An Innovative Procedure Using a Sublimable Solid to Align Lipid Bilayers for Solid-State NMR Studies

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ABSTRACT Uniaxially aligned phospholipid bilayers are often used as model membranes to obtain structural details of membrane-associated molecules, such as peptides, proteins, drugs, and cholesterol. Well-aligned bilayer samples can be difficult to prepare and no universal procedure has been reported that orients all combinations of membrane-embedded components. In this study, a new method for producing mechanically aligned phospholipid bilayer samples using naphthalene, a sublimable solid, was developed. Using ^{31}P-NMR spectroscopy, comparison of a conventional method of preparing mechanically aligned samples with the new naphthalene procedure found that the use of naphthalene significantly enhanced the alignment of 3:1 1-palmitoyl-2-oleoyl-phosphatidylethanolamine to 1-palmitoyl-2-oleoyl-phosphatidylglycerol. The utility of the naphthalene procedure is also demonstrated on bilayers of many different compositions, including bilayers containing peptides such as pardaxin and gramicidin. These results show that the naphthalene procedure is a generally applicable method for producing mechanically aligned samples for use in NMR spectroscopy. The increase in bilayer alignment implies that this procedure will improve the sensitivity of solid-state NMR experiments, in particular those techniques that detect low-sensitivity nuclei, such as ^{15}N and ^{13}C.

INTRODUCTION

Solid-state NMR has yielded many insights concerning the structure and function of lipid bilayers, membrane-associated peptides, and proteins (Cross and Opella, 1994; Bechinger et al., 1993; Kovacs et al., 2000; Ramamoorthy et al., 1995; Gasset et al., 1988; Hori et al., 1999; Zhou et al., 1999; Fenske and Cullis, 1992; Marassi and Opella, 1998; Murphy et al., 2001; Smith et al., 1996; McDowell and Schaefer, 1996). Additionally, the ability of peptides to change lipid phases has been probed using ^{31}P-NMR (Epand, 1998; Fenske and Jarrell, 1991; Gasset et al., 1988; Keller et al., 1996; Killian and de Kruijff, 1985; Liu et al., 2001; Morein et al., 1997, 2000). In these studies, structural details of membrane components were elucidated using mechanically aligned phospholipid bilayers or multilamellar dispersions. Mechanically aligned samples offer several advantages when compared to multilamellar dispersions. A commonly exploited benefit of aligned samples is the determination of a peptide's orientation with respect to the bilayer normal. Aligned samples also exhibit greater resolution and allow for smaller quantities of lipid and peptides to be used because of the sensitivity enhancement gained from uniform orientation. However, preparation of well-aligned samples is difficult, and one of the most common problems is the presence of unaligned bilayers within the aligned sample. Unaligned lipids can easily compose 30–50% of a sample, greatly reducing the spectral intensity of the desired signal. These problems are exacerbated by the presence of certain membrane components, including peptides. Unfortunately, some of these membrane components are important for constructing good model membranes.

The ideal model membrane is one that mimics the composition of the relevant cellular membrane, which is often very complex. Closely modeling natural biological membranes is especially important when determining the structure and orientation of peptides in bilayers (Yang et al., 2001; Roux et al., 1994). Two components regularly found in cellular membranes, but seldom included in aligned samples due to technical difficulties, are lipids with phosphatidylethanolamine headgroups and cholesterol. In addition to poor alignment, improper hydration of samples containing these components can result in the formation of lipid phases other than the desired phase (Webb et al., 1993; Gunstone et al., 1994). Therefore, development of a protocol to reliably produce fully hydrated, well-aligned bilayers containing multiple components would allow for systems that better mimic cell membranes to be studied. In this work we demonstrate an innovative method for producing aligned samples that reduces sample preparation time and improves the alignment of lipid bilayers with a variety of components, including peptides.

MATERIALS AND METHODS

Materials

Fmoc-amino acids were purchased from PerSeptive Biosystems (Foster City, CA) and Advanced ChemTech (Louisville, KY). 1,2-Dimyristoyl-phosphatidylcholine (DMPC), 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC), 1-palmitoyl-2-oleoyl-phosphatidylethanolamine (POPE), and 1-palmitoyl-2-oleoyl-phosphatidylglycerol (POPG) were purchased from Avanti Polar Lipids (Alabaster, AL). Gramicidin and cholesterol were purchased from Sigma (St. Louis, MO) and naphthalene was purchased from Fisher Scientific (Pittsburgh, PA). Chloroform and methanol were

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purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI). All chemicals were used without further purification or analysis.

Synthesis of pardaxin

The carboxy-amide of pardaxin (P1a) was synthesized using standard Fmoc-based solid-phase methods with an ABI 431A Peptide Synthesizer (Applied Biosystems, Foster City, CA). The sequence of the synthesized pardaxin is identical to the peptide isolated from *Pardachirus marmoratus*, G-F-F-A-L-I-P-K-I-I-S-S-P-L-F-K-T-L-L-S-A-V-G-S-A-L-S-S-S-G-G-Q-E (Shai et al., 1988).

Sample preparation

All of the samples discussed in this paper were aligned on glass plates using either the newly developed naphthalene procedure or a conventional method for comparison. All samples contained ≈4 mg of lipids. For the naphthalene procedure, the lipids and peptides were dissolved in an excess of 2:1 $CHCl_3/CH_3OH$. The lipid-peptide solution was dried and then redissolved in 200 μl 2:1 $CHCl_3/CH_3OH$ containing a 1:1 molar ratio of naphthalene to lipid-peptide. The solution was then spread and dried on two thin glass plates (11 mm × 22 mm × 50 μm, 100 μl/plate). To remove the naphthalene and any residual organic solvent, the samples were vacuum dried overnight. (The removal of naphthalene was confirmed by ^{1}H solution NMR after redissolving the lipid-peptide film in $CDCl_3$.) The samples were indirectly hydrated at 37°C (93% relative humidity using a saturated $NH_4H_2PO_4$ solution (National Research Council (U.S.) 1926) for 1–2 days, after which 28 mol of 4°C water/mol of lipid-peptide were added to the edges of the glass plates. We found that adding water to the center of the glass plates reduced the quality of the sample's alignment in some cases, such as 3:1 POPE/POPG. The plates were stacked and then sealed in plastic, and equilibrated at 4°C for an additional 1–2 days. For comparison, a 3:1 POPE/POPG sample (mol/mol ratio) was prepared using the naphthalene procedure, except that no naphthalene was added.

A "conventional method" was used to prepare several samples for comparison to the naphthalene procedure. However, there is no standard method that is used to prepare aligned samples and many different solvent systems and hydration methods have been reported. For simplicity, we selected an easy method of preparation as outlined below. In our "conventional method" the lipids and peptides were codissolved in 2:1 $CHCl_3/CH_3OH$, the solution was dried on glass plates, and the resultant lipid-peptide film was vacuum-dried overnight. Enough water was added to achieve a 25:1 ratio of H_2O to lipid-peptide and the sample was allowed to remain for 14 days at 37°C in 93% relative humidity. The conventional procedure used to prepare 3% P1a in 3:1 POPE/POPG was identical, except a little more water was added (28:1 water to lipid-peptide) and the sample was equilibrated at 4°C for 10 days after the addition of the water. ^{31}P-NMR was then used to determine the degree of sample alignment. Adequate signal-to-noise for the ^{31}P chemical shift spectra was typically obtained by signal averaging ≈2000 transients.

The NMR experiments were performed on a Chemagnetics/Varian Infinity 400 MHz spectrometer operating at a field of 9.4 T with resonance frequencies of 400.14 MHz and 161.979 MHz for ^{1}H and ^{31}P, respectively. The spectra of mechanically aligned samples were obtained using a home-built (flat-coil) double-resonance probe with a four-turn square coil (14 mm × 14 mm × 4 mm) constructed from 2 mm wide flat-wire with a spacing of 1 mm between turns. All samples were oriented with their bilayer normal parallel to the external magnetic field. The ^{31}P chemical shift spectra were obtained using a spin-echo sequence (Schmidt-Rohr and Spiess, 1994) (90°-τ-180°-τ, τ = 100 μs) and the second half of the spin-echo was acquired with a proton decoupling field of 50 kHz. The ^{31}P chemical shift spectral width was 25 kHz and the recycle delay was 3–5 s. The ^{31}P chemical shift spectra of mechanically aligned samples were referenced relative to 85% H_3PO_4 at 30°C that was placed between glass plates (0 ppm). A large difference (4.3 ppm) was found between the ^{31}P reference frequency of 85% H_3PO_4 between glass plates when compared to 85% H_3PO_4 in a capillary tube. This change in chemical shift frequency may be attributed to a difference in sample shapes (Belorizky et al., 1990); therefore, it is essential to use a reference sample of similar dimensions to the sample under study. All experiments were conducted at 30°C after a minimum equilibration time of 10 min.

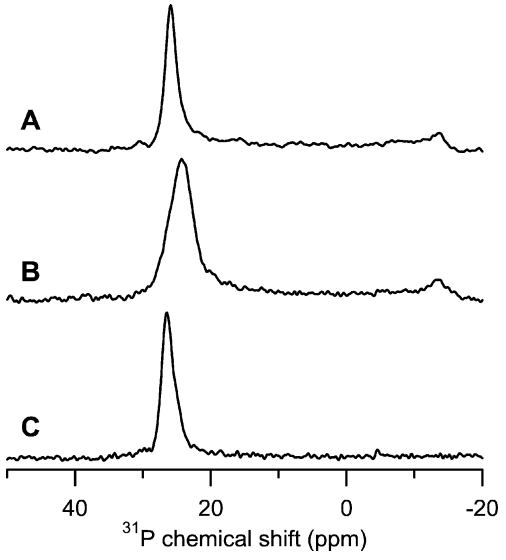

FIGURE 1 ^{31}P chemical shift spectra of aligned lipid bilayers composed of 3:1 POPE/POPG prepared using (*A*) the conventional method, (*B*) a method similar to the naphthalene procedure but without naphthalene, and (*C*) the naphthalene procedure. The sample preparation procedures are described in the text.

RESULTS AND DISCUSSION

Bilayers that mimic bacterial membranes are important for studying the structure and function of antimicrobial peptides. 3:1 POPE/POPG is a simple model that approximates the important features of bacterial membranes, but this combination of lipids can be difficult to align. Fig. 1 compares the effects of different preparation methods on 3:1 POPE/POPG samples. Fig. 1 *A* shows the ^{31}P chemical shift spectrum of a sample prepared using the conventional method. The spectrum can be divided into two components: a peak at 25.9 ppm indicative of aligned lipids and an unaligned component that extends from 26 ppm to −13 ppm. Integration reveals that the peak from aligned lipids contains 70% of the total spectral intensity, while the unaligned component composes 30%. Fig. 1 *B* shows the ^{31}P chemical shift spectrum of 3:1 POPE/POPG prepared using a method identical to the naphthalene procedure, except that the naphthalene was omitted. Like Fig. 1 *A*, it has significant residual powder pattern (25% of total intensity), but the peak has broadened and shifted to 24.3 ppm. Close comparison of the two spectra reveals that the ^{31}P CSA spans measured from the residual powder patterns are similar within experimental error (~40 ppm in Fig. 1 *A* and ~42 ppm in Fig. 1 *B*), but the most intense peak in Fig. 1 *B* has a full-width at

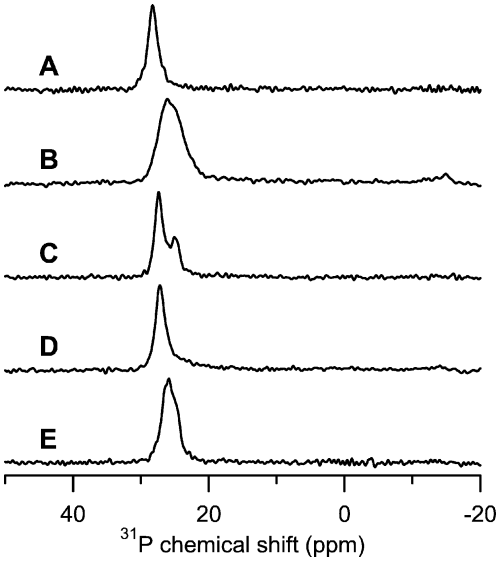

FIGURE 2 ^{31}P chemical shift spectra of aligned lipid bilayers composed of (*A*) POPC, (*B*) 4:1 POPC/cholesterol, (*C*) 3:1 POPC/POPG, (*D*) POPE, and (*E*) 4:1 POPE/cholesterol. All of these samples were prepared with naphthalene as outlined in the text.

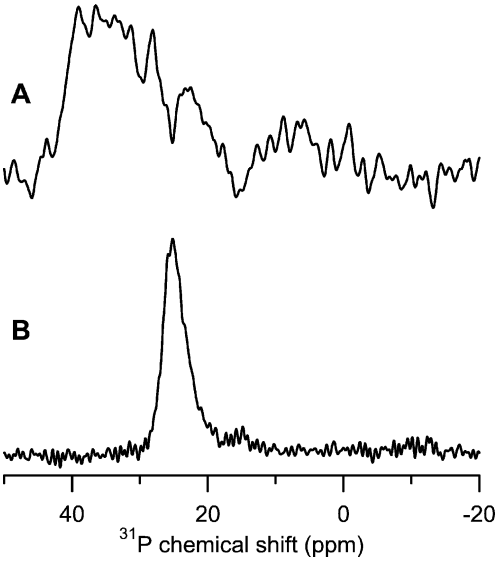

FIGURE 3 ^{31}P chemical shift spectra of aligned lipid bilayers composed of 3% P1a in 3:1 POPE/POPG prepared (*A*) using the conventional method and (*B*) with the naphthalene procedure.

half-maximum (FWHM) height of 4.5 ppm, compared to a FWHM of 2.0 ppm in Fig. 1 *A*. The cause of this difference is not currently understood. Fig. 1 *C* is the ^{31}P spectrum of 3:1 POPE/POPG prepared using the naphthalene procedure outlined above. The spectrum has a peak with a FWHM of 2.0 ppm and a negligible residual powder pattern, which corresponds to a 36% increase in sample alignment compared to 3:1 POPE/POPG prepared using the conventional method.

Other types of membranes, including those of mammalian cells, are also of interest. Bilayers mimicking many types of membranes are frequently difficult to hydrate and align, particularly those containing cholesterol. To demonstrate that this procedure works for bilayers with a variety of compositions, bilayers composed of POPC, 4:1 POPC/cholesterol, 3:1 POPC/POPG, POPE, and 4:1 POPE/cholesterol were prepared using this procedure and were well-aligned, as evidenced by the ^{31}P chemical shift spectra in Fig. 2. The compositions selected include bilayers that mimic both mammalian (POPC, 4:1 POPC/cholesterol) and bacterial membranes (3:1 POPC/POPG, POPE); 4:1 POPE/cholesterol was tested because it is difficult to hydrate and has a propensity to form inverted lipid phases instead of fluid lamellar phase (L_α). Fig. 2, *A* and *B* show that POPC bilayers exhibit a narrow peak (FWHM = 1.7 ppm), unlike 4:1 POPC/cholesterol (FWHM = 4 ppm). The ^{31}P chemical shift spectrum of 3:1 POPC/POPG bilayers is intriguing because it has two peaks (Fig. 2 *C*), probably due to the different components within the bilayer. The spectrum of POPE bilayers (Fig. 2 *D*) has a comparable FWHM to POPC bilayers (Fig. 2 *A*). The lineshape of the 25.9 ppm peak observed from 4:1 POPE/cholesterol bilayers (Fig. 2 *E*) suggests that it is composed of multiple peaks, with similar, but not identical, chemical shifts. The causes of these spectral differences are not understood and their study is beyond the scope of this work. However, these spectra demonstrate the resolution mechanically aligned samples can provide. The above differences would not be observable in spectra of multilamellar dispersions, even the two peaks observed from 3:1 POPC/POPG might not be apparent in an unoriented sample, which would consist of two overlaying powder patterns. Even without understanding the causes of the spectral lineshapes, these results confirm that the naphthalene procedure produces well-hydrated and aligned lipid bilayers containing a variety of components, and can be used for fundamental studies of lipids. However, aligned lipid bilayers used in NMR studies often contain peptides for structure and orientation determination. To investigate the advantages of the naphthalene procedure in preparing bilayers containing peptides, aligned samples were prepared containing two different peptides, pardaxin and gramicidin.

Pardaxin is a 33-residue membrane-associating peptide isolated from *Pardachirus marmoratus* that functions as a shark repellent in nature (Shai et al., 1988). Pardaxin's amphipathic, α-helical secondary structure and ability to disrupt cell membranes suggest it has some pharmaceutical potential as an antimicrobial peptide (Shai, 1994). However, difficulty in preparing aligned bilayers thwarted our initial attempt to study this peptide. The ^{31}P chemical shift spectrum of 3% P1a (mol %) in 3:1 POPE/POPG prepared using a conventional method is shown in Fig. 3 *A*; the broad peak spanning from 40 to 20 ppm indicates that the sample prepared by the conventional method was poorly hydrated and unusable for structural studies. Another 3% P1a in 3:1 POPE/POPG was prepared, but this time the naphthalene procedure was used and the results are shown

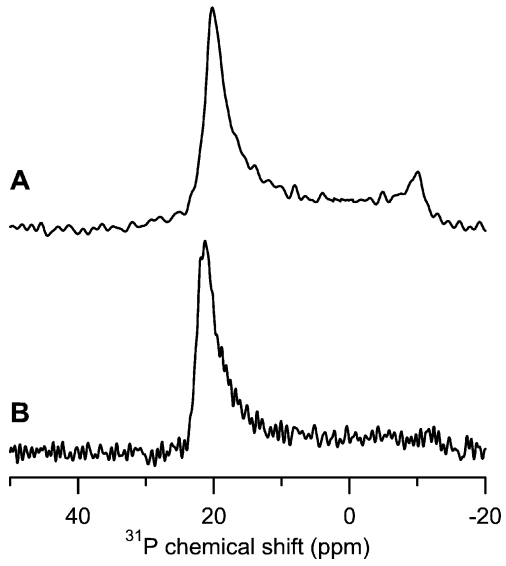

FIGURE 4 ^{31}P chemical shift spectrum of aligned lipid bilayers composed of 1:8 gramicidin/DMPC. (*A*) The sample was prepared conventionally. (*B*) The sample was prepared using the naphthalene procedure.

in Fig. 3 *B*. The sample prepared with the naphthalene procedure was well-aligned and hydrated, ready for investigation. By using the naphthalene procedure, we have been able to study the effect of pardaxin on lipid bilayers of many compositions (unpublished results).

Gramicidin is a 15-residue transmembrane peptide that has been extensively studied using solid-state NMR and aligned bilayers (Nicholson et al., 1987; Ketchem et al., 1993; Moll and Cross, 1990), and we selected it to explore the robustness of the naphthalene procedure. The ^{31}P chemical shift spectra of 1:8 gramicidin/DMPC samples are shown in Fig. 4. In Fig. 4 *A*, the sample was prepared using the conventional method outlined in Materials and Methods, and Fig. 4 *B* shows the ^{31}P spectrum of a sample prepared in 2 days using the naphthalene procedure. Comparison of these two spectra shows that the naphthalene procedure aligns lipid bilayer samples containing hydrophobic transmembrane peptides. It should be mentioned that there are published procedures optimized for producing aligned gramicidin-DMPC samples (Moll and Cross, 1990; Cotten et al., 1999), and we did not directly compare these preparations with the naphthalene procedure. However, the naphthalene procedure significantly reduced the sample preparation time and produced a sample with alignment comparable to the conventional method. Further reduction in sample preparation times may be possible, but experiments exploring this were not attempted.

We postulate that these improvements in sample preparation are caused by naphthalene randomly distributing among the lipids and peptides when the 2:1 chloroform/methanol solution is dried on the glass plates. The removal of naphthalene by overnight vacuum-drying leaves a porous lipid-peptide film that allows for more rapid and complete hydration. This is particularly beneficial for films containing hydrophobic components such as cholesterol and gramicidin, and for bilayers containing phosphatidylethanolamine lipids. However, the use of naphthalene does not guarantee a 100% aligned sample; naphthalene only produces a lipid-peptide film that is amenable to hydration. Some of the ^{31}P spectra shown have low-intensity broad components, notably Fig. 4 *B* and to a lesser extent Fig. 2, *B* and *D*. These samples are well-aligned for the system being studied; it may not be possible to orient 100% of the lipids in every sample. Even for such samples, the naphthalene procedure is beneficial because it makes the sample more amenable to hydration, making preparations simpler in all cases, and possible in very difficult cases (such as pardaxin) where conventional methods failed (compare Fig. 3, *A* and *B*). Although the sample preparation method outlined above functioned well for the wide variety of samples tested, other samples may require variations on the procedure (indirect hydration temperature, amount of water added, temperature of water added, etc.) to maximize their alignment. Once this is accomplished, additional improvements such as minimizing mosaic spread can be attempted, although the improvement gained from these latter optimizations is not as significant as reducing the percentage of unaligned lipids. We suspect that other solids that sublime at room temperature and reasonable pressures (100 mTorr) may be substituted for naphthalene. We began this study using phenol and its use resulted in bilayers with better alignment, but the phenol was not completely removed by vacuum-drying and its use was discontinued.

In conclusion, this work demonstrates that a wide variety of aligned samples can be prepared with the naphthalene procedure described here. When compared to more conventional methods, our procedure produces samples that are more amenable to hydration. This reduces sample preparation time and improves alignment of lipid bilayers, which will aid NMR studies of peptides in aligned bilayers. Improved alignment would benefit solid-state NMR studies examining peptides isotopically labeled with low-sensitivity nuclei, such as ^{15}N and ^{13}C (Lee et al., 1999; Marassi et al., 1997). Extrapolation from our ^{31}P spectra (see Fig. 1) suggests that a 36% improvement in sample alignment would lead to a corresponding increase in the S/N. Therefore, using this naphthalene procedure could increase the signal-to-noise ratio of low-sensitivity experiments, reducing the acquisition time. However, it is important to note that the sensitivity of ^{15}N (or ^{13}C) solid-state NMR experiments depends on many factors, including the degree of alignment of lipid bilayers, efficacy of the cross-polarization and proton decoupling pulse sequences, the inherent mosaic spread of the peptide (caused by multiple conformations, orientations etc.), and the time-scale of peptide or protein dynamics. Therefore, the improvements in lipid bilayer alignment observed using ^{31}P-NMR may not indicate the degree of alignment of the peptide or protein present in the same sample. However, an essential first step in preparing lipid bilayer samples containing isotopically labeled peptides is the formation of fully hydrated, well-aligned bilayers, which can

be accomplished using the naphthalene procedure. In addition to NMR studies, the naphthalene procedure may also benefit other techniques that use aligned samples (Katsaras, 1997; Smith et al., 1994).

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