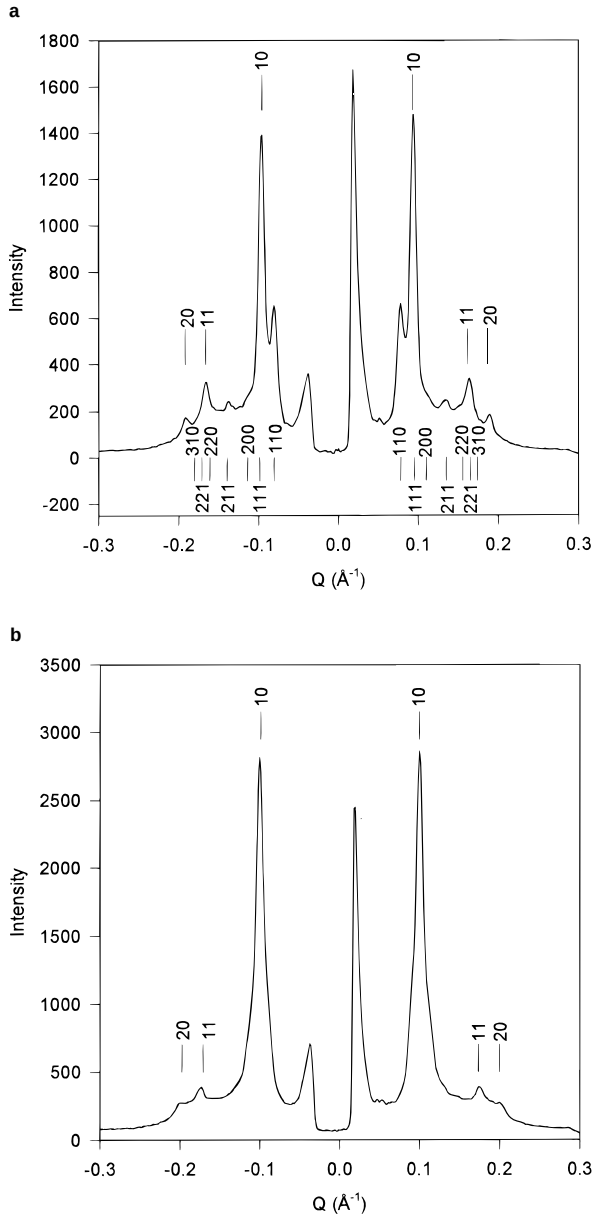

Figure 5. Azimuthal integration of the two-dimensional X-ray diffraction patterns upon heating of a hydrated 1:1 lipid to MilliQ water mixture: (a) 25 °C, coexistence of double diamond (112.6 Å) and inverse hexagonal (76.1 Å) phases; (b) 60 °C, hexagonal phase (72.6 Å). Tick marks indicate the expected positions of the appropriate corresponding lattice.

55 Å was observed below −20 °C. Further characterization of the low-temperature behavior was complicated by the partial freezing of sample water. At temperatures above 40 °C the H_{II} phase was found in a pure state. The measured lattice parameters for the hexagonal and the cubic phases decreased on heating in a reversible manner, varying between 81.1 Å at 0 °C and 69.3 Å at 80 °C and 121.2 Å at 0 °C and 108.2 Å at 40 °C, respectively. Figure 5 shows the corresponding azimuthal integrations of the two-dimensional X-ray diffraction patterns in each of the regions along this isopleth.

Polymerization of the H_{II} Phase. Polymerization studies of the H_{II} phase were performed at both low and high concentrations. Redox-initiated radical polymerization using $K_2S_2O_8$/L-cysteine (1/1) as the initiator was employed for the H_{II} assemblies at low concentration (100 mg/mL). The polymerization was performed at 60 °C in the absence of oxygen, with a ratio of monomer to initiator of 8. This redox system generates hydroxyl radicals which are free to diffuse into the nonlamellar assemblies where they react with the polymerizable dienoyl groups. Redox polymerization of similar dienoyl lipids in lamellar assemblies afforded polymers having a degree of polymerization of 30.[24] The solubility of **1** in organic solvents was tested before and after polymerization. The unpolymerized lipid **1** was soluble in several organic solvents at room temperature, whereas the poly-**1** was insoluble in organic solvents including 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP). This dramatic change in solubility confirmed that the polymer obtained was cross-linked.[19] The conversion to polymer was determined by methanol extraction of a known weight of freeze-dried lipid and subsequent observation of the intensity of the dienoyl group at 265 nm. The UV spectrum showed that the conversion was greater than 90% (Figure 6).

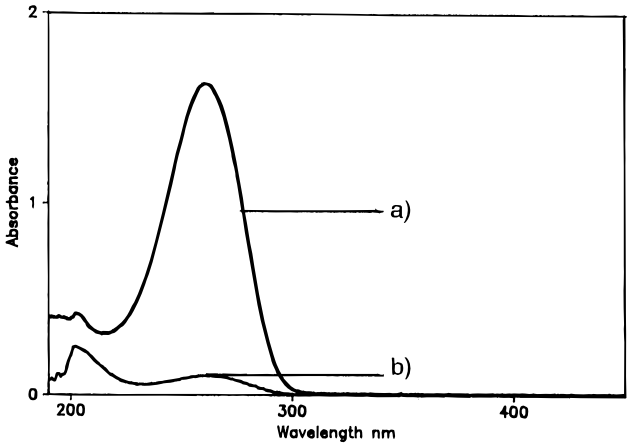

Figure 6. Absorption spectra of methanol extractions of samples of **1** before (a) and after (b) polymerization with potassium persulfate/L-cysteine as described in the Experimental Section.

Hydrogen peroxide (30% in water) was employed as a redox initiator for polymerization of the H_{II} phase at a lipid/water weight ratio of 1. After addition of the same weight deoxygenated H_2O_2 solution to **1**, the sample was centrifuged at rt and kept at 5 °C for 1 day to allow the sample to equilibrate. At this time, the UV spectrum of the lipid showed no decrease in the absorbance of the dienoyl peak at 265 nm; therefore, little or no polymerization took place during this initial incubation period. The sample was then incubated at 60 °C under Ar(g) for 2 days to perform the polymerization. The UV spectrum of the methanol-extracted polymerized lipid showed that the conversion to polymer was ca. 90%. The polymers obtained were insoluble in organic solvents including HFIP, the same as those obtained at low concentration. A control experiment with DOPE and the redox chemistry showed that the cis-double bonds in each acyl chain did not react under these conditions.

Phase Study of Polymerized Lipid 1. Lipid **1** was hydrated with MilliQ water at a concentration of 100 mg/mL. An opaque solid in excess water was observed over the temperature range 5−90 °C. The proton-decoupled ^{31}P-NMR specrtum of **1** at 25 °C showed the characteristic line shape of an inverted hexagonal assembly in excess water with a width of 26 ppm. The line shape is indicative of a multidomain hexagonal assembly which is expected upon hydration in excess water. The excess water at the edge of the assembly somewhat distorts the line shape (Figure 7a). The characteristic ^{31}P-NMR line shape for an inverted hexagonal assembly was retained after redox polymerization, but with a slightly broader width of 27−30 ppm (Figure 7b). Addition resonances within the baseline were observed on the sides of the hexagonal line shape.

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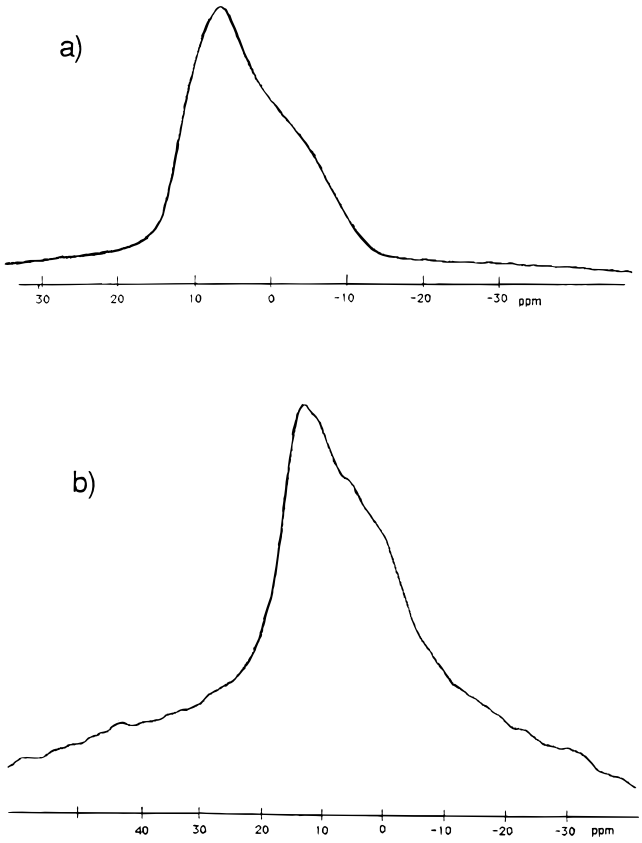

Figure 7. ^{31}P-NMR spectra of hydrated lipid **1** (100 mg/mL) before (a) and after (b) polymerization as described in the Experimental Section.

Presumably this is due to the decrease in rotational and translational motions of the lipids upon polymerization of the assembly unit cells.

A sample of lipid **1** in aqueous hydrogen peroxide in the H_{II} phase at 60 °C (being the only phase along this isopleth) was polymerized using thermal initiation of the hydrogen peroxide. Polymerization was followed in-situ using X-ray diffraction over a period of 48 h. The pattern was observed to weaken over the course of the reaction, but still indexed to a hexagonal phase. After 48 h the pattern no longer evolved. At this point a temperature cycle showed that the pattern remained unaltered on decreasing temperature while maintaining the same lattice parameter (70.6 Å as compared with 72.6 Å for the unpolymerized sample at 60 °C), unlike that of the unpolymerized phase where the value increased with decreasing temperature. The sample remained stable without further alteration. The spectrum characteristic of the double diamond phase was not observed to form. Figure 8 shows the diffraction patterns of the polymerized H_{II} phase at 60 and 20 °C. The diffraction suggests that the hexagonal phase is more poorly ordered after than before polymerization. The predominant diffraction is consistent with a hexagonal phase, even though the asymmetry of the (1,0) peaks suggests that the sample may contain some small fraction of material in either a different phase or different lattice spacing.

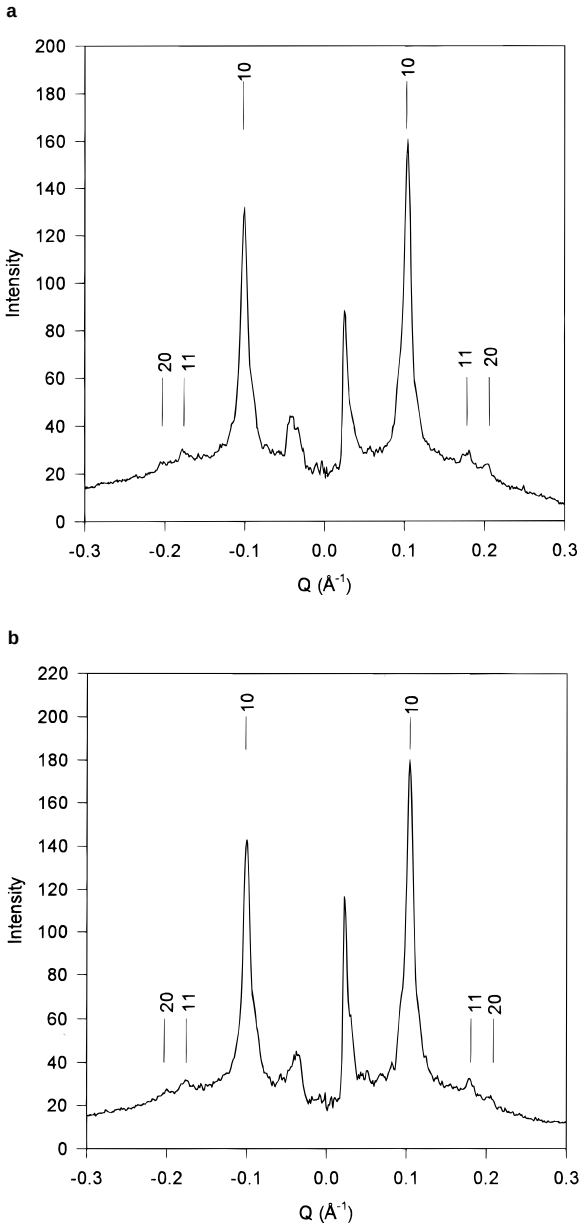

Figure 8. Azimuthal integration of the two-dimensional X-ray diffraction patterns of polymerized H_{II} phase following 48 h incubation at 60 °C in a 30% H_2O_2 solution such that lipid to total solvent is 1:1: (a) 60 °C, with hexagonal lattice parameter equal to 70.6 Å and (b) corresponding pattern after the temperature is decreased to 20 °C, lattice parameter 71.0 Å. Note weakening of the pattern in comparison to that of the unpolymerized sample at 60 °C and the lack of formation of the double diamond phase and constancy of the pattern on reducing temperature as compared with a similar sequence for the unpolymerized system. Tick marks indicate the expected positions of the corresponding hexagonal lattice.

Conclusions

Phosphoethanolamines are the most thoroughly studied lipids that readily form the H_{II} phase.[2] These lipids when hydrated usually show a thermotropic transition from the gel (L_β) phase to the liquid-crystalline (L_α) phase (designated T_m) and a higher temperature, lower enthalpy transition from the L_α to the H_{II} phase. This literature provides an excellent starting point for the design of lipids for the polymerization of the H_{II} phase. The synthetic route employed for the preparation of **1** was straightforward with generally high yields for each step. This procedure allows the preparation of research quantities of the polymerizable lipid in high purity, and it can be readily adapted for the preparation of lipids with other substitution patterns in the fatty acid tails. The critical design features of **1** include the following: a relatively poorly hydrated PE head group to decrease the head group size and permit the membrane contact required for lipid reorganization during the lamellar to nonlamellar transition; *cis*-double bonds in each lipid tail to enhance chain disorder;[39] and long lipid tails (22 carbons) to lower the lamellar to nonlamellar phase transition temperature.[40] The

relative significance of these features in nonpolymerizable lipids was examined by Lewis et al.[40] We are currently exploring the effect of lipid tail structure on the phase behavior of polymerizable PEs.

Although the molecular design was successful for this study, the coexistence of a bicontinuous cubic and H_{II} phases over part of the temperature range investigated was somewhat surprising. Whether this is a consequence of the water to lipid ratio (1:1 by weight), which is an excess of water required for the H_{II} phase, or is intrinsic to this lipid remains to be determined. The water to lipid ratio was selected to facilitate the addition of the initiator after the formation of the H_{II} phase. The initiator chemistry yields radicals that can diffuse from the water into the lipid. Other polymerization protocols that could be employed without excess water are also being examined.

The successful polymerization of the H_{II} phase of **1** at 60 °C effectively fixed the diameter of the hexagonal unit cell at 70.6 Å even after the sample was cooled to 20 °C. In the absence of polymerization the diameter would have increased to 77 Å. This observation is consistent with the previous observation of Tate and Gruner that the change of the lattice vector with temperature is primarily due to variation in the water channel size.[5] Furthermore it suggests the dimensions of the unit cell of a given polymerizable lipid in the H_{II} phase can be varied by temperature prior to polymerization and then fixed by polymerization at a desired temperature. It has not escaped our notice that this characteristic provides a means to prepare lipid tubes of selected dimension that have an aqueous interior and a hydrophobic exterior. This is a consequence of the polymerization of the unit cell of the H_{II} phase without polymerization of the whole assembly. We have already demonstrated that the polymerized H_{II} phase of **1** can be dispersed by selected organic solvents to yield supramolecular polymers. The nature of these polymers is currently under study and will be reported in due course.

Experimental Section

Methods and Materials. All chemicals were obtained from Aldrich Chemical Corp., except trimethyl 4-phosphonocrotonate, which was purchased from Lancaster Synthesis Inc. Solvents were dried and distilled prior to use. Compounds that contained UV-sensitive groups were handled under yellow light. The reactions were monitored by TLC visualized by UV light and/or phosphomolybdic acid reagent. NMR spectra were recorded on a Bruker AM-250 magnetic resonance spectrometer in chloroform-*d* with TMS as an internal reference.

Synthesis of 1. 9-(*Z*)-Octadecenal (4). Pyridinium dichromate (PDC, 4.2 g, 11.1 mmol) was added to a solution of oleyl alcohol (2.0 g, 7.4 mmol) in 30 mL of dichloromethane. The reaction was stirred at rt for 18 h and then filtered through silica gel to remove the used PDC. The filtrate was concentrated, and the crude product was purified by column chromatography (hexane/EtOAc, 95/5) (75% yield). ^{1}H-NMR ($CDCl_3$): 9.77 (m, 1H), 5.34−5.32 (m, 2H), 2.43−2.39 (m, 4H), 2.02−2.00 (m, 4H), 1.63−1.60 (2H), 1.30−1.27 (b, 18H), 0.90−0.85 (t, J = 6.09 Hz, 3H) ppm.

Methyl 2,4,13-(*Z*)-Docosatrienoate (5). A solution of trimethyl 4-phosphonocrotonate (2.0 g, 9.6 mmol) in 10 mL of tetrahydrofuran was added slowly to a suspension of sodium hydride (60% dispersion in mineral oil, 0.4 g) in 5 mL of tetrahydrofuran at 0 °C. After 1 h, oleyl aldehyde (2.0 g, 7.5 mmol) in 15 mL of THF was added dropwise at 0 °C. The reaction was then allowed to warm to rt and monitored by TLC using hexane/EtOAc (95/5) as the mobile phase. After the reaction was completed, excess NaH was destroyed by slow addition of cold water. After evaporation of THF, the residue was diluted by diethyl ether and extracted several times with water and brine. The organic layer was dried with anhydrous $MgSO_4$, then concentrated. The crude product was purified by column chromatography using hexane/EtOAc (95/5) to give methyl ester **5** in 75% yield. ^{1}H-NMR ($CDCl_3$): 7.32−7.22 (m, 1H), 6.17−6.12 (m, 2H), 5.82−5.76 (d, J = 15.38 Hz, 1H), 5.37−5.33 (m, 2H), 3.74 (s, 3H), 2.18−2.00 (m, 6H), 1.42−1.27 (b, 22H), 0.91−0.85 (t, J = 6.52 Hz, 3H) ppm.

2,4,13-(*Z*)-Docosatrienoic Acid (3). The methanolic solution of methyl ester **5** (2.0 g, 5.7 mmol in 100 mL) was treated with 1.5 mol equiv of 85% aqueous solution of KOH. The mixture was refluxed gently until the reaction was finished (about 5 h) as determined by TLC using hexane/ethyl acetate (95/5) as the mobile phase. The methanolic solution was concentrated and then diluted with ether. After the solution was acidified to pH 3 with dilute HCl solution, it was extracted several times with water. The organic layer was dried with anhydrous $MgSO_4$ and then concentrated, affording the crude dienoic acid **3** in 85% yield.

2,4,13-(*E*,*E*,*Z*)-Docosatrienoic Acid (3a). A well-stirred solution of urea (5.0 g, 83 mmol) in methanol (100 mL) was treated with a solution of acid **3** (2.0 g, 5.7 mmol) in methanol (100 mL). The solution was then kept at 0 °C overnight. The needle-like crystals were filtered, washed many times with methanol, and then dried under vacuum. These crystals were dissolved in ether and washed several times with dilute HCl solution and water. The organic layer was combined and dried with anhydrous $MgSO_4$. After concentration, the crude acid was purified by recrystallization from hexane at −30 °C, giving the ester **3a** as colorless needles in 80% yield. ^{1}H-NMR ($CDCl_3$): 7.39−7.31 (m, 1H), 6.25−6.14 (m, 2H), 5.90−5.72 (d, J = 15.22 Hz, 1H), 5.37−5.28 (m, 2H), 2.34−2.01 (m, 6H), 1.44−1.31 (b, 22H), 0.92−0.86 (t, J = 6.77 Hz, 3H) ppm.

1,2-Bis[2,4,13-(*E*,*E*,*Z*)-docosatrienoyl]-3-(4-methoxybenzyl)-*sn*-glycerol (6). 4-Methoxybenzyl-protected glycerol (0.3 g, 1.4 mmol), acid **3a** (0.6 g, 1.8 mmol), and DMAP (0.2 g, 1.8 mmol) were dissolved in 20 mL of chloroform, and then 0.4 g (1.8 mmol) of DCC in 10 mL of chloroform was added. After being stirred at rt overnight, the urea was filtered and the filtrate was concentrated. The crude product was purified by column chromatography using hexane/EtOAc (9/1) to give the protected glyceride **6** in 50% yield. ^{1}H-NMR ($CDCl_3$): 7.19−7.15 (m, 4H), 6.81−6.77 (d, J = 8.69 Hz, 2H), 6.10−6.05 (m, 4H), 5.76−5.65 (dd, J = 12.65, 15.20, 2H), 5.37−5.20 (m, 2H), 5.25−5.23 (m, 1H), 4.42−4.40 (d, J = 13.62 Hz, 2H), 4.31−4.25 (m, 2H), 3.72 (s, 3H), 3.55−3.53 (d, J = 5.14 Hz, 2H), 2.30−2.00 (m, 12H), 1.50−1.30 (b, 44H), 0.83−0.78 (m, 6H) ppm.

1,2-Bis[2,4,13-(*E*,*E*,*Z*)-docosatrienoyl]-*sn*-glycerol (2). A solution of the protected glycerol **6** (0.1 g, 0.1 mmol) in dry dichloromethane at −78 °C was treated with dimethylboron bromide (0.1 mL, 1.2 mmol) by addition through a syringe under argon. The reaction mixture was stirred for 15 min, while the dry-ice bath was removed. The reaction was then diluted with ether and quenched with water slowly. The organic layer was washed many times with water until the aqueous part was neutral. After evaporation of the solvent, the crude glyceride **2** was purified by column chromatography using hexane/EtOAc (8/1) and then (1/1) (80% yield). ^{1}H-NMR ($CDCl_3$): 7.35−7.23 (m, 2H), 6.18−6.15 (m, 4H), 5.84−5.76 (m, 2H), 5.35−

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5.19 (m, 4H), 5.19−5.15 (m, 1H), 4.62−4.37 (d, J = 5.08 Hz, 2H), 3.81−3.77 (m, 2H), 2.48 (b, 1H), 2.20−2.13 (m, 12H), 1.45−1.20 (b, 44H), 0.91−0.85 (m, 6H) ppm.

***N*-[(2,2,2-Trichloroethoxy)carbonyl][1,2-bis[2,4,13-(*E*,*E*,*Z*)-docosatrienoyl]-*sn*-glycerol]-3-phosphoethanolamine 7.** The solution of **2** (50 mg, 0.07 mmol) and pyridine (0.03 mL) in chloroform (5 mL) was transferred slowly into the solution of dichloro-[[*N*-[(2,2,2-trichloroethoxy)carbonyl]-2-amino]ethyl]-phosphinic acid (42 mg, 0.12 mmol) in benzene (2 mL) under Ar(g) at 0 °C. The reaction was allowed to warm to rt and stirred overnight. After dilution with ether, it was extracted with water, dilute HCl solution, saturated $NaHCO_3$ solution, and then brine. The organic layer was dried with anhydrous $MgSO_4$ and concentrated. The crude product was purified by column chromatography using hexane/EtOAc (1/1) and then chloroform/methanol/water (65/25/4) (70% yield). ^{1}H-NMR ($CDCl_3$): 7.59−7.25 (m, 2H), 6.78−6.53 (b, 1H), 6.21−6.19 (m, 4H), 5.83−5.75 (m, 2H), 5.39−5.25 (m, 5H), 4.76 (s, 2H), 4.49−3.95 (m, 6H), 3.49−3.45 (b, 2H), 2.37−2.00 (m, 12H), 1.42−1.03 (b, 44H), 0.89−0.83 (m, 6H) ppm.

[1,2-Bis[2,4,13-(*E*,*E*,*Z*)-docosatrienoyl]-*sn*-glycerol]-3-phosphoethanolamine (1). Zinc dust (0.2 g, 3.2 mmol) was carefully added to the solution of **7** (0.4 g, 0.4 mmol) in glacial acetic acid (6 mL) and diethyl ether (3 mL). The suspension was stirred under Ar(g) at rt overnight. The mixture was then diluted with ether, and zinc metal was filtered off. The filtrate was washed many times with water, saturated $NaHCO_3$ solution, and then brine. The organic layer was dried with anhydrous $MgSO_4$ and concentrated. The crude product was purified by column chromatography using chloroform/methanol/water (65/25/4). Bis-PE lipid **1** was obtained as a white solid in 80% yield. ^{1}H-NMR ($CDCl_3$): 8.40−8.12 (b, 3H), 7.69−7.15 (m, 2H), 6.13−6.04 (m, 4H), 5.91−5.70 (m, 2H), 5.37−5.20 (b, 5H), 4.34−3.78 (b, 6H), 3.27−3.15 (m, 2H), 2.30−1.97 (m, 12H), 1.36−1.18 (b, 44H), 0.86−0.82 (m, 6H) ppm. FAB-MS: 849.4 (M^+), 707.6 ($M^+ - [OPO_2CH_2CH_2NMe_2]$), 360.3 (707.6-dienoyl acid). Elemental analysis. Calcd: C, 69.40; H, 10.15; N, 1.65; O, 15.12; P, 3.66. Found: C, 69.73; H, 9.85.

Phase Behavior of Hydrated Lipid 1. ^{31}P NMR. A hydrated PE lipid sample was sealed in a 5 mm NMR tube. The sample temperature was controlled to ±0.2 °C. The proton-decoupled ^{31}P-NMR spectra were acquired on a Varian-Unity 300 spectrometer operating at 121.4 MHz (7.0 T). A phase cycled pulse sequence (90°−τ_1−180°−τ_2−acquisition) was used with a 90° pulse of 14 μs, the delay τ_1 of 100 μs, the time before acquisition τ_2 of 60 μs, and the delay between sequences of 0.5 s. At low lipid concentration, the free induction decays consisted of 80 000−90 000 acquisitions and were multiplied by a line broadening of 100 Hz after Fourier transform. At high lipid concentration, the free induction decays consisted of 5000 acquisitions and were multiplied by a line broadening of 50 Hz after Fourier transform.

The polymerized sample was handled in the same manner except that the NMR tube and sample were flushed with argon to provide an oxygen free environment. Polymerization was initiated by the addition of a 13 μL aliquot of potassium persulfate/sodium bisulfite (1/1) initiator (see below) to the NMR sample. After polymerization the NMR spectrum was obtained as above.

X-ray Diffraction. Phase behavior of **1** was determined along a single isopleth over the temperature range −40 to 80 °C at a concentration of 1:1 water to lipid. All samples were made directly into X-ray transparent capillaries under an argon atmosphere: failure to either store the lipid under argon or make the liquid crystalline samples under argon resulted in decomposition of the lipid over time and with increase in temperature. Capillaries were made by injection of a solution of the lipid in cyclohexane which was then lyophilized, and the capillaries were weighed to determine the amount of lipid and the corresponding amount of deionized water was added. Capillaries were then flame sealed. Samples were made and kept in the dark.

X-ray diffraction data were obtained using a Rigaku RU-200 rotating anode X-ray diffractometer equipped with a microfocus cup. The generated Cu Kα X-rays were focused via bent mirror optics. Two-dimensional X-ray images were collected with the Princeton PM area detector.[41] The digital powder diffraction images were azimuthally integrated along an arc of ±15° from the meridional axis to generate plots of scattered intensity versus $Q = 4\pi \sin d/1.54$ Å, where $2d$ is the angle between the incident and scattered beam directions.

Thermal polymerization was affected by replacing the deionized water with a 30% solution of H_2O_2, which was used as the source of hydroxyl radicals at elevated temperatures, which diffuse into the hydrocarbon region to react with the dienoyl moieties. Samples were incubated at 60 °C for 2 days.

Polymerization of Hydrated Lipid 1. Potassium Persulfate/L-Cysteine (1/1) Initiator. Lipid **1** (6 mg) was hydrated with an argon-flushed $K_2S_2O_8$ buffer solution (60 μL) through several freeze−thaw−vortex cycles and then incubated at 60 °C. Potassium persulfate (300 mg, 1.1 mmol) and L-cysteine (174 mg, 1.1 mmol) were diluted to 10 mL with deoxygenated MilliQ water. A volume of this solution was added to the thermally incubated lipid sample to give [M]/[I] = 8. After polymerization under Ar(g) at 60 °C for 2 days, the sample was freeze-dried. The methanol extract of a known weight lipid was then characterized using UV spectroscopy. The absorbance of dienoyl peak at 265 nm was measured for the calculation of percent conversion to polymer. The residue of sample after extraction with methanol was tested for its solubility in organic solvents including chloroform, dichloromethane, tetrahydrofuran, and HFIP.

H_2O_2 Initiator. Aqueous hydrogen peroxide solution (30% H_2O_2 in water, 6 μL) was flushed with argon then added to 6 mg of lipid **1**. The sample was centrifuged at rt and equilibrated at 5 °C for 1 day. The polymerization was then performed at 60 °C under Ar (g) for 2 days, after which the sample was freeze-dried. The isolated polymer was then characterized as described above.

DOPE Control. A sample of DOPE (60 mg) was hydrated with an argon-flushed K_2SO_4 buffer solution (2 mL) and incubated at 60 °C for a day to form the H_{II} phase. Potassium persulfate solution (200 mM) and L-cysteine solution (200 mM) were then added to the hydrated lipid to give [M]/[I] = 8. After incubation of the reaction mixture for 2 days at 60 °C, the sample was freeze-dried and then partly dissolved in chloroform. The chloroform extract was concentrated, and its ^{1}H-NMR spectrum was taken in chloroform-*d*. The ratio of integration area between vinyl and terminal methyl protons on the lipid chains was to 2:3, the same as that obtained for DOPE prior to the reaction. The chloroform-insoluble residue was totally soluble in water, precluding the presence of lipid polymers in this fraction.

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