Lipid Polymorphism

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1 INTRODUCTION

Lipids are a structurally diverse class of biological molecules that are defined on the basis of their solubility in organic solvents. Lipids are involved in numerous metabolic processes including energy metabolism and hormone biosynthesis. They are also important structural components of biological membranes. Biological membranes are fluid, noncovalent assemblies of lipid and protein which form the external boundary and internal compartments of eukaryotic cells, thereby allowing local environments appropriate to particular functions. Membranes are also selective permeability barriers which provide a means of regulating cellular concentrations of ions and metabolites. The essential structural features of membranes are described by the fluid mosaic model, which was proposed by Singer and Nicholson over 20 years ago. In this model, the lipid components are arranged in lamellar sheets with a thickness of two molecules (about 4 nm), providing a fluid matrix into which proteins can insert or associate at the surface. The lipid bilayers form spontaneously in water, as a result of the amphipathic nature of the lipid molecules.

Membranes contain a remarkable diversity of lipids which exceed the requirements for basic structural functions. For
example, although a single lipid such as phosphatidylcholine can form fluid semipermeable bilayers, biological membranes typically contain over 100 lipid components. That the synthesis and transport of these lipids requires significant metabolic expenditure suggests specific biochemical or physicochemical roles for the individual components of membranes. Efforts to clarify these roles have been a focus of membrane biology for over three decades, with the task still ongoing. Much insight has been gained through investigations of the physicochemical properties of model systems formed from different membrane lipids. The pioneering X-ray diffraction studies of Luzzati et al. revealed that isolated membrane lipids could, upon hydration, adopt fluid phases (known as mesophases) other than the bilayer, a phenomenon referred to as lipid polymorphism. While elucidation of the structure of many of these mesophases was accomplished using X-ray diffraction, a great deal of information pertaining to the phase behavior and physical properties of different lipid mesophases, for both pure and mixed lipid assemblies, has come from solid state $^{31}$P and $^2$H NMR. This review will discuss the information provided by magnetic resonance techniques, and attempt to place these data within a biological framework. The importance of lipid polymorphism stems not only from the interesting biophysics of lipid-water assemblies, but from the insight which it provides into many aspects of membrane function.

2 LIPID POLYMORPHISM AND NMR

2.1 Lipid Structure and Diversity

The main classes of lipids found in eukaryotic membranes include the glycerophospholipids, the sphingolipids, and cholesterol. Lipids found in the former group include phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), and cardiolipin (CL). The type of phospholipid is determined by the polar headgroup, which is attached to position 3 of the glycerol backbone; fatty acyl chains occupy the other two positions. The structures of a representative PC and PE are shown in Figure 1 (see legend for details). The two lipids differ only at the headgroup nitrogen, which is protonated in PE and trimethylated in PC. The sphingolipids are derivatives of the long-chain base sphingosine. The headgroup can be the same as that of PC (giving sphingomyelin), or a hydroxyl group (giving ceramide), or can contain a variety of sugars (giving the glycosphingolipids). A single fatty acyl chain attaches to sphingosine via an amide bond. Cholesterol is a sterol; a hydroxyl group is found at one end of its rigid ring system and a branched hydrocarbon chain at the other.

This brief summary of the main lipid groups illustrates the heterogeneous nature of biological membranes. This is further compounded by variation in fatty acid chain length and degree of unsaturation, as well as the presence of minor lipid components.

2.2 Lipid Polymorphic Phases

Lipids pack within a bilayer such that the headgroups form the aqueous interface of the membrane surface, while the fatty acyl chains form the hydrophobic membrane interior [Figure 2(a)]. Lipid bilayer models include multilamellar vesicles (MLVs), oriented multibilayers, and large and small unilamellar vesicles (LUVs and SUVs, respectively). For NMR studies of polymorphism, MLVs have been utilized almost exclusively. These large quasispherical structures (1-10 μm diameter) are

Figure 1 Chemical structures of representative phospholipids: (a) 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and (b) 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE). The stereospecific numbering of the glycerol backbone is shown in (a). POPC has a cylindrical shape which favors the bilayer phase, whereas POPE has a cone shape which favors the $H_{II}$ phase.

Figure 2 Schematic cross-sectional representation of (a) the liquid crystalline bilayer and (b) hexagonal $H_{II}$ phases, showing how these phases favor the packing of cylindrical and cone shaped lipids, respectively. The $H_{II}$ cylinder axis is normal to the plane of the paper.
easily formed by dispersing lipid in water with vortexing and (often) several cycles of freezing and thawing. In addition, they are large enough so that tumbling of the MLV, or lateral diffusion of lipids around the MLV do not result in significant motional averaging effects. As a result, broadline $^{31}$P and $^2$H spectra are observed.

Some membrane lipids, PE in particular, adopt the hexagonal H$_n$ phase in isolation [Figure 2(b)].\textsuperscript{3,10} This nonbilayer phase is composed of hexagonally packed lipid cylinders, where the phospholipid headgroup points towards the center of the cylinder, forming an aqueous channel with a diameter of approximately 2 nm. Most lipids that adopt the H$_n$ phase can also adopt the bilayer phase if the temperature is low enough; transitions between the two phases are rapid and reversible, although little exchange of lipid between these phases appears to occur when they coexist.\textsuperscript{11}

Other nonbilayer phases include micellar structures and cubic phases, in which the lipid aggregate forms a three-dimensional lattice.\textsuperscript{12} The structures of the cubic phases are quite complex, and will not be described here. In general, neither the H$_n$ nor cubic phases are observed as long-lived structures in vivo. The reason these phases are of interest in a biological context stems from evidence suggesting that some of the intermediates of bilayer to nonbilayer transitions are involved in processes such as membrane fusion (see Section 3).\textsuperscript{13,14}

2.3 Phosphorus-31 NMR

Phosphorus-31 NMR has been widely used in the study of membrane structure and lipid polymorphism.\textsuperscript{3,4} Phosphorus-31 is a spin-1 nucleus with a natural abundance of 100%, found in membranes only in the headgroups of phospholipids and sphingolipids. All of the common phospholipids give rise to broad $^{31}$P NMR spectra, where the spectral width can range from 20 to 200 ppm depending on the mesophase being examined and the extent of hydration. Because $^{31}$P NMR spectra are almost always acquired with broadband decoupling (to remove $^1$H-$^{31}$P dipolar interactions), the observed lineshape depends on the types of motions which average the shielding anisotropy of the phosphate group. The nonaxially symmetric shielding tensor gives rise to three principal components, which are observed in spectra of anhydrous lipid, having widths of the order of 200 ppm.\textsuperscript{11} In liquid crystalline MLVs, rapid axial rotation averages the components of the shielding tensor in the plane of the membrane, giving rise to the classic ‘powder pattern’ lineshape [Figure 3(a)] with a width of 40-50 ppm.\textsuperscript{3} The origin of this lineshape is discussed by Cullis and DeKruijff and by Seeleg.\textsuperscript{4,15}

In the H$_n$ phase, rapid diffusion of lipid about the cylinder long axis provides an additional mechanism of motional averaging.\textsuperscript{3,10,15} The result is a powder pattern which, compared with that of the bilayer, is reduced by a factor of two and has the opposite sign [Figure 3(b)]. This allows transitions between the two phases to be conveniently and accurately monitored. The correlation between phase determinations by $^{31}$P NMR and small-angle X-ray diffraction has been shown to be excellent.\textsuperscript{16}

The cubic phases, although structurally diverse, allow the phospholipid molecules to sample all angles in a period short on the NMR timescale, i.e. they allow isotropic averaging of the powder lineshape.\textsuperscript{3,12} This results in a narrow peak at the isotropic chemical shift, which is difficult to differentiate from that produced by micelles, SUVs, or some LUVs [Figure 3(c)]. Lipid mixtures containing nonbilayer lipids often exhibit isotropic peaks,\textsuperscript{12} which are said to originate from ‘isotropic phase’ lipid.

Phosphorus-31 NMR is clearly a convenient tool for monitoring the polymorphic phase preferences of pure and mixed lipid assemblies. Although techniques such as X-ray diffraction and freeze-fracture electron microscopy have arguably been of equal importance,\textsuperscript{6,17} the advantages of NMR, primarily in convenience and rapidity, have made it the technique of choice for characterizing a variety of different lipids and their response to variation in parameters such as pH, temperature, and ion concentrations. These results are summarized in the next section.

2.4 Lipid Polymorphic Phase Preferences

A comprehensive tabulation of the polymorphic phase preferences of a large variety of pure and mixed lipid systems is given in Cullis \textit{et al.}\textsuperscript{18} A number of interesting observations have emerged from these data. In single-component systems, a few lipids favor the bilayer phase almost exclusively: these include the PCs, PI, sphingomyelin, diglucosyldiglyceride (DGlcDG), and digalactosyldiglyceride (DGalDG). Other lipids can adopt either the lamellar or H$_n$ phase depending on the conditions: these include PE, PS, phosphatidic (PA), phosphatidylglycerol (PG), and CL. Two of the glycolipids [monoglucosyldiglyceride (MGlcDG) and monogalactosyldiglyceride (MGalDG)] adopt the H$_n$ phase exclusively.

Of the nonbilayer phase lipids, PE has been most extensively studied.\textsuperscript{3,10,14,18} PE forms stable bilayers at sufficiently low temperatures, undergoing a transition to the H$_n$ phase as the temperature is raised above some critical value. Increasing the level of acyl chain unsaturation favors the formation of the H$_n$ phase. Other lipids, such as PS and phosphatidic acid, will
only form the HII phase below pH 3.5, where the negative charge on the headgroup is neutralized. Similarly, phosphatidic acid and CL will adopt the HII phase in the presence of Ca2+, which neutralizes the negative surface charge. These observations have biological relevance, as they indicate possible mechanisms (control of pH and divalent ion concentration) available to the cell to regulate bilayer-nonbilayer transitions in an isothermal environment.

Phosphorus-31 NMR has also proven useful in characterizing the polymorphism of multicomponent systems, which bear a greater resemblance to biological membranes than pure lipid systems. This has resulted in two important observations. Firstly, mixtures of bilayer and nonbilayer lipids will progressively favor the bilayer phase as the proportion of bilayer forming lipids is increased; sometimes as little as 20 mol% of the bilayer lipid will completely stabilize the lamellar phase.3,14 Secondly, cholesterol may play a role in regulating bilayer to nonbilayer transitions in complex systems. Cholesterol can induce the formation of HII phase in PE-containing bilayer systems that have been stabilized by PC.14

The substantial body of data on lipid polymorphic behavior has led to theoretical insight into the factors which determine the phase preference of a given lipid system. One of the most successful approaches, developed by Israelachvili et al.,19 involves consideration of the molecular shapes of individual lipid molecules. The basic idea is that molecules with different shapes will preferentially pack in phases of different symmetry. As illustrated in Figure 1, lipids such as PC which favor the lamellar phase have similar average cross-sectional areas in the headgroup and acyl chain regions, and are thus said to be ‘cylindrical’. Lipids such as PE have a cone shape, where the headgroup occupies a smaller cross-sectional area than the acyl chains, thereby imparting a curvature to the assembly that favors the HII phase (see Figure 2). For detergent-like lipids which form micellar structures the geometry is reversed, giving an inverted cone where the headgroup cross-sectional area is greater than that of the chains. To see how this can provide insight into polymorphic phase transitions, consider the lamellar to hexagonal transition of dioleoyl-PE (DOPE). At temperatures less than 10°C the lipid chains are sufficiently ordered to impart a cylindrical molecular geometry [see Figure 1(a)] that favors the bilayer phase [see Figure 2(a)]. Elevating the temperature increases the extent of acyl chain molecular motion, resulting in a greater cross-sectional area of the hydrocarbon chains relative to the headgroup. This results in a cone geometry [see Figure 1(b)] that favors the HII phase at room temperature [see Figure 2(b)]. Similar considerations explain why neutralizing the charge of the headgroup, either via pH or divalent cations, leads to the formation of the HII phase in other lipids. Charged headgroups repel and are therefore larger; neutralizing the charge reduces their effective size thereby imparting cone geometry to the lipid.

A more quantitative approach to understanding the factors involved in the formation of nonbilayer phases has come from the ‘curvature’ concept introduced by Gruner.20 In this model, the lamellar to hexagonal transition is described as a tendency for nonbilayer lipids to force a monolayer to curl into cylinders with a spontaneous radius of curvature RH; the magnitude of this parameter, which can be measured by X-ray diffraction, provides a measure of the polymorphic tendencies of a lipid system. A lipid with a strong tendency to form the HII phase will have a small radius of curvature, whereas a system with less tendency, such as a PC/PE mixture, will have a larger radius of curvature. Studies on DOPE and its methylated derivatives reveal that while systems with small and large RH form hexagonal and lamellar phases, respectively, those with intermediate values have a strong tendency to form cubic phases.21

2.5 Deuterium NMR

Deuterium NMR is a powerful technique for the study of molecular order and dynamics in model and biological membranes.5-7 The theory and application of 2H NMR have been extensively reviewed; we will summarize only those aspects relevant to the study of polymorphism. Deuterium is a quadrupolar nucleus (I = 1) whose behavior is dominated by the quadrupolar interaction, and is therefore sensitive primarily to intramolecular motions of the C−2H bond. In anisotropic dispersions such as MLVs, each C−2H bond gives rise to a ‘powder pattern’ lineshape, also known as a ‘Pake doublet’. The separation between the two maxima is the quadrupolar splitting ΔνQ, which, for deuterons on the acyl chain carbons, is related to the carbon-deuterium bond order parameter $S_{CD}$ by

$$\Delta\nu_Q = \frac{3}{4}xS_{CD} \quad (1)$$

where x is the quadrupolar coupling constant. The order parameter provides a measure of the angular excursions of the acyl chains about the surface normal. For lipids containing several deuterons, a superposition of Pake doublets is obtained [Figure 4(a)]. This is problematic for measurements of $S_{CD}$ only if the splittings cannot be assigned.

Deuterium NMR can be used to assess lipid polymorphic preferences, and in this way provides analogous information to the phosphorus-31 NMR analysis.

![Figure 4 Deuterium NMR as a technique for assessing lipid polymorphic preferences. All of the spectra are equimolar dispersions of ceramide : cholesterol : palmitic acid-d31, which approximates the composition of mammalian stratum comeum. (a) Liquid ordered membranes (maximum $S_{CD} = 0.4$) acquired at pH 5.2 and 50°C. (b) Hexagonal HII phase at 75°C, acquired after adjustment of the pH to 7.4. (c) Isotropic phase at 75°C and pH 5.2. (We thank Dr Jennifer L. Thewalt and Dr Neil Kitson for providing the spectra)](image)

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to $^{31}$P NMR. In addition, the use of selectively deuterated lipids allows monitoring of the phase behavior of individual components of mixed phospholipid systems, as exemplified by the Ca$^{2+}$ triggered lamellar to H$_{II}$ transition for $[^{2}$H$]d$ioleoyl-PS ([HIDOPS) in DOPE/dioleoyl-PC/[H]DOPS/cholesterol (1: 1: 1: 3).\textsuperscript{22}

A fascinating polymorphic system recently investigated by $^{2}$H NMR models the intercellular lipid lamellae of mammalian stratum comeum, the uppermost layer of skin.\textsuperscript{23} The model, composed of ceramide, cholesterol, and perdeuterated palmitic acid (1: 1: 1), displays a complex polymorphism, with the palmitic acid partitioning into solid, gel, liquid ordered, hexagonal, and isotropic phases depending on the temperature and pH.\textsuperscript{23} The $^{2}$H NMR spectrum obtained at 50°C and pH 5.2, where the palmitic acid is neutral, reveals liquid ordered membranes (plateau $S_{CD} = 0.4$), which are significantly more ordered than liquid crystalline bilayers but have the same line-shape [Figure 4(a)]. Heating to 75°C induces an isotropic phase [Figure 4(c)]. However, if the pH is adjusted to 7.4, where the palmitic acid is negatively charged, the same temperature increase triggers a transition to the H$_{n}$ phase [Figure 4(b)]. In this case the charge on the palmitic acid (i.e. size of the headgroup) regulates the phase induced at elevated temperatures. Furthermore, if the system is prepared with sphingomyelin instead of ceramide (where the former contains a phosphocholine headgroup compared with the hydroxyl group of the latter), then at low pH the membranes are liquid ordered over a wide temperature range and no polymorphism is observed.\textsuperscript{23}

In addition to phase preferences, $^{2}$H NMR can provide information concerning variation in orientational order in the hydrocarbon region of lamellar and hexagonal phases. For lipids containing a perdeuterated acyl chain, dePaking and integration methodology allows the complete order profile to be derived from a single spectrum.\textsuperscript{24} This has been applied to dispersions of 1-[$^{2}$H$_{31}$]palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC-$d_{31}$) and POPE-$d_{31}$, and mixtures thereof.\textsuperscript{25-27} Significant differences are observed in the lamellar and H$_{II}$ phases (Figure 5). The variation of $S_{CD}$ in bilayers shows little change from carbons 2 to 10 (the plateau region), followed by a rapid drop in order toward the center of the bilayer. In the inverted hexagonal phase, the magnitude of the order parameters is decreased by at least a factor of two, and the plateau region is essentially nonexistent, giving a relatively linear decrease in order down the length of the chain. This results from looser packing of carbons 2 through 8 in the H$_{II}$ phase.\textsuperscript{25,26} The results indicate that order parameters are sensitive to the lipid phase symmetry, suggesting a relationship between the order profile and lipid polymorphic tendencies. This is further supported by the ability of nonbilayer lipids to modulate the order profile of a lamellar matrix. The introduction of POPE into POPC (or DOPE into DOPC) increases the order of the bilayer lipid.\textsuperscript{25,27,28} Whether the changes in order parameters are as predictive of polymorphic preferences as changes in parameters such as $R_{0}$ remains to be seen.

3 BIOLOGICAL IMPLICATIONS OF LIPID POLYMORPHISM

Although nonbilayer lipids are constrained to the lamellar phase in biological membranes, the study of lipid polymorphism has provided insight into several aspects of membrane function. Nonbilayer lipids may be involved in the regulation of certain membrane enzymes, and in some cases this regulation may involve modulation of membrane order, which can be achieved by controlling the ratio of bilayer to nonbilayer lipids. One example comes from $^{2}$H NMR studies on the microorganism Acholeplasma laidlawii strain B, where perdeuterated palmitic acid was biosynthetically incorporated into the cell membrane. Growth of the microorganism could only occur over a fairly narrow range of order (0.14 < $S_{CD}$ < 0.18).\textsuperscript{29} Maintenance of the order profile was attributed to the ratio of MGlcDG to DGlcDG, which in isolation prefer the H$_{II}$ and bilayer phases, respectively. The failure of the organism to thrive outside this range of order suggests an order requirement for optimum membrane protein function.

Nonbilayer lipids may play an important role in membrane fusion. LVUs made of charged and nonbilayer lipids (such as PE and PS) can often be induced to fuse in the presence of Ca$^{2+}$ to form larger lamellar structures which then rearrange to the H$_{II}$ phase.\textsuperscript{14} When these same systems are studied as MLVs, they undergo lamellar to H$_{II}$ transitions. Certain lipid soluble fusogens, such as monoolein, are capable of inducing H$_{II}$ or cubic phase structure in model and biological membranes.\textsuperscript{12,14} Clearly, membrane fusion cannot occur without local, transient departures from bilayer morphology at the fusion interface. These observations provide strong evidence that fusion proceeds via nonbilayer intermediates.\textsuperscript{13}

Our understanding of the link between lipid polymorphism and membrane fusion owes much to the work of Siegel,\textsuperscript{13} who has developed a unified description of both lamellar to hexagonal and lamellar to cubic transitions. Siegel has proposed that the first fusion intermediates are either inverted micellar intermediates (IMIs) or stalk structures, which form between apposed bilayers at appropriate temperatures, and then assemble into either H$_{II}$ phase or cubic

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Figure 5 Smoothed order parameter profile of bilayer phase POPC-$d_{31}$(□) and hexagonal phase POPE-$d_{31}$(□), obtained by integration of dePaked spectra acquired at 30 and 70°C respectively. The two profiles are normalized, but the absolute order parameters of the H$_{II}$ phase at C-2 are approximately half those of the bilayer phase. (We thank Dr M. A. Monck for providing the data)

For list of General Abbreviations see end-papers
phase precursors [the latter are also referred to as interlamellar attachments (ILAs)]. The particular path chosen is a function of the spontaneous radius of curvature of the membrane, but only the latter path (ILAs) leads to membrane fusion. The highly curved ILA structure explains the narrow 'isotropic' resonances often observed in lipid mixtures by $^{31}$P NMR. Liposome fusion has been observed to occur only over the same narrow temperature range where isotropic $^{31}$P NMR resonances are observed. 

4 RELATED ARTICLES

Bilayer Membranes: Deuterium & Carbon-13 NMR, Glycolipids; Membrane Lipids of Acholeplasma laidlawii Membranes: Carbon-13 NMR; Membranes: Deuterium NMR; Membranes: Phosphorus-31 NMR; Molecular Motions: $T_1$ Frequency Dispersion in Biological Systems.

5 REFERENCES


Biographical Sketches
