Membrane Properties of Archæal Macrocyclic Diether Phospholipids

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Abstract: Several biophysical properties of four synthetic archæal phospholipids [one polyprenyl macrocyclic lipid **A** and three polyprenyl double-chain lipids (**B**, **C**, **D**) bearing zero, one or four double bonds in each chain] were studied using differential scanning calorimetry, electron and optical microscopies, stopped-flow/light scattering and solid-state ^{2}H-NMR techniques. These phospholipids gave a variety of self-organized structures in water, in particular vesicles and tubules. These assemblies change in response to simple thermal convection. Some specific membrane properties of these archæal phospholipids were observed: They are in a liquid-crystalline state over a wide temperature range; the dynamics of their polyprenyl chains is higher than that of *n*-acyl chains; the water permeability of the membranes is lower than that of *n*-acyl phospholipid membranes. It was also found that macrocyclization remarkably improves the barrier properties to water and the membrane stability. This may be related to the adaptation of *Methanococcus jannaschii* to the extreme conditions of the deep-sea hydrothermal vents.

Keywords: archæa · macrocycles · membranes · phospholipids · vesicles

Introduction

Archæa, the third major kingdom of living organisms, have cell structures and biocomponents markedly different from those found in bacteria and eucaryotes. Archæal membrane lipids have in particular characteristic chemical structures: Their hydrophobic part is composed of C_{20-25} polyprenyl chains, more or less saturated, linked by ether functions to a variety of head groups based on glycerol or other polyols, which in turn are linked to phosphoric acid derivatives. They sometimes comprise macrocyclic structures obtained by ω,ω'-cyclization of a dimeric C_{20} chain into a C_{40} one; the termini of those are linked either to the same, or to both head groups, thus giving either 36- or 72-membered rings (Figure 1).[1–3]

A number of models for the archæal membrane lipids have been synthesized, and the physicochemical properties of the natural or synthetic lipids, such as their thermotropic properties, phase behaviour or enzyme susceptibilities, have been studied by various methods, including electron microscopy, X-ray diffraction, differential thermal analysis, conductance and capacitance measurements, infrared spectroscopy, Langmuir monolayer studies and enzyme assays on liposomes.[4–19]

We have become interested in studying the biophysical significance of the characteristic macrocyclic ring structure of the archæal lipids, in particular the 36-membered ring, the presence of which in a deep-sea hydrothermal vent methanogen, *Methanococcus jannaschii* was reported by Comita et al.[3] The σ-bond formation between the termini of the adjacent chains could affect the conformation (*trans*/*gauche* ratios), rotational motions and lateral diffusion of the chains. These could consequently lead to changes in the 3D structure, lipid packing, membrane permeability and stability. We have recently synthesized a phosphatidylcholine **A** bearing the natural archæal C_{36} macrocyclic glycerol skeleton,[20, 21] and we reported the thermal analysis and monolayer studies of this synthetic lipid in a short communication.[22] The results showed that the macrocyclic lipid **A** aggregates into more tightly packed structures than its acyclic congener **B**. We

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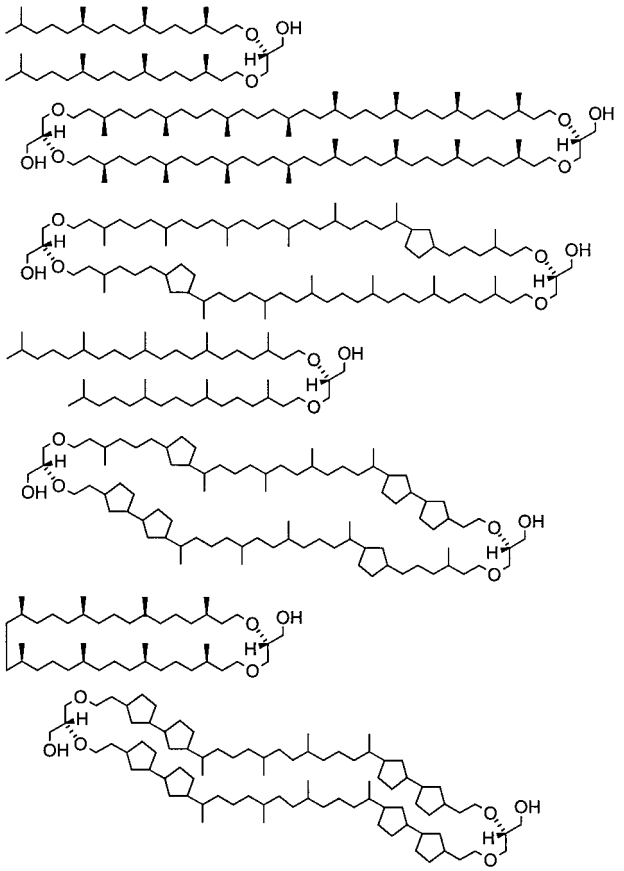

Figure 1. Typical basic structures of archæal membrane lipids.

present here the physicochemical properties of bilayer systems of the macrocyclic phospholipid **A**, and their comparison with those of its noncyclized analogues **B, C** and **D**, bearing different numbers of double bonds. Structure **B** carries totally saturated diphytanyl diethers, the most frequent skeleton of membrane lipids in *Halobacteria*,[2] whereas structure **D**, bearing unsaturated digeranylgeranyl diethers derived directly from the isoprenoid biosynthesis, has so far been found only in one archæa, *Methanopyrus kandleri* (Figure 2).[23]

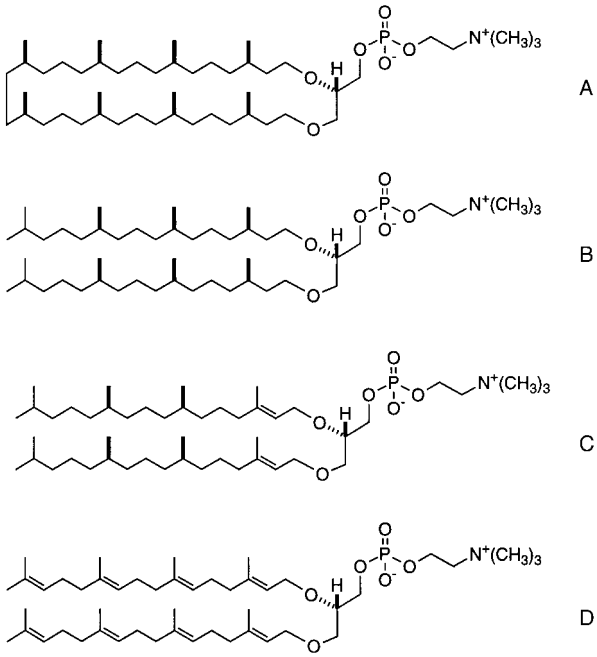

Figure 2. Structures of archæal type phosphocholines studied.

Abstract in French: *Les propriétés biophysiques de quatre phospholipides archéobactériens de synthèse (un lipide **A** possédant une seule chaîne macrocyclique polyprényle et trois lipides, **B**, **C**, **D**, possédant deux chaînes avec zéro, une ou quatre double-liaisons sur une des deux chaînes) ont été étudiées par calorimétrie différentielle, microscopies électronique et optique, flux bloqué/diffusion de la lumière et spectroscopie de RMN du deutérium à l'état solide. Ces lipides donnent une variété des structures auto-organisées dans l'eau, notament des vésicules et des tubules, transformées l'une en l'autre par simple convection thermique. Quelques propriétés membranaires spécifiques pour ces phospholipides archéobactériens ont été observées: ils sont à l'état de cristaux liquides sur une zone très vaste de température. La dynamique de leurs chaînes est plus grande que celle des n-acyl phospholipides. La perméabilité à l'eau de leurs membranes est moins grande que celle des n-acyl phospholipides. La macrocyclisation améliore remarquablement la barrière membranaire à l'eau et la stabilité de la membrane. Ceci peut-être lié à l'adaptation de Methanococcus jannaschii aux conditions extrêmes des évents hydrothermiques sous-marins.*

We first describe the synthesis of the novel synthetic lipids **C** and **D**. The phase properties of all phospholipids **A, B, C** and **D** in water are then described: The spontaneous formation of vesicles and microtubular structures from these phospholipids as well as the transformation between diverse self-organized structures were observed by means of microscopic techniques (cryo-electron microscopy, optical, fluorescent and confocal microscopies).

To study the membrane properties of these phosphocholines, two nonperturbative methods have been used: a stopped-flow/light scattering method[24, 25] and a solid-state ^{2}H-NMR technique.[26–28] The former permits the evaluation of the water permeability of unilamellar vesicles, while the second offers information about the lipid chain dynamics at different depths in bilayers.

Results and Discussion

Thermal analysis: Previous studies have shown that a methyl branchings into alkyl chains lowers the phase transition temperature (T_m),[29] and that *Halobacterium* lipids bearing several methyl-branched terpenyl chains exhibit no detectable gel-to-liquid crystalline phase transition over a wide range.[30] In our case the liposomes of archæal lipids (macrocyclic lipid **A**, diphytanylphosphatidylcholine **B**, diphytylphosphatidylcholine **C** and digeranylgeranylphosphatidylcholine **D**) also did not show any phase transition between -40 and 60 °C.[22] These lipids are in a liquid-crystalline state over

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an unusually wide temperature range, as compared with eucaryotic or bacterial lipids. The feeble thermal response suggests that the branched polyprenyl chains of archæal lipids perform a regulatory function by themselves on the fluidity of the membranes, as does cholesterol in eucaryotic membranes. The liposomes formed by starting from the binary mixture archæal lipid (**A**, **B**, **C** or **D**)/dimyristoylphosphatidylcholine (DMPC 1:1 mol/mol) also did not show any phase transition between 4 and 80 °C.

Electron microscopy: Two different samples (archæal phospholipids **A** and **B**) were observed by cryo-transmission electron microscopy (cryo-EM). They were obtained by hydration of the lipid film, followed by a mechanical shaking ("vortexing" to obtain small enough vesicles). Neither sonication nor extrusion were employed. Cryo-EM is the least perturbing method which allows the observation of vesicles in their native state.[31–33] Suspensions of the macrocyclic phosphatidylcholine **A** form mainly tubular vesicles (Figure 3), but vesicles showing a hemifusion were sometimes observed (data not shown). Suspensions of the two-chain phosphatidylcholine **B** present unilamellar vesicles of various sizes and shapes (Figure 4). In addition, we confirmed the formation of vesicular structures from the aqueous suspension of archæal lipids, using the techniques of metal shadowing and negative staining microscopies (figures not shown).

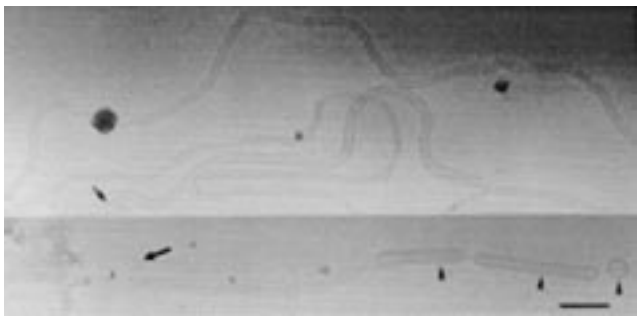

Figure 3. Macrocyclic phosphocholine **A** vesicle suspension observed in the frozen hydrated state. Mainly unilamellar tubules several micron long and around 300 Å in diameter are observed. As the images were not taken under low dose conditions the bilayer appears as a single line. A few unilamellar vesicles are observed (small arrow). Sometimes several microtubules are connected to each other through a narrow tether, the microtubule is fused with a spherical vesicle (large arrow) or includes a second vesicle (arrow heads). Scale bar: 200 nm.

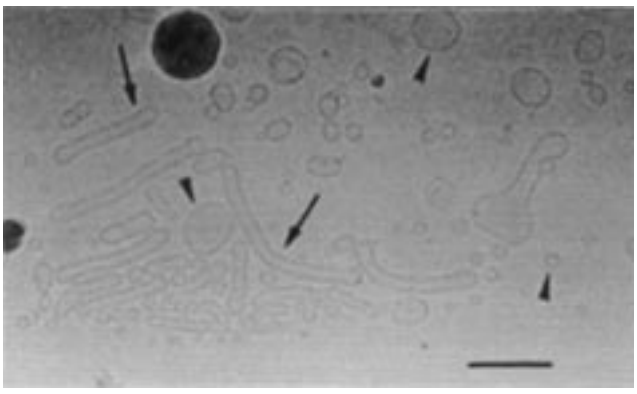

Figure 4. Cryo EM of the two-chain phosphocholine **B** vesicles suspension. Two kinds of structures are observed. Tubular vesicles (arrows) and spherical vesicles (arrow heads). The round shaped vesicles have a size between 180 Å and 1200 Å. The length of the tubular vesicles is up to 0.5 μm. Scale bar: 200 nm.

To further analyze the shape and shape changes of these assemblies of archæal phospholipids, we used optical microscopy.

Optical microscopies: Giant vesicles with a diameter of 10 μm or more have sizes similar to those of biological cells, and therefore renders them useful model compounds. They are visible in situ, in water, and with an optical microscope, so that no preparations are required for electron microscopy (which is of course required for smaller vesicles). Using a differential interference enhanced-contrast optical video-microscope (Nomarski technique), their shape and shape changes can be easily observed on the monitor and registered in real time. We observed that different types of self-organized structures were formed spontaneously when dry films of archæal phospholipids (**A**, **B**, **C**, **D**) swell in excess of water or buffer at 298 K. Uni- and multilamellar vesicles (diameter up to 20 μm), oligovesicular vesicles, microtubules, membrane aggregates and their interactions (figure not shown) were observed.

With an acetate buffer (pH 4.5) or with a phosphate buffer (pH 4.5 to 8.2), the amphiphiles **A**, **B**, **C** and **D** afforded mostly spherical multilamellar structures, accompanied by discoid multilamellar vesicles (Figures 5 and 6). However,

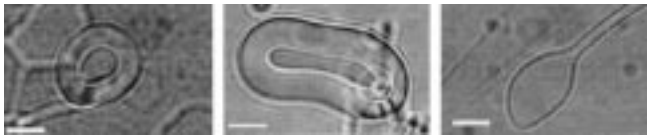

Figure 5. Differential interference contrast optical microscopy observations of self-organized structures of the archæal lipid **B** ($T = 298$ K). Left: spherical vesicle (pH 4.5, acetate buffer). Middle: discoid shape vesicle (pH 7.0, phosphate buffer). Right: microtubule (pH 8.85, borate buffer). Scale bar 10 μm.

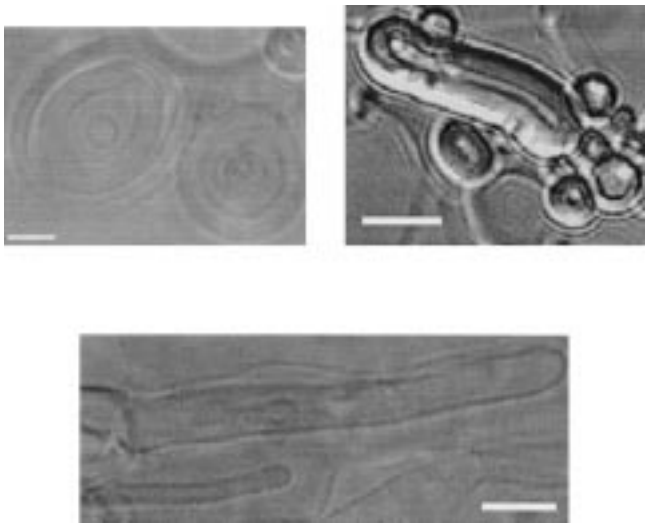

Figure 6. Differential interference contrast optical microscopy observations of archæal type lipid membranes (**C** and **D**) ($T = 298$ K). Top left: multilamellar vesicles of archæal lipid **C** (pH 4.5, acetate buffer). Top right: multilamellar vesicles of archæal lipid **D** (pH 7.0, phosphate buffer). Bottom: microtubules of archæal lipid **D** (pH 8.85, borate buffer). Scale bar 10 μm.

microtubular structures are predominant among the self-organized structures formed in a borate buffer suspension (pH 8.85) of amphiphiles **A**, **B**, **C** or **D** (Figures 5 and 6).

By the addition of Nile Red (Figure 7), a neutral fluorescent hydrophobic membrane probe, we could follow its selective incorporation into the lipidic bilayers using fluorescence microscopy, and follow more precisely various types of morphological changes.[34] With an aqueous suspension of the archæal lipid **A**, we observed the endocytosis-like shape

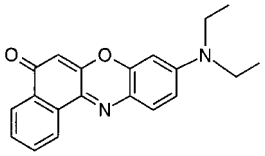

Figure 7. Structure of Nile Red; $\lambda_{abs} = 485$ nm (heptane).

change of a vesicle (Figure 8). The shape transformation of the same vesicle from a discoid to a two-vesicle shape runs through a stomatoid, whereby one might have been triggered by thermal convection due to the slow evaporation of water in our unsealed samples,[35] because while observing the same samples in sealed sample holders, under the same experimental conditions, no similar shape transformations were detected.

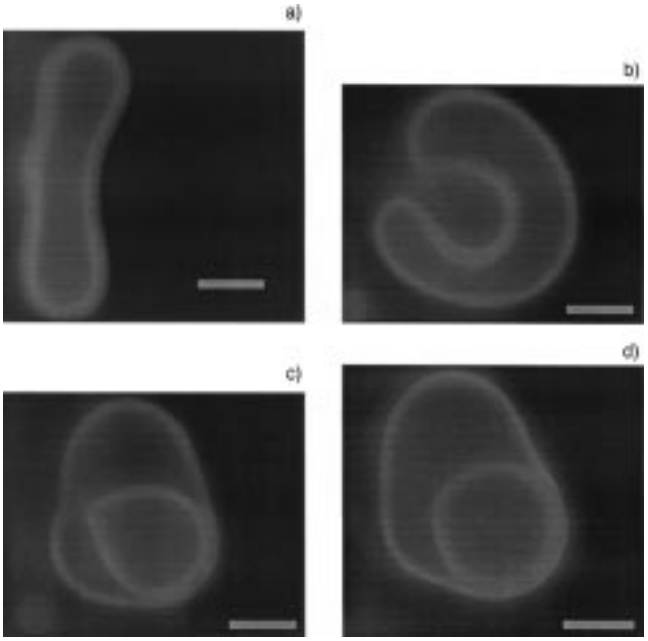

Figure 8. Observation by differential interference contrast fluorescence optical microscopy of the shape transformation of vesicle of lipid **A** suspended in a phosphoric buffer ($T = 298$ K, pH 7.0, $\lambda = 485$ nm, Nile Red 1% mol). a) $t = 0$ s. b) 17 s. c) 25 s. d) 32 s. Scale bar 10 μm. A series of the transformations from a discoid shape to a two-vesicle structure, reminiscent of the process of endocytosis.

Sackmann et al.[36] had already shown that a shape transformation of a pure DMPC giant vesicle from a quasispherical to a two-vesicle shape can be triggered simply by slight mechanical agitation of the vesicle kept between microslides. Discoid–stomatoid shape transitions of a DMPC vesicle in water had also been observed when the temperature varied from 43.6 to 43.8 °C.[37] This was explained by asymmetric thermal expansivity of the inner and outer monolayers in accordance with the bilayer-coupling model of Svetina and Zeks.[38]

Besides the shape transformations, we have observed the hemifusion of spherical vesicles in an aqueous suspension of the archæal phospholipid **A** in a phosphate buffer (pH 7) without any additive (Figure 9). Hemifusion, an intermediate stage between two separate membranes and fusion, was already reported in vesicle-planar lipid bilayers systems when lipids known to promote opposite spontaneous curvatures, lysophosphadidylcholine and arachidonic acid[39] had been added, or in two giant liposome systems of opposite charges.[40] Several models have been recently developed for lipid bilayer hemifusion.[41, 42]

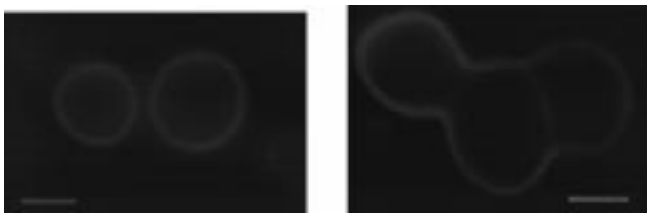

Figure 9. Observation by differential interference contrast fluorescence optical microscopy: before (left) and after (right) double hemifusion of three vesicles of archæal lipid **A**. Two vesicles are observed in the center of the image and the third one is situated on the right side $t = 21$ s (left figure $t = 0$ s). After the double hemifusion, no structural change was observed for 12 h ($T = 298$ K, pH 7.0, $\lambda = 551$ nm, Nile Red 1% mol). Scale bar 10 μm.

Furthermore, using confocal microscopy we could obtain 3D-reconstruction images of diverse types of self-organized structures of archæal lipids and differentiate more easily multilamellar and oligovesicular vesicles, and microtubules. We show herein one example in Figure 10, confirming the ellipsoidal structure of a vesicle of archæal lipid **B**.

Water permeability: In a previous study, we have shown that the stopped-flow/light scattering method is well adapted to the evaluation of the vesicle permeability to water.[24] Water permeability was measured at 33.0 ± 0.1 °C. The swelling rate is slightly affected by the vesicle size in a such way that the half-time ($t_{1/2}$) increases with increasing vesicle diameters (see Table 1, sample **B** 1 and 2), and the values are compared for different vesicles having approximately the same size:[24] one group of vesicle diameter of about 200 nm (upper part, Table 1) and the other group of about 100 nm (lower part, Table 1). A light scattering intensity/time exponential recording obtained for lipid **A** (branched macrocyclic phosphatidylcholine) vesicles in swelling experiments is shown in Figure 11.

Table 1 reports the results obtained with the four phosphatidylcholines (**A, B, C, D**). These can be summarized as follows:

1) The barrier properties of vesicles to water are strikingly better for macrocyclic lipid **A**, relative to the two-chain lipid **B** (upper part).

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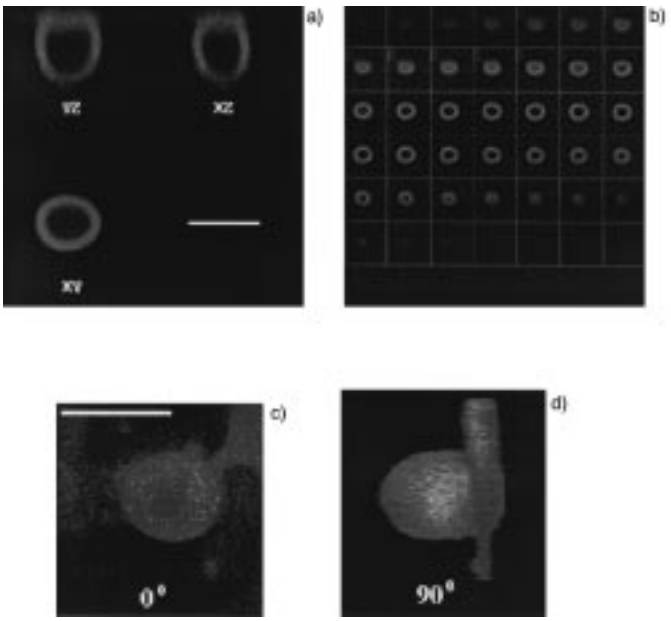

Figure 10. Fluorescence confocal microscopy photographs of an ellipsoidal vesicle (5 μm small axis, 8 μm large axis) of the archæal lipid **B** ($T = 298$ K, pH 7.0, $\lambda = 551$ nm, Nile Red 1% mol). Scale bar 5 μm. a) Perpendicular vesicular confocal sections in the (XY), (YZ) and (ZX) planes. b) IR serial vesicular optical sections of 0.2 μm, three depth in the (XY) plane. c) and d) Face (0°) and profile (≈90° rotation) views of the three-dimensional reconstruction. The ellipsoidal vesicle is not yet detached from the silde surface.

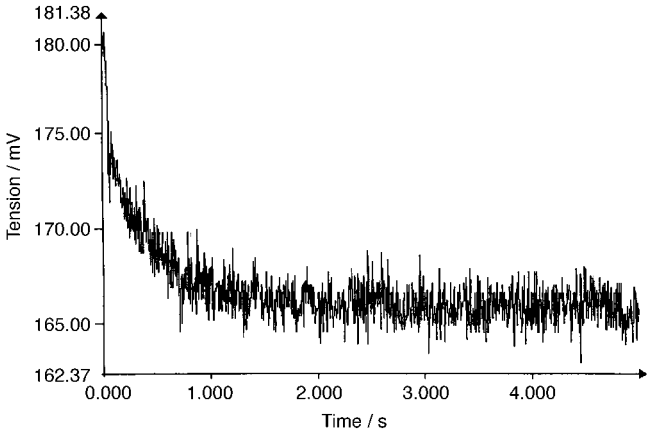

Figure 11. Vesicle swelling upon osmotic shock: experimental scattered light variation vs. time: vesicles from lipid **A**, average diameter 179 nm, $T = (33 \pm 0.1)$ °C; $\lambda = 400$ nm.

Table 1. Water permeability ($t_{1/2}$) for vesicles of the phosphocholines studied at $T = (33.0 \pm 0.1)$ °C.[a]

Phosphocholines	⟨diameter⟩ (SD) [nm]	k [s^{-1}]	$t_{1/2}$ [ms]
A	179 (narrow)	3.0 ± 0.1	228.0 ± 9.0
B (sample 1)	193 (52)	10.2 ± 0.3	68.1 ± 1.7
DMPC (sample 1)	186 (38)[a]	26.4 ± 1.2	26.3 ± 1.1
B (sample 2)	105 (28)	11.8 ± 0.4	58.5 ± 2.0
C	107 (narrow)	9.7 ± 0.1	71.3 ± 0.8
D	94 (26)	25.9 ± 0.8	26.7 ± 0.8
DMPC (sample 2)	88 (26)	36.1 ± 0.9	19.2 ± 0.5

[a] The same order of the value was obtained in our previous experiments.[24]

2) The water permeability of vesicles of lipid **C** (one double bond per chain) is slightly lower than that of lipid **B** (totally saturated chains) (lower part).
3) The presence of four double bonds on a phytanyl chain (lipid **D**) increases the water permeability of vesicles as compared with those of the lipids **B** and **C** (lower part).
4) The water permeability of DMPC (as an example of eucaryotic membrane lipid) is of the same order as that of the unsaturated lipid **D** (lower part).

The improvement of the barrier properties of the macrocyclic compound **A** is of course linked to the presence of the bond at the termini of the chains, which prevents them from engaging in independent motions and ensures a more close-packed assembly in the liquid crystalline state. These results are in a good agreement with those obtained in our monolayer study of lipid **A**,[22] with those of Menger et al.[14] or those of Ladika et al.[16] for nonbranched macrocyclic phosphatidylcholines, and with those obtained by us for the nonbranched macrocyclic phosphates.[19]

The comparable level of water permeability observed for lipid **D** and for DMPC is somewhat surprising, because it was shown that the introduction of a methyl branching into alkyl chains lowers T_m and ΔH by reducing the total number of van der Waals interactions.[29] Similar effects had already been described by Yamauchi et al.,[43, 44] who reported that liposomes from di-(3*RS*,7*R*,11*R*)-phytanylphosphatidylcholine (diastereomers of compound **B**) or di-(3*RS*,7*R*,11*R*)-phytanylphosphatidic acid were very stable even in 5 M aqueous NaCl, and retained Na^+ or Cl^- ions as well as carboxyfluorescein in the aqueous interior at temperatures as high as 70 °C. The liposomes were also highly resistant to proton permeation; by contrast, liposomes of ordinary diester lipids such as dipalmitoylphosphatidylcholine (DPPC) and egg-yolk lecithin were less stable and more permeable than those of the diphytanyl lipids. Extreme membrane stability, high tolerance to salt, as well as a lower permeability to proton, water and ions is therefore somehow due to the isoprenoid chains. NMR studies on the polar lipids of *Halobacterium halobium* suggested that the segmentary motion at the tertiary carbons was hindered as a result of the presence of the methyl groups on the side chains.[45] ESR studies also showed that archæal lipid membranes are in a "rigid" state.[46–48] Yamauchi and Kinoshita[49] suggest that the "steric hindrance" (we propose rather the "lateral interdigitation") in polyisoprenoid membranes might be one of the key factors of a slower permeation of ions or solutes, though the mechanism by which water or proton permeability is lowered in archæal lipid membranes cannot be exactly defined.

In addition, it had been shown that the incorporation of bacterioruberins, the major red carotenoids of *Halobacterium*, further lowers water permeability of the vesicles of diphytanylphosphatidylcholine (compound **B**) and those of the total polar lipids of *Halobacterium*.[50]

^{2}H-NMR spectroscopy: Solid-state ^{2}H-NMR studies provide an excellent method to investigate lipid-chain dynamics in bilayers; by using oriented multibilayers, the sensitivity of the method is greatly increased.[51] We had successfully used ^{2}H-NMR spectroscopy to investigate the role of terpenoids as

membrane reinforcers.[52, 53] We have now used this method to analyze the effect of macrocyclization and unsaturation on the lipid-chain dynamics of the phospholipids studied here, on oriented multibilayer samples prepared from the 1:1 molar mixture of [D_{27}]DMPC and a phosphatidylcholine (**A, B, C, D**), which will allow us to compare the effects of all compounds in the same model system. We are fully aware that the deuterated lipid, when used in such a high proportion, cannot be considered as a genuine "probe". However, this had to be accepted to obtain strong enough signals. The quality and homogeneity of the results obtained appears to vindicate this deviation from a more reasonable procedure.

Figure 12 shows the S_{mol} profiles for [D_{27}]DMPC and four samples of the 1:1 molar mixture of [D_{27}]DMPC (1-myristoyl-2-[$^2H_{27}$]myristoyl-*sn*-glycero-3-phosphocholine) and a phosphatidylcholine (**A**, **B**, **C**, or **D**). The segmental order

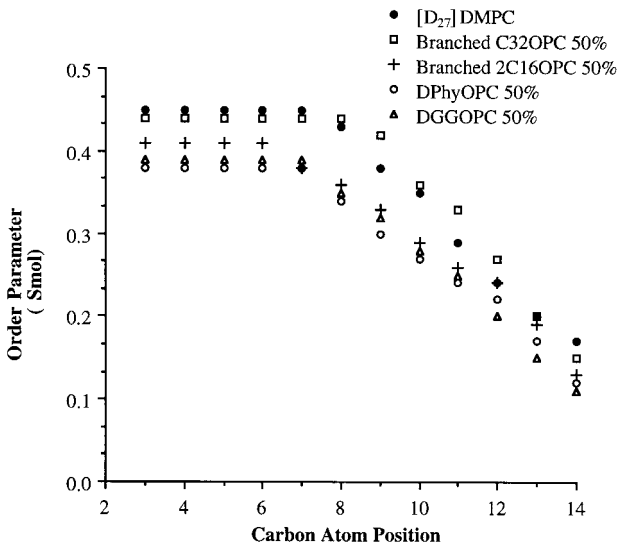

Figure 12. Order parameter profiles for various systems ($T = (306 \pm 4)$ K): pure [D_{27}]DMPC, [D_{27}]DMPC+**A** (branched C32OPC) (1:1), [D_{27}]DMPC+**B** (branched 2C16OPC) (1:1), [D_{27}]DMPC+**C** (DPhyOPC) (1:1) and [D_{27}]DMPC+**D** (DGGOPC) (1:1). The S_{mol} experimental error is about ± 0.002 for carbon atom positions 3 to 13 and ± 0.005 for the carbon atom position 14: estimated accuracy of the angular settings between the bilayer and the magnetic field: $\Delta\theta = \pm 2°$; estimated experimental error of the quadrupolar splitting: $\Delta(\Delta\bar{\nu}) = 244.14$ Hz by considering that the peaks maxima digital assignation is realized with the precision of ± 122.07 Hz (122.07 Hz^{-1}: points number per Hertz fixed in our spectral acquisition program).

parameter S_{mol} was calculated from the ^{2}H-NMR spectra of the 90 °C oriented multibilayers recorded at 306 ± 4 K. The peaks of the oriented spectra were assigned on the basis of their intensity and on the assumption that S_{mol} decreases monotonically from position 3 to the terminal methyl group as in the case of pure DMPC bilayers.[26]

All S_{mol} profiles exhibit a characteristic shape with a plateau from position 3 to position 6 of the DMPC *sn*-2 acyl chain and a gradual decrease when going towards position 14; this indicates the highly dynamic character of the mid section of the bilayer.[26] The S_{mol} value of pure [D_{27}]DMPC and that of the 1:1 molar mixture of [D_{27}]DMPC and a phosphatidylcholine (**A, B, C, D**) were compared. In the presence of 50 mol % **A**, the S_{mol} profile is not much changed at the plateau (with a 2.4 % decrease from the value for pure [D_{27}]DMPC), and it is shifted towards a little higher values for the positions 6 – 12, followed by going down to a lower value at C-14. The presence of 50 mol % noncyclic polyprenyl analogues **B**, **C**, **D** decreases S_{mol} in the sequence (for the plateau region): **B** (diphytanyl: 9 %) < **C** (diphytyl: 16 %) ≈ **D** (digeranylgeranyl: 14 %).

The order parameter at 60 °C with all phosphatidylcholines decreases for all the positions, relative to 33 °C. The presence of 50 mol % of any of the other lipids decreases S_{mol} for all the positions except C-14: The order parameter of the plateau region is decreased by 5 % (**A**), by 7 % (**B**), 22 % (**C**), 12 % (**D**) (data not shown).

Among the 1:1 molar mixtures tested of [D_{27}]DMPC with different compounds, the macrocyclic phosphatidylcholine **A** is the only compound which hardly perturbed DMPC bilayers at 33 °C ($T > T_m$ of DMPC), while any of other compounds increases the disorder of the DMPC chains. These results imply that the mobility of each position in the chains of archæal lipids **B**, **C**, **D** is microscopically high, though we have not checked the results with the pure compound. Smith et al.[30] have already shown by ^{2}H-NMR spectrometry analysis that the branched chains in diphytanylglyceryl lipids are more disordered than the *n*-alkyl or *n*-acyl chains of conventional lipids. Yamauchi et al.[54] showed in monolayer experiments that the saturated isoprenyl chain occupies 50 – 60 Å^2 per chain, which is much more than for the straight chain (30 – 40 Å^2 per chain). They also observed a low surface tension of polyprenyl lipids, which might result from their unordered state and their large cross-sectional area.[55] The low permeability to water (in the present study) and to ions or to a fluorescent molecule (as observed by Yamauchi et al.[43, 44]) for archæal lipid vesicles is apparently not related to the fluidity of the polyprenyl chains (as was observed in NMR studies). We showed previously in a monolayer study that the film area of the macrocyclic lipid **A** was significantly smaller than that of the acyclic lipid **B**.[22] This implies that lipid **A** aggregates into a more closely packed structure, which accounts for the water permeability of membranes composed of the lipid **A** to be lower than that of membranes composed of acyclic lipids.

Conclusion

In conclusion, we have first shown, using optical and electron microscopies, that vesicles of archæal type phosphatidylcholines could exhibit different shapes of self-organized structures (vesicles, tubules, etc.) in their aqueous suspensions, and that they can be transformed into each other simply by thermal convection. We have then presented the results of the water permeability measurement of membranes made of these lipids by stopped-flow/light scattering method. Finally, we compared the effect of their incorporation on the acyl chain ordering of DMPC by using a solid state ^{2}H-NMR spectroscopy.

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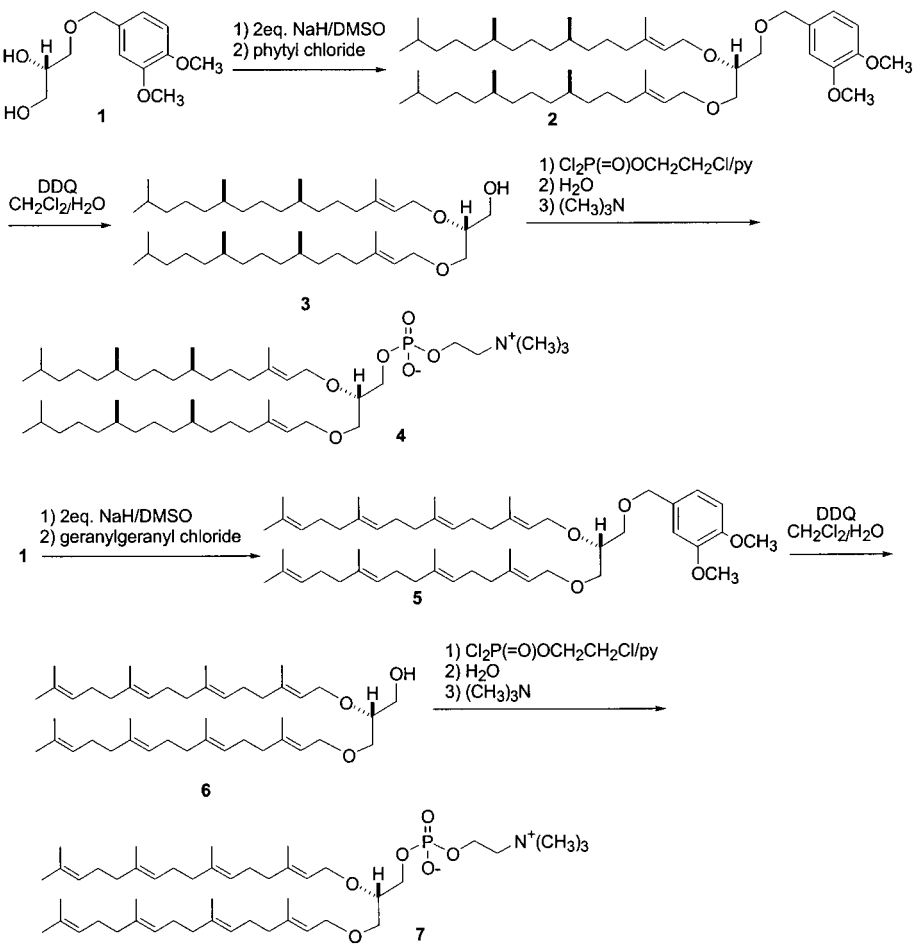
Taking the structural and functional data into account, some specific membrane properties of archæal polyprenyl lipids appear:

1) The macrocyclization remarkably improves the membrane barrier to water and the membrane stability, probably due to a more closely packed structure;
2) the vesicle permeability to water, ions and other molecules is lower with archæal lipids than with straight-chains lipids;
3) the dynamics of the polyterpenyl chains however, is microscopically higher than that of *n*-acyl chains, and
4) these archæal-type lipids remain in the liquid-crystalline state over an unusually wide range, as compared with eucaryotic or procaryotic lipids.



Scheme 1. Synthesis of archæal type lipids **C** (compound **4**) and **D** (compound **7**).

Experimental Section

Materials

All chemicals used were commercially available reagents unless otherwise stated. IR spectra were measured on a Hitachi 285 infrared spectrometer. ^{1}H- and ^{13}C-NMR spectra were recorded on a JEOL LA-300 spectrometer. Deuteriochloroform (99.8% atom enriched, Aldrich) was used as NMR solvent throughout, unless otherwise stated. ^{1}H- and ^{13}C-NMR chemical shifts are reported in δ values based on internal TMS ($\delta_H = 0$), or solvent signal ($CDCl_3$, $\delta_C = 77.0$) as reference. Mass spectra were measured on a JEOL AX-505 mass spectrometer. Column chromatography was carried out with a Kieselgel 60 (70–230 mesh, Merck) column.

Synthesis

The phosphatidylcholines **A** and **B** were synthesized as described in a previous paper.[21] Synthesis of compounds **C** and **D** was performed according to Scheme 1. One of the important points in their synthesis is the choice of a suitable protecting group for the glycerol moiety: Dipolyprenyl glycerols **3** and **6** should be released from their protected precursors without affecting the double bonds. After a failure in the deprotection of their *p*-methoxybenzyl ethers, the use of a 3,4-dimethoxybenzylether[56, 57] as a protective group allowed to overcome the difficulties encountered in the cleavage, as the latter protecting group had been already successfully employed in the synthesis of phospholipids.[58] On the other hand, the dialkylation of the monoprotected glycerol **1a** with a polyprenyl chloride was achieved in a good yield by the use of NaH in DMSO.

1-*O*-(3,4-Dimethoxybenzyl)-2,3-di-*O*-[(7*R*,11*R*)-3,7,11,15-tetramethylhexadec-2-en-1-yl]-*sn*-glycerol (2): A solution of 1-*O*-(3,4-dimethoxybenzyl)-*sn*-glycerol[58] (**1**) (750 mg, 3.10 mmol) in DMSO (2 mL) was added to a suspension of prewashed (*n*-hexane) NaH (186 mg, 7.75 mmol) in DMSO (10 mL). After 1 h, a solution of phytyl chloride (2.44 g, 7.75 mmol) in DMSO (3 mL) was added. The mixture was stirred at room temperature overnight. Saturated aqueous NH_4Cl (15 mL) was added and the mixture was extracted with EtOAc. The organic phase was washed with water, dried (Na_2SO_4), filtered and concentrated to dryness. The residue was purified by flash chromatography on silica gel with hexane/EtOAc (8:1) to give **2** (2.19 g, 88%) as an oil. ^{1}H NMR (300 MHz): $\delta = 0.84$ (d, $J = 7.0$ Hz, 12H), 0.87 (d, $J = 7.0$ Hz, 12H), 1.63 (s, 6H), 1.95 (br t, $J = 7.0$ Hz, 4H), 3.50–3.72 (m, 5H), 3.87 (s, 3H), 3.88 (s, 3H), 4.00 (d, $J = 7.0$ Hz, 2H), 4.16 (d, $J = 7.0$ Hz, 2H), 4.49 (s, 2H), 5.34 (m, 2H), 6.79–6.92 (m, 2H), 7.38 (s, 1H); ^{13}C NMR (75 MHz): $\delta = 16.37$, 16.41, 19.69, 19.73, 22.61, 22.70, 24.46, 24.78, 25.15, 27.96, 32.69, 32.78, 36.72, 36.77, 37.28, 37.36, 37.43, 39.36, 39.94, 55.78, 55.90, 66.82, 67.87, 70.05, 70.26, 73.24, 76.90, 110.85, 111.00, 120.15, 120.70, 121.05, 131.04, 140.13, 140.13, 140.40, 148.50, 148.97; IR (neat): $\tilde{\nu} = 1033$, 1099, 1138, 1159, 1238, 1265, 1365, 1277, 1463, 1515, 1592, 2867, 2927, 2952 cm^{-1}; anal. calcd for $C_{52}H_{94}O_5$: C 78.14, H 11.85; found: C 78.39, H 11.82.

2,3-Di-*O*-[(7*R*,11*R*)-3,7,11,15-tetramethylhexadec-2-en-1-yl]-*sn*-glycerol (3):[59] A mixture of **2** (2.01 g, 2.50 mmol) and 3,4-dichloro-5,6-dicyanobenzoquinone (852 mg, 3.77 mmol) in CH_2Cl_2 (20 mL) and H_2O (0.5 mL) was stirred for 2.5 h at room temperature. The mixture was passed through a short column of silica gel (10 g) and the column was eluted with $CHCl_3$. The eluent was concentrated to dryness and the residue was purified by flash chromatography on silica gel with hexane/EtOAc (20:1) to give **3** (988 mg, 61%) as an oil. ^{1}H NMR (300 MHz): $\delta = 0.84$ (d, $J = 7.0$ Hz, 12H), 0.87 (d, $J = 7.0$ Hz, 12H), 1.63 (s, 6H), 1.99 (br t, $J = 7.0$ Hz, 4H), 3.44–3.80 (m, 5H), 4.00 (d, $J = 7.0$ Hz, 2H), 4.10 (dd, $J = 12.0$, 7.0 Hz, 1H), 4.17 (dd, $J = 12.0$, 7.0 Hz, 1H), 5.34 (m, 2H); ^{13}C NMR (75 MHz): $\delta = 16.37$, 16.41, 19.69, 19.72, 22.60, 22.69, 24.43, 24.77, 25.09, 27.93, 32.66, 32.77, 37.26, 37.41, 39.38, 39.91, 63.05, 66.51, 67.98, 69.98, 77.42, 120.31, 120.52, 140.93; IR (neat): $\tilde{\nu} = 1066$, 1101, 1376, 1461, 1670, 2867, 2925, 2952, 3453 cm^{-1}; anal. calcd for $C_{43}H_{84}O_3$: C 79.56, H 13.04; found: C 79.38, H 13.17.

2,3-Di-*O*-[(7*R*,11*R*)-3,7,11,15-tetramethylhexadec-2-en-1-yl]-*sn*-glycero-1-phosphocholine (4): β-Chloroethyl phosphoryl dichloride (0.26 mL, 2.06 mmol) was added at 0 °C to a solution of **3** (890 mg, 1.37 mmol) in pyridine (4 mL). The mixture was stirred for 2 h at 10 °C. Water (5 mL) was added and the mixture was stirred at room temperature for 1 h. Next, the mixture was acidified by addition of 2N HCl and extracted four times with $CHCl_3$. The combined organic layers were dried (Na_2SO_4), filtered and concentrated to dryness. The residue obtained was dissolved in toluene

(5 mL) and CH_3CN (5 mL) and the solution was transferred into a pressure bottle. The mixture was cooled to $-78\,^{\circ}C$ and dry trimethylamine (10 mL) was introduced. The mixture was heated at 50–60 °C for 3 d. After the solvent was removed in vacuo, the residue was purified by flash chromatography on silica gel with $CHCl_3$/methanol (8:1) to $CHCl_3$/methanol/H_2O (65:15:1) to give **4** (365 mg, 33%) as a hygroscopic wax. ^1H NMR (300 MHz, $CDCl_3/CD_3OD$ 1:1): $\delta = 0.86$ (d, $J = 7.0$ Hz, 12H), 0.88 (d, $J = 7.0$ Hz, 12H), 1.70 (s, 3H), 2.00 (t, $J = 7.0$ Hz, 4H), 3.25 (s, 9H), 3.42–3.73 (m, 5H), 3.92 (m, 2H), 4.03 (d, $J = 7.0$ Hz, 2H), 4.18 (d, $J = 7.0$ Hz, 2H), 4.26 (m, 2H), 5.34 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3/CD_3OD$ 1:1): $\delta = 16.50, 19.93, 19.98, 22.79, 22.86, 24.86, 25.15, 25.67, 25.70, 28.35, 33.14, 33.21, 37.26, 37.72, 37.83, 37.85, 39.83, 40.37, 40.41, 54.62, 59.35$ (d, $J = 5.0$ Hz), 65.76 (d, $J = 5.5$ Hz), 67.14, 67.22, 68.35, 70.16, 77.65 (d, $J = 7.5$ Hz), 121.07, 121.36, 140.91, 141.00; HR FAB-MS: calcd for $C_{48}H_{97}O_6NP$ $[M+H]^+$: 814.7054, found: 814.7080.

1-*O*-(3,4-Dimethoxybenzyl)-2,3-di-*O*-[3,7,11,15-tetramethylhexadeca-2,6,10,14-tetraen-1-yl]-*sn*-glycerol (5): A solution of 1-*O*-(3,4-dimethoxybenzyl)-*sn*-glycerol[58] (**1**) (960 mg, 3.96 mmol) in DMSO (5 mL) was added to a suspension of prewashed NaH (*n*-hexane) (212 mg, 8.80 mmol) in DMSO (5 mL). After 2 h, a solution of geranylgeranyl chloride[60] (2.72 g, 8.80 mmol) in DMSO (3 mL) was added. The mixture was stirred at room temperature overnight. Saturated aqueous NH_4Cl (15 mL) was added and the mixture was extracted with EtOAc. The organic phase was washed with water, dried (Na_2SO_4), filtered and concentrated to dryness. The residue was purified by flash chromatography on silica gel with hexane/EtOAc (20:1) to give **5** (2.10 g, 60%) as an oil. ^1H NMR (300 MHz): $\delta = 1.60$ (s, 18H), 1.65 (s, 6H), 1.68 (s, 6H), 3.49–3.72 (m, 5H), 3.88 (s, 3H), 3.89 (s, 3H), 4.01 (d, $J = 7.0$ Hz, 2H), 4.16 (d, $J = 7.0$ Hz, 2H), 4.60 (s, 2H), 5.11 (m, 6H), 5.35 (m, 2H), 6.79–6.91 (m, 2H), 7.36 (s, 1H); ^{13}C NMR (75 MHz): $\delta = 15.99, 16.51, 17.67, 25.68, 26.39, 26.64, 26.76, 39.64, 39.70, 55.79, 55.90, 66.81, 67.87, 70.08, 70.24, 73.25, 77.21, 110.86, 110.98, 120.17, 120.88, 121.24, 123.86, 123.90, 124.08, 124.20, 124.38, 131.01, 131.24, 134.93, 135.26, 139.79, 140.05, 148.51, 148.97$; IR (neat): $\tilde{\nu} = 1031, 1097, 1238, 1265, 1380, 1450, 1515, 1592, 1670, 2856, 2917, 2964\ cm^{-1}$; anal. calcd for $C_{52}H_{82}O_5$: C 79.34, H 10.50; found: C 79.39, H 10.72.

2,3-Di-*O*-[3,7,11,15-tetramethylhexadeca-2,6,10,14-tetraen-1-yl]-*sn*-glycerol (6): A mixture of **5** (953 mg, 1.21 mmol) and 3,4-dichloro-5,6-dicyanobenzoquinone (550 mg, 2.42 mmol) in CH_2Cl_2 (20 mL) and H_2O (0.5 mL) was stirred for 3 h at 0 °C. The mixture was passed through a short column of silica gel (10 g) and the column was eluted with $CHCl_3$. The eluent was concentrated to dryness and the residue was purified by flash chromatography on silica gel with hexane/EtOAc (20:1) to give **6** (458 mg, 60%) as an oil. ^1H NMR (300 MHz): $\delta = 1.60$ (s, 18H), 1.68 (s, 12H), 3.45–3.80 (m, 5H), 4.01 (d, $J = 7.0$ Hz, 2H), 4.10 (dd, $J = 12.0$, 7.0 Hz, 1H), 4.19 (dd, $J = 12.0$, 7.0 Hz, 1H), 5.10 (m, 6H), 5.35 (m, 2H); ^{13}C NMR (75 MHz): $\delta = 16.00, 16.50, 16.53, 25.69, 26.30, 26.61, 26.74, 39.60, 39.67, 39.70, 63.06, 66.51, 67.93, 70.02, 77.42, 120.52, 120.74, 123.78, 124.16, 124.37, 131.24, 134.93, 135.36, 140.55$; IR (neat): $\tilde{\nu} = 1045, 1074, 1110, 1280, 1448, 1668, 2856, 2919, 2965, 3446\ cm^{-1}$; anal. calcd for $C_{43}H_{72}O_3$: C 81.07, H 11.39; found: C 81.31, H 11.27.

2,3-Di-*O*-[3,7,11,15-tetramethylhexadeca-2,6,10,14-tetraen-1-yl]-*sn*-glycero-1-phosphocholine (7): β-Chloroethyl phosphoryl dichloride (0.12 mL, 0.94 mmol) was added at 0 °C to a solution of **6** (400 mg, 0.63 mmol) in pyridine (4 mL) and the mixture was stirred for 2 h at the same temperature. Water (5 mL) was added and the mixture was stirred at room temperature for 1 h. Then, the mixture was acidified by addition of 2N HCl and extracted four times with $CHCl_3$. The combined organic layers were dried (Na_2SO_4), filtered and concentrated to dryness. The obtained residue was dissolved in toluene (5 mL) and CH_3CN (5 mL) and the solution was transferred into a pressure bottle. The mixture was cooled to $-78\,^{\circ}C$ and dry trimethylamine (5 mL) was introduced. The mixture was heated at 65 °C for 3 d. After the solvent was removed in vacuo, the residue was chromatographed on silica gel with $CHCl_3$/methanol (10:1) to $CHCl_3$/methanol/H_2O (65:15:1) to give **7** (210 mg, 42%) as hygroscopic wax. ^1H NMR (300 MHz, $CDCl_3/CD_3OD$ 1:1): $\delta = 1.60$ (s, 18H), 1.68 (s, 12H), 3.24 (s, 9H), 3.48–3.73 (m, 5H), 3.93 (m, 2H), 4.02 (d, $J = 7.0$ Hz, 2H), 4.17 (d, $J = 7.0$ Hz, 2H), 4.27 (m, 2H), 5.11 (m, 6H), 5.34 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3/CD_3OD$ 1:1): $\delta = 16.20, 16.66, 16.70, 17.81, 25.81, 26.94, 27.08, 27.10, 27.21, 40.08, 40.10, 40.11, 40.17, 54.61, 59.44$ (d, $J = 5.0$ Hz), 65.73 (d, $J = 5.5$ Hz), 67.05, 67.19, 68.34, 70.19, 77.68 (d, $J = 8.0$ Hz), 121.31, 121.59, 124.31, 124.69, 124.86, 131.45, 135.28, 135.72, 135.74, 140.48; HR FAB-MS: calcd for $C_{48}H_{85}O_6NP$ $[M+H]^+$: 802.6115, found: 802.6159.

Techniques

DSC (Differential scanning calorimetry) thermograms: A thin lipid film was prepared by dissolving the preweighed components in a small volume of $CHCl_3$ and removing the solvent first in an argon stream and then in vacuo. 100 µL of "Ultrapure" water was added and the mixture was heated at 60 °C for 1 h. After stirring on a vortex mixer, the suspensions were incubated overnight at 37 °C. Approximately 60 µL of the mixture was sealed in an inox vial and weighed. All calorimetric scans were performed using the Perkin–Elmer DSC-2B calorimeter, usually at $1.0\,K\,min^{-1}$ (heating). With pure DMPC, a concentration of $100\,mg\,mL^{-1}$ was used, for the pure compounds **A**, **B**, **C**, **D** a concentration of $110\,\mu g\,mL^{-1}$ and for the mixtures of lipids, concentrations of $60-300\,\mu g\,mL^{-1}$.

Microscopies

Cryo-transmission electron microscopy (cryo-EM):[34] A phosphocholine (5 mg) was dissolved in $CHCl_3$ (1 mL). After evaporation of the solvent, the film was dried under vacuum overnight. The film was then hydrated by addition of 1 mL of buffer (for example, phosphate buffer, pH 7.0) and vortexed for 2 min at room temperature. The preparation was observed in the frozen hydrated state by cryo-EM. 700 mesh copper grids were dipped into lipid stock solution ($\approx 5\,mg\,mL^{-1}$), the excess liquid was blotted out with a piece of filter paper (Whatman no. 2) and the grids were plunged into liquid ethane or liquid propane cooled with liquid nitrogen. The grids were mounted under liquid nitrogen in a Gatan 626 Cryoholder, transferred into the electron microscope, and observed at approximately $-170\,^{\circ}C$. Observations were made in a CM 12 Philips microscope operating at 100 kV. The microscope was equipped with an additional anti-contamination device. Images were not taken under low-dose conditions, as pure lipid solutions are not as sensitive to beam irradiation as proteins. Images were recorded on Kodak SO163 films and developed under standard conditions.

Optical and confocal microscopies—*Differential interference contrast microscopy and image analysis*: Inverted optical microscope: Axiovert 135, 63×/1.40 Plan Achromat Oil DIC objective, ×2.5 insertion lens, Carl Zeiss. Light sources: Hg (HBO 100, bandpass 450–490 blue filter, bandpass 546–558 green filter, Carl Zeiss) and halogen lamps (12 V, 100 W, Carl Zeiss). Temperature control (TRZ 3700, room temperature +3.0 °C–50.0 °C/±0.2 °C, Carl Zeiss). Video system: charge-coupled device (CCD) camera (C 2400-75H, Hamamatsu Photonics), image processor (Argus 20, Hamamatsu Photonics), S-VHS video recorder (SVO-9500MDP, Sony), trinitron color video monitor (PVM-1443MD, Sony), microcomputer (Power MacIntosh 7500/100, Apple), video printer (VP-1800 EPM, Sony) and digital image recorder (Focus). Improvement of the quality of optical microscopy images is ensured by increasing the ratio optical signal/background noise by appropriate softwares (N.I.H. Image 1.55 and Photoshop 4.0).

Observations: A typical procedure was as follows: A phosphocholine (3 mg) was dissolved in 300 µL of 2:1 (*v*/*v*) mixture of chloroform and methanol. An aliquot (15 µL) of the solution was dropped on a cover glass (0.17 mm thick). After 10 min of drying at room temperature, the lamellar solid remaining on the slide was brought into focus and 500 µL of a buffer (sodium acetate/acetic acid bufffer: pH 4.5, Na_2HPO_4/NaH_2PO_4 buffer: pH 4.5 to 8.2, or borate buffer: pH 8.85) at 25 °C was added. Samples were unsealed. Vesicles were observed to grow from the edges of the solid. Beside multilamellar structures shown in the figure, unilamellar vesicles and tubules were observed. Membranes appear to be thick because of the diffraction-limited resolution of the optical system.

Laser scanning microscopy and image analysis: Laser inverted confocal microscope (LSM Invert 410, 63×/1.40 Plan Apo oil DIC objective, ×2.5 insertion lens, Carl Zeiss), laser sources Ar (488 nm, bandpass 515–565 filter, Carl Zeiss) and He/Ne (543 nm, Longpass 595 filter), image processor (Matrox 4MW), color monitor (FT 3420 ETKL, Mitsubishi), microcomputer (PC 486-488PX), video printer (VP-1800 EPM, Sony) and digital image recorder (Focus). Vesicles were subjected to optical serial sectioning to produce images in the X–Y plane. Each optical section was scanned eight times to obtain an averaged image which was used for analysis. 3D imaging was constructed from IR serial optical sections of 0.2 µm depth. The images were recorded digitally in a 768×576 pixel format and saved on an erasable magneto optical disk.

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Water permeability of vesicles measured by stopped-flow osmotic shock method—*Preparation of unilamellar vesicles of homogeneous size*: Vesicles of the phosphocholines (**A, B, C, D**) were prepared by the freeze-thaw method[61] using the following sea water pH 7.86 adjusted buffer A): 10 mM TRIS · HCl, 5 mM NaN_3, 1 mM EDTA, 150 mM NaCl. For the stopped-flow experiments, we used buffer B): 10 mM TRIS · HCl, 5 mM NaN_3, 1 mM EDTA, 0 mM NaCl, pH 7.86. The artificial sea water composition is:[62] 0.725 g KCl, 26.5 g NaCl, 5.3 g $MgCl_2 \cdot 6H_2O$, 6.799 g $MgSO_4$, 0.193 g $NaHCO_3$, 1.46 g $CaCl_2 \cdot 2H_2O$ in 1 L Milli-Q water, pH 7.86). The vesicle suspension was then extruded ten to forty times through two polycarbonate membranes (pore size 200 nm or 100 nm, Nucleopore, Corning Costar) under 10–15 atm N_2 pressure and the sample was finally diluted to 25 mL with the same buffer. The vesicle size distribution and its dispersity were evaluated by photon correlation spectroscopy (PSC) on a Coulter–Counter N4MD instrument, using laser light scattering at 33 °C with a 90° scattering angle and the following parameters: viscosity 0.006 poise, refraction index 1.33, for supposedly infinitely diluted vesicular solutions ($\approx 10^{-4}$ M) in low concentration aqueous buffers (concentrations of components $\approx 10^{-4}$ M). The average diameter measured by PSC is for example 179 nm (lipid **A**) for the vesicles filtered through the filter of pore size 200 nm and 107 nm (lipid **C**) for the vesicles filtered through the filter of pore size 100 nm.

Stopped-flow experiments: The variation of scattered light intensity (I) versus time (t) upon osmotic shock thermostated at $T = (33.0 \pm 0.1)$ °C (MT/2 Lauda thermostat) was followed at the fixed wavelength of 400 nm (entrance and exit slits width 2 mm) on a Biosequential DX-17MV stopped-flow ASVD spectrofluorimeter (2 mm pathlength cuvette, Applied Photophysics). Analysis of data: Bio-Kine Analysis V 3.14 software (Bio-Logic). The vesicle dispersions were subjected to the osmotic shock 60 min after their preparation. The stability of the samples was checked by comparison of the average size of the vesicles (PSC) just after their preparation and their average size 5 h later. A sample was deemed stable if it remained monodispersed and the average size of the vesicles was constant. An aliquot of vesicle dispersions prepared with buffer A was rapidly mixed with the same volume of the hypomolar buffer B in the stopped-flow instrument: $\Delta(NaCl)_{int-ext} = 75$ mM (just after the mixing). Just before their introduction into the stopped-flow syringes, the vesicle sample and the hypotonic buffer B were filtered through Millipore 0.5 mm PTFE filters and de-aerated by passing through a gentle flow of argon for 10 min at the temperature of 33 °C. To achieve thermal equilibrium, they were left at (33 ± 0.1) °C in the drive syringes for at least 10 min before the beginning of the kinetic swelling experiments.[24] Each rate constant value k was calculated by averaging rate constant values obtained in turn by computerized fitting of average curves I versus t derived from the superimposition of several experimental curves (typically 10–15 for each average curve). Each experimental curve (1000 points) was obtained by monitoring the change in scattered light intensity following the rapid mixing ($t \leq 3$ ms) of equal volumes (100 μL) of sample and hypertonic buffer. Typically 10 to 15 injections provided independent experimental kinetic curves which were superimposed, averaged, and numerically treated by the Biokine software, which uses a factor analysis method and a Simplex algorithm. The results of 10 to 15 runs of experiments were then averaged: The corresponding k values and standard deviation are given in Table 1. The values of the first-order rate constants k determined for the theoretical exponential model [Equation (1)] measure the H_2O permeability of the vesicles.[24, 63] The half-time is defined as the Equation (2).

$$I(t) = I_o - (I_\infty - I_o)\, e^{-kt} \quad (1)$$

$$t_{1/2} = (\ln 2)/k \quad (2)$$

^{2}H-NMR spectroscopy on oriented multibilayers—*Preparation of oriented samples*: Cover glasses (Polylabo no. 0/1: $20 \times 20 \times 0.15$ mm) were first cleaned by immersion in fuming HNO_3 during 2 d, then thoroughly washed with MilliQ water and pure acetone, and dried in an oven overnight. They were cut into 6–9 mm strips. A phosphocholine (10 mg) was dissolved in 2-propanol (900 μL). The organic solution was applied dropwise on the glass strips and dried under vacuum (0.01 atm) over P_2O_5. The strips were stacked in a 10 mm (o.d.) NMR tube (45 to 50 strips). To hydrate the sample, about 50 μL of deuterium-depleted water (deuterium content $10^{-2} \times$ natural abundance, Sigma) was added, and the sample was kept at 35 °C for 24 h in the saturated water atmosphere before the tube was sealed. The bilayer normal was set perpendicular (90°) to the magnetic field by positioning manually the NMR tube in the probe holder. The oriented samples were prepared from 10 mg (14.2 mM) [D_{27}]DMPC and an equimolar amount of the additive, according to previously published procedures.[52, 53]

^{2}H-NMR spectroscopy: All NMR measurements were carried out on a Bruker MSL-300 spectrometer; ^{2}H NMR spectra were recorded at 46.053 MHz with the quadrupolar echo sequence: $(90°_y - t - 90°_x - \tau)$-acquire, with $t = 25$ μs and a 90° pulse of about 7 μs (10 mm coil) or 4 μs (5 mm coil). The recycle time was 1 s unless otherwise stated. A spectral width of 250 kHz was used for oriented samples. The observed quadrupolar splitting of a C–D bond having an axially symmetric motion is given by:

$\Delta\tilde{\nu}_Q = \frac{3}{2} \cdot (e^2qQ/h) \cdot S_{C-D} \cdot (3\cos^2\theta - 1)/2$

with e^2qQ/h as the static quadrupolar coupling constant (168 kHz for aliphatic C–D bond), S_{C-D} the C–D bond-order parameter, and θ the angle between the symmetry axis for the motion and the magnetic field direction.

It has been shown that the bilayer normal is the director of motional averaging for lipids in various membranes, especially in DMPC bilayers.[51] In these experiments the bilayer normal was set perpendicular to the magnetic field ($\theta = 90°$) by manually positioning the NMR tube into the probe; the estimated accuracy of the angular settings was $\pm 2°$ as determined by multiple settings of the 90° orientation.

A segmental order parameter S_{mol} can be assigned to each labelled position and is expressed as follows:[26, 64]

$S_{mol} = -2S_{C-^2H}$ for the methylene groups,

$S_{mol} = -6S_{C-^2H}$ for the terminal methyl group.

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