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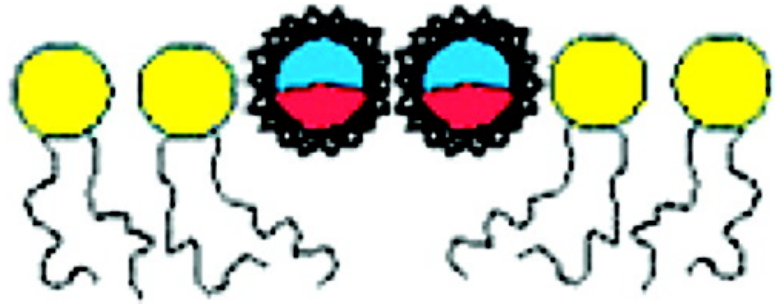

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Nitrogen-14 Solid-State NMR Spectroscopy of Aligned Phospholipid Bilayers to Probe Peptide−Lipid Interaction and Oligomerization of Membrane Associated Peptides

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Abstract: Characterization of the oligomerization of membrane-associated peptides is important to understand the folding and function of biomolecules like antimicrobial peptides, fusion peptides, amyloid peptides, toxins, and ion channels. However, this has been considered to be very difficult, because the amphipathic properties of the constituents of the cell membrane pose tremendous challenges to most commonly used biophysical techniques. In this study, we present the application of a simple ^{14}N solid-state NMR spectroscopy of aligned model membranes containing a phosphatidyl choline lipid to investigate the oligomerization of membrane-associated peptides. Since the near-symmetric nature of the choline headgroup of a phosphocholine lipid considerably reduces the ^{14}N quadrupole coupling, there are significant practical advantages in using ^{14}N solid-state NMR experiments to probe the interaction of peptide or protein with the surface of model membranes. Experimental results for several membrane-associated peptides are presented in this paper. Our results suggest that the experimentally measured ^{14}N quadrupole splitting of the lipid depends on the peptide-induced changes in the electrostatic potential of the lipid bilayer surface and therefore on the nature of the peptide, peptide-membrane interaction, and peptide−peptide interaction. It is inferred that the membrane orientation and oligomerization of the membrane-associated peptides can be measured using ^{14}N solid-state NMR spectroscopy.

Introduction

There is considerable current interest and research activity focused on characterizing and understanding lipid−peptide and peptide−peptide interactions that play a vital role in the folding, secondary structure, dynamics, topology, oligomerization, and function of membrane-associated peptides.[1–10] While various biophysical techniques have been used to characterize lipid−peptide interactions at low resolution, it has been challenging to determine electrostatic interactions such as intermolecular hydrogen bonding and peptide−peptide interactions which lead to the oligomerization of peptides in bilayers. Instead, molecular dynamics simulations[11] are employed to probe such interactions at atomistic-level resolution, which are often subject to questions until proven experimentally. The amphipathic properties of the cell membrane components are not suitable for most biophyiscal techniques. Particularly, lipids and membrane-associated peptides and proteins are difficult to solubilize in water for high-resolution solution NMR studies and difficult to crystallize for X-ray diffraction studies. Even if these are feasible, the results obtained from solution or single crystals do not reveal the peptide or protein interaction with other components of the cell membrane. Equilibrium dialysis, analytical ultracentrifugation, and other biochemical experiments have been used to characterize the oligomerization of peptides in membranes.[12] However, the measurements using these techniques are limited in accuracy and have been difficult to apply to a significant number of biological systems. Therefore, there is a significant need for the development of new high-resolution techniques to probe lipid−peptide and peptide−peptide interactions that lead to the oligomerization process. On the other hand, solid-state NMR studies on model membranes have provided a wealth of high-resolution information about cell membranes and also addressed significant biological questions. In this study, we demonstrate a ^{14}N NMR approach to investigate the electrostatic interaction between lipid and cationic peptides, and oligomerization of peptides, in phospholipid bilayers.

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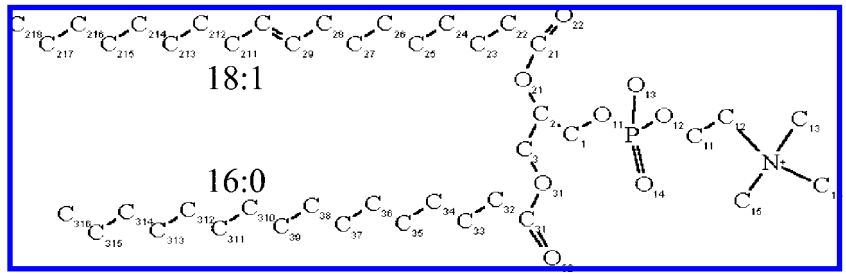

Figure 1. Molecular structure of a phospholipid, POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine), which contains a choline group in the headgroup. The near-tetrahedral symmetry around the ^{14}N nucleus considerably reduces the electric field gradient and therefore the ^{14}N quadrupole coupling. Axial rotation and diffusion of the lipid in the liquid crystalline lamellar phase further averages the quadrupole interaction. Two acyl chains of POPC are labeled as 18:1 and 16:0 to indicate the number of C atoms and double bonds in each acyl chain.

LL-37:	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES
MSI-78 (or pexiganan):	GIGKFLKKAKKFGKAFVKILKK-NH_2
Gramicidin :	VGALAVVVWLWLWLW
GABA$_A$-TM2 :	SVPARTVFGVTTVLTMTTLSISAR

Figure 2. Amino acid sequences of membrane-associated peptides used in this study.

Nitrogen-14 is a spin 1 quadrupole nucleus with a high natural abundance. Several studies have reported the measurement of the fundamental ^{14}N NMR transition frequency of chemical and biological molecules under static and MAS experimental conditions.[13–23] Since the span of the fundamental ^{14}N NMR transition frequency of an amide site of a peptide or protein in solid state is quite large (typically on the order of MHz), it has been very difficult to use this nucleus for studies on biological solids. This has been the main factor that limited the applications of ^{14}N NMR experiments to model systems like single crystals.[24] To overcome this difficulty, ^{14}N overtone NMR and indirect detection experiments have been proposed.[24–27] While these techniques have certain unique advantages over the fundamental ^{14}N NMR, they are still at an early stage for biological applications. On the other hand, the ^{14}N quadrupole interaction becomes significantly small when the electric field gradient surrounding the nucleus is reduced due to symmetry like in the case of a choline moiety in phosphatidylcholine (POPC) lipid (Figure 1) where ^{14}N is in a near-tetrahedral symmetry environment.[2,14,19,20] For example, the ^{14}N quadrupole interaction of a dry POPC powder sample is about 101.25 kHz. It becomes even smaller when the lipid is fully hydrated due to fast axial rotational motion of the lipid molecule in addition to the rotation of the choline group around the $C_\beta-N$ bond.[14] Because of these reasons, the fundamental ^{14}N NMR experiments have been used to study aligned and unaligned model membranes containing the PC lipid such as POPC and DMPC.[14–17,19–21]

In this study we present the applications of the fundamental ^{14}N solid-state NMR spectroscopy to probe lipid−peptide interactions, orientation, and oligomeric condition of membrane-associated peptides using aligned model phospholipid membranes. Two different channel-forming peptides (gramicidin and GABA$_A$-TM2) and two different antimicrobial peptides (LL-37 and MSI-78) are used to examine the performance of this technique. Amino acid sequences of these peptides are given in Figure 2. Previous studies have shown that the 15-residue gramicidin (no charge at neutral pH) forms a head-on β-helical dimer that has a transmembrane orientation and conducts K^+ ions across the membrane.[28,29] The 24-residue GABA$_A$-TM2 is a channel-forming transmembrane α-helical peptide (net

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charge = +2 at neutral pH).[16] A 37-residue human LL-37 (net charge = +6 at neutral pH)[30] and a 22-residue MSI-78 (or pexiganan, net charge +10 at neutral pH)[31] are cationic, amphipathic, helical antimicrobial peptides. Previous NMR studies have shown that these two antimicrobial peptides are oriented on the surface of POPC bilayers and interact with the lipid headgroup.[32,33] These peptides were specifically chosen for the present study as they all form stable α-helical structures in a membrane environment and their membrane orientations have also been reported in the literature. Therefore, the interpretation of ^{14}N NMR spectra in terms of membrane orientation of these peptides can be compared with the already available information in the literature. Our results on the membrane orientation of these peptides determined from ^{14}N NMR experiments are in excellent agreement with previous studies on these peptides. The oligomerization of a human antimicrobial peptide, LL-37, in phospholipid membranes is also examined using ^{14}N NMR experiments.

Experimental Section

Materials. All phospholipids were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Chloroform, methanol, and Gramicidin were procured from Aldrich Chemical Inc. (Milwaukee, WI). Naphthalene was purchased from Fisher Scientific (Pittsburgh, PA). Peptide synthesis and cleavage reagents were purchased from Applied Biosystems (Foster City, CA) and Aldrich (Milwaukee, WI), respectively. Fmoc-protected amino acids were from Advanced ChemTech (Louisville, KY). All the chemicals were used without further purification. Peptides were synthesized and purified as explained elsewhere.[16,31–33]

Sample Preparation. All mechanically aligned lipid samples were prepared using a naphthalene procedure developed in our laboratory as mentioned below.[34] A 5 mg amount of a phospholipid (POPC or DMPC) and an appropriate amount of a peptide were dissolved in 2:1 $CHCl_3/CH_3OH$. All the peptides used in this study were soluble in this solvent mixture. The resultant solution was dried under N_2 gas and redissolved in a solution containing 2:1 $CHCl_3/CH_3OH$ and a 1:1 molar ratio of naphthalene to lipid−peptide. The clear solution was slowly spread on 2−4 thin glass plates (11 mm × 22 mm × 50 μm, Paul Marienfeld GmbH & Co., Bad Mergentheim, Germany). Samples on the glass plates were gently dried under a stream of N_2 gas first and then under vacuum for at least 10 h to remove any residual organic solvents and naphthalene. After drying, the glass plate samples were indirectly hydrated in a hydration chamber at 93% relative humidity using a saturated $NH_4H_2PO_4$ solution for 2 days at 37 °C, after which approximately 3 μL of H_2O were gently misted onto the surface of the lipid−peptide film on the glass plates. The glass plates containing well-hydrated lipid−peptide samples were then stacked and further equilibrated at 4 °C for 6−24 h. The sandwich sample was wrapped using parafilm and sealed in plastic bags (Plastic Bagmart, Marietta, GA) for solid-state NMR experiments. MLVs were prepared by mixing 25 mg of lipid and the desired amount of peptide in 2:1 $CHCl_3/CH_3OH$, but no naphthalene was used. The samples were dried under N_2 gas, vacuum-dried overnight, and transferred to an 8-mm OD glass tube, and 50 wt % water was added to the dry peptide−lipid mixture. The samples were gently vortexed and subjected to 10 freeze−thaw cycles. The glass tubes were cut off a short distance above the sample and sealed using Teflon stoppers, paraffin, and parafilm. All MLV samples were stored at −20 °C prior to NMR experiments.

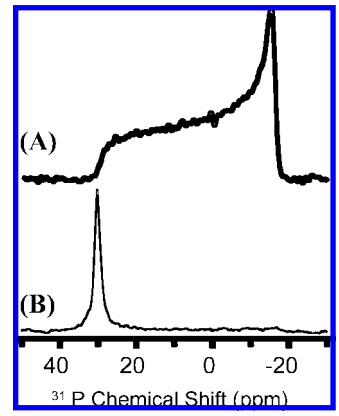

Figure 3. Phosphorus-31 chemical shift spectra of POPC obtained from (A) unaligned multilamellar vesicles and (B) mechanically aligned lipid bilayers.

Quality of Lipid Bilayer Samples. ^{31}P chemical shift spectra were first recorded at 37 °C to examine the quality of each sample. Spectra of pure and peptide-containing MLVs showed similar motionally averaged ^{31}P chemical shift powder patterns with a span of 48 ppm (Figure 3A). On the other hand, ^{31}P spectra of mechanically aligned glass-plates sandwich samples showed a single sharp line at a frequency position corresponding to the parallel edge of the powder pattern observed from MLVs (Figure 3B). This observation suggested that the lipid bilayer samples were well hydrated, present in a fluid lamellar phase, and the presence of peptide did not disrupt the lipid bilayer structure. Since antimicrobial peptides such as LL-37[35] and MSI-78[36] have been shown to disrupt model phospholipid membranes at a relatively higher concentration, it was important to confirm the integrity of the lipid bilayers at the peptide concentration used in this study by ^{31}P NMR.

Solid-State NMR. All solid-state NMR experiments were performed on a Chemagnetics/Varian Infinity 400 MHz solid-state NMR spectrometer operating at resonance frequencies of 400.138, 161.978, 61.46, and 28.899 MHz for ^{1}H, ^{31}P, ^{2}H, and ^{14}N nuclei, respectively. Unless otherwise noted, all experiments were performed at 37 °C. A Chemagnetics/Varian temperature controller unit was used to maintain the temperature of the sample in the probe, and each sample was equilibrated for at least 45 min before starting the experiment. All experiments on oriented samples were performed with the bilayer normal parallel to the external magnetic field using a home-built double resonance probe. ^{31}P chemical shift spectra were obtained using a spin−echo sequence (90°−τ−180°−τ−acq with τ = 125 μs),[37] 5 μs 90°-pulse length, 45 kHz proton-decoupling rf field, 50 kHz spectral width, and recycle delay of 5 s. The ^{31}P chemical shift spectra are referenced relative to 85% H_3PO_4 on thin glass plates (0 ppm). A quadrupole echo pulse sequence (90°−τ−90°−τ−Acquisition; τ = 80 μs)[37] with a recycle delay of 2 s was used to record ^{14}N NMR spectra of lipid bilayer samples. All experimental data were processed using the Spinsight (Chemagnetics/Varian) software on a Sun Sparc workstation.

Results

Fundamental Nitrogen-14 NMR Experiments on Mechanically Aligned POPC Lipid Bilayers. As described in the Experimental Section, the quality of mechanically aligned and

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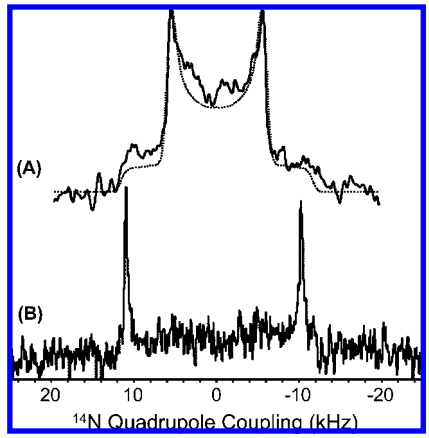

Figure 4. Nitrogen-14 quadrupole coupling spectra of POPC lipid bilayers: (A) unaligned multilamelar vesicles and (B) mechanically aligned lipid bilayers. The trace with dotted lines in (A) is the simulated spectrum.

unaligned lipid bilayers used in this study was examined using ^{31}P NMR experiments. These experiments suggested that all bilayer samples used for ^{14}N NMR experiments were in a fluid lamellar phase and all lipids in the mechanically aligned samples were well-aligned. The fundamental ^{14}N NMR spectrum of POPC multilamellar vesicles (MLVs) displays a ^{14}N quadrupole coupling powder pattern (Figure 4). The span of the ^{14}N powder pattern of POPC is temperature dependent and motionally averaged to about 22 kHz at a physiologically relevant temperature (37 °C), which is a characteristic of the lamellar phase of the model membranes used. On the other hand, spectra of mechanically aligned bilayers show two narrow peaks, corresponding to two different ^{14}N fundamental transition frequencies, separated by the quadrupole coupling. The narrowness of these peaks (full width at half-maximum is about 250 Hz) suggests that all lipid molecules are well-aligned along the external magnetic field as indicated by ^{31}P NMR spectrum of the sample (data not shown). It should be noted that the signal-to-noise ratio (*S*/*N*) observed in an aligned sample is significantly larger than that of unaligned MLVs. In fact, our experiments suggested that the amount of POPC lipids required for the measurement of a reasonable *S*/*N* ^{14}N NMR spectrum from an aligned sample is about 10 times less than that of unaligned MLV samples.

Effect of Peptides on ^{14}N NMR of POPC Lipid Bilayers. The observed quadrupole splitting is sensitive to the changes in the electrostatic interactions surrounding ^{14}N. Therefore, the quadrupole splitting provides an easy measure of the size of the electrostatic potential on the membrane surface.[14,19–21] Experiments were carried out on aligned POPC bilayers containing peptides to examine if the peptide−lipid interaction alters the electrostatic interaction surrounding ^{14}N. Spectra measured from mechanically aligned POPC bilayers containing antimicrobial peptides (LL-37 and MSI-78) and channel-forming peptides (gramicidin and GABA$_A$-TM2) are given in Figure 5. Since the signal from an amide-^{14}N of a peptide is too broad (~MHz), it does not appear in the ^{14}N spectra of bilayers containing peptides as presented in Figure 5. Incorporation of LL-37 and MSI-78 considerably decreases the ^{14}N quadrupole splitting of POPC bilayers. On the other hand, there is no measurable change in the observed ^{14}N quadrupole splitting for bilayers containing the channel-forming gramicidin (Figure 5C) and GABA$_A$-TM2 (Figure 5B) peptides. These results are discussed in the next section.

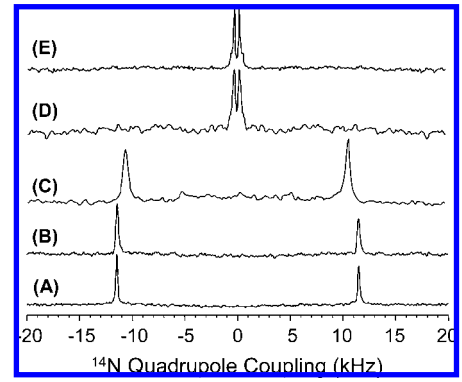

Figure 5. Nitrogen-14 NMR spectra of mechanically aligned POPC bilayers at room temperature: pure lipids (A) and bilayers containing 3 mol % GABA$_A$-TM2 (B), gramicidin (C), MSI-78 (D), and LL-37 (E).

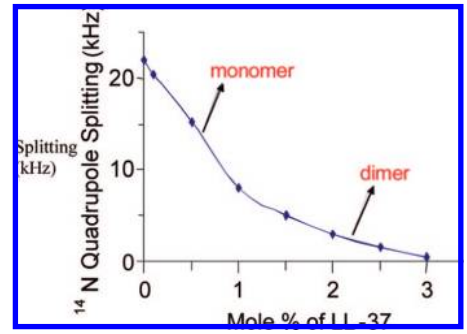

Figure 6. Nitrogen-14 quadrupole splitting measured from mechanically aligned POPC bilayers containing LL-37 at room temperature.

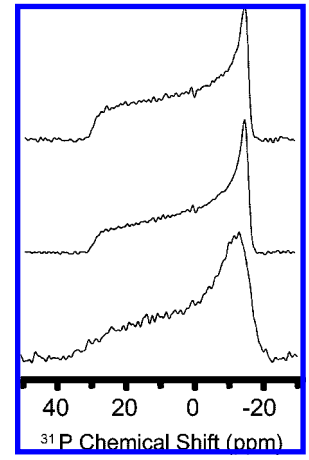

Figure 7. Phosphorus-31 chemical shift spectra of unaligned multilamellar vesicles: pure POPC (top), 1 mol % LL-37 (middle), and 5 mol % LL-37 (bottom) at room temperature.

Effect of LL-37 Concentration on ^{14}N Quadrupole Splitting of POPC Lipid Bilayers. Quadrupole splitting was measured from POPC bilayers containing different concentrations of LL-37 to understand the effect of increasing surface potential; the results are given in Figure 6. As mentioned in the Experimental Section, ^{31}P chemical shift spectra of MLVs containing LL-37 were also obtained (Figure 7) to test the peptide-induced changes in the lipid bilayer structure. These spectra suggest that the lipids

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in the presence of the peptide still remain in the fluid liquid crystalline lamellar phase and no fragmentation of lipid bilayers or nonlamellar phase lipids was observed. The increased reduction in the quadrupole coupling with the peptide concentration is because at a higher peptide concentration more lipids interact with the peptide. The reduction in the quadrupole splitting predicts a peptide-induced change in the size of the electrostatic potential on the lipid bilayer surface. The extent of reduction at different concentrations of a peptide depends on the structure, dynamics, and folding of the peptide, and also on the peptide−lipid and peptide−peptide interactions. For example, the dependence of the observed quadrupole coupling on the concentration of LL-37 is not linear, while an antidepressant, such as desipramine, induced a linear change.[19] The data in Figure 6 suggest that the variations for 0 to 1 and 1 to 3 mol % of LL-37 are linear but significantly differ in slopes. This observation could be attributed to the oligomerization of LL-37 in lipid bilayers as discussed in the next section.

Discussion

High natural abundance of ^{14}N and high sensitivity of ^{14}N quadrupole coupling of POPC lipid to electrostatic potential are the main advantages of fundamental ^{14}N solid-state NMR spectroscopy. The location of ^{14}N nuclei in the lipid is such that it can be a useful probe to measure the dynamics of a lipid headgroup and also changes in the electrostatic potential near the lipid bialyer surface. Studies have shown that the information obtained from ^{14}N NMR are complementary to ^{31}P NMR of lipids.[14,15,19–21] Several previous studies have demonstrated the advantages of ^{14}N of phosphocholine containing model membranes to understand the lipid−ligand interactions.[14–17,19–21] Both static experiments on aligned lipid bilayers and MAS experiments on MLVs have been reported.[19–21] In this study, we have demonstrated the significance of ^{14}N NMR to determine the membrane orientation of peptides and also to understand the oligomerization of a cationic human antimicrobial peptide, LL-37.

Nitrogen-14 NMR Reveals the Membrane Orientation of Peptides. Four different membrane-associated peptides were used to test the applicability of ^{14}N experiments to determine the membrane orientation of peptides (Figure 2). A 37-residue human LL-37 (net charge = +7)[30] and a 22-residue MSI-78 (net charge = +9)[31] are cationic, amphipathic, helical antimicrobial peptides. Previous NMR studies have shown that these peptides are oriented on the surface of POPC bilayers and interact with the lipid headgroup.[32–36] Gramicidin[28,29] and the second transmembrane segment of the $GABA_A$ receptor ($GABA_A$-TM2)[16] are channel-forming transmembrane peptides. The 15-residue gramicidin forms a head-on β-helical dimer that has a transmembrane orientation and conducts K^+ ions across the membrane.[28] The 24-residue $GABA_A$-TM2 is a channel-forming transmembrane α-helical peptide.[16] In pure lipid bilayers, the electrostatic interaction between N^+ of one lipid's choline moiety and the O^- of a nearby lipid's phosphate group cross-links lipids. Since LL-37 (+9) and MSI-78 (+5) are highly cationic and oriented with their helical axes nearly parallel to the bilayer surface, the positively charged residues electrostatically interact with the lipid phosphate group. Such a peptide−lipid interaction breaks the cross-linkage of lipids, which increases the tetrahedral symmetry around ^{14}N. This results in a reduced electric field gradient surrounding the ^{14}N nucleus in POPC and therefore a decrease in the observed quadrupole splitting (Figure 5D and 5E). On the other hand, a transmembrane peptide does not significantly alter the electrostatic interaction around the ^{14}N nucleus, and therefore, within the concentration ranges used in this study, there is no measurable change in the quadrupole splitting for bilayers containing gramicidin (Figure 5C) and $GABA_A$-TM2 (Figure 5B) peptides. The observation for MSI-78 confirms the prediction of a recent molecular dynamics simulations study that showed a hydrogen bonding interaction between the lysine residue of MSI-78 and the O^- in the phosphate headgroup of POPC.[11] The change in the electrostatic interactions around ^{31}P nuclei, due to the peptide-induced breakdown of lipid−lipid cross-linking, does not influence the ^{31}P CSA (Figure 7). This effect is compensated by the presence of a hydrogen bond or electrostatic interactions between the peptide and the phosphate group, and therefore there is no drastic change in the ^{31}P CSA (Figure 7).

Solid-state NMR spectroscopy has been a powerful tool to determine the topology of membrane-associated proteins and peptides.[38–49] Typically, NMR spectra of aligned model membranes, such as mechanically aligned lipid bilayers[34,38] or magnetically aligned bicelles,[50] containing a protein or peptide labeled with an isotope (like ^{15}N, ^{13}C, ^{2}H or a combination of these isotopes) are used to determine the membrane orientation. High-resolution structural topologies of membrane proteins have been determined using multidimensional solid-state NMR techniques. These studies have provided powerful insights into the function of membrane-associated molecules. While these sophisticated techniques have tremendous advantages, they require isotopically labeled samples and significant spectrometer time which are relatively expensive. On the other hand, our results presented in this paper suggest that ^{14}N NMR of lipid bilayers containing a phosphocholine lipid, such as POPC or DMPC, is useful to determine the orientation of cationic peptides

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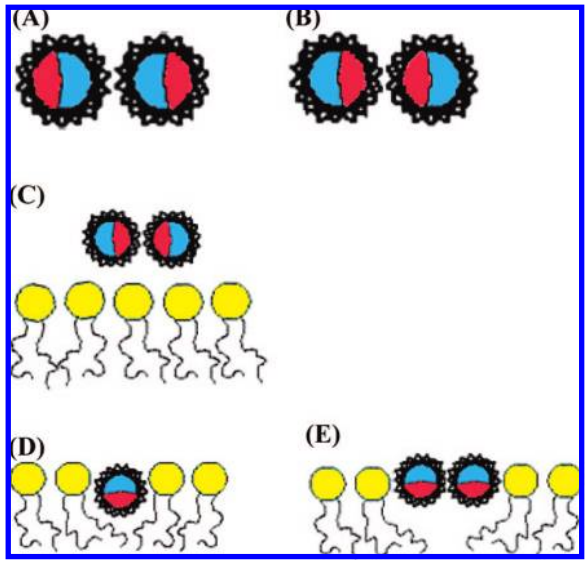

Figure 8. Schematic representations of models of LL-37 in the hydrophobic/hydrophilic interface at the surface of the lipid bilayer: hydrophilic interaction (A) and hydrophobic interaction (B) between peptides; hydrophilic and hydrophobic faces of the peptide are represented by blue and red colors, respectively; (C) a dimer formed through hydrophobic interaction between peptides in a lipid bilayer; monomer (D) and dimer (E) locations in lipid bilayers consistent with NMR data. Helical wheels are used to represent the amphipathic peptide with the hydrophobic face shaded. Only a monolayer of the lipid bilayer is shown.

and possible the cationic structural domains of membrane proteins as well. While this is the first study that report on the membrane orientation of peptides using ^{14}N NMR, application of this technique could be limited to charged molecules and it will be challenging to apply this technique to a complex membrane protein. Nevertheless the main advantages of ^{14}N NMR approach are the high natural abundance, high sensitivity of ^{14}N quadrupole splitting to electrostatic potential on the membrane surface, and samples are inexpensive. Therefore, this approach will be valuable in studying the biochemical and biophysical aspects of membrane association of charged molecules.

Nitrogen-14 reveals the oligomerization of LL-37. Previous studies reported that LL-37 is helical peptide, oriented near the surface of the lipid bilayers, and forms oligomeric structures in lipid bilayers. Since ^{14}N quadrupole splitting is highly sensitive to changes in the electrostatic potential near the phosphocholine lipid headgroup, in this study, we explored the possibility of using ^{14}N NMR to understand the oligomerization of cationic LL-37 in POPC bilayers. An oligomer of amphipathic, helical LL-37 could mainly result from two different types of peptide−peptide interactions: 1) hydrophilic-hydrophilic (Figure 8A) or 2) hydrophobic-hydrophobic (Figure 8B) sides of the helices. In case 1, the resultant LL-37-oligomer would have a reduced number of charged residues exposed to lipids and therefore may slightly alter the surface potential of the lipid bilayer. In case 2, the oligomer would have a large charged exterior surface, which would significantly increase the positive surface potential of the lipid bilayer. Therefore, one would observe small (in case 1) and large (in case 2) reductions in the ^{14}N quadrupole splitting. Based on the observed quadrupole splitting values (Figure 6), case 1 can be ruled out for LL-37; also, case 1 should result in significant insertion of the oligomer in the hydrophobic core of the lipid bilayer which was not observed in our previous ^{15}N NMR study.[32] While case 1 is not possible and the oligomerization occurs through hydrophobic-hydrophobic interaction between LL-37 helices, there are several possible models for case 2 as discussed below.

In case 2, the oligomer with a lot of charged residues exposed outside the helical bundle could significantly alter the bilayer structure or result in the formation of a nonlamellar phase such as inverted hexagonal/cubic phases or reverse micelles. ^{31}P NMR spectra in Figure 7 suggest that the peptide neither induces nonlamellar lipid structures nor fragments lipid bilayers. In another possibility, the oligomers of LL-37 can be immersed in the water phase without much interaction with bilayers (Figure 8C). But this situation cannot explain the observed decrease in the ^{14}N quadrupole splitting, which obviously results from the peptide−lipid interactions. In addition, the previous solid-state NMR study using ^{15}N-labeled LL-37[35] and ^{2}H-labeled lipids[32] predicted the location of the peptide in lipid bilayers (Figure 8D). Therefore, a model that could explain the observed reduction in the ^{14}N quadrupole splitting in Figure 6 is the membrane-mediated oligomerization of LL-37 (Figure 8E). In this model, the oligomers are stabilized partly by hydrophobic−hydrophobic and hydrophilic−hydrophilic interaction between helices and partly by the interaction of remaining parts of the helices with the lipid bilayer (Figure 8E). In this model, the hydrophilic surface area of the helix that interacts with lipid headgroups is decreased due to peptide−peptide interaction as shown in Figure 8E. This model can be used to explain our data. However, depending on the experimental conditions, a combination of these models (particularly the ones shown in Figure 8C−E) could also be used to explain the experimental data. While applying such a combination, dynamical exchanges between these models and an appropriate population ratio need to be considered for accurate results.

The linear decrease in the observed quadrupole coupling up to 1 mol % of LL-37 indicates the monomeric nature of the peptide as the number of positive charges on the lipid bilayer surface increases with the peptide concentration. On the other hand, the formation of an oligomer (Figure 8E) would reduce the number of positive charges from each peptide that influence the electrostatic potential around ^{14}N nuclei. This suggests that the formation of oligomers decreases the quadrupole splitting with a slower slope than the monomers. Therefore, above 1 mol % of LL-37, in addition to the existence of monomers, the formation of oligomers (possibly a dimer) could explain the decrease in the slope of the variation of ^{14}N quadrupole splitting in Figure 6. While these results are highly valuable and difficult to obtain using other approaches, further studies are required to completely determine the high-resolution oligomeric 3D structure of LL-37. Nevertheless, these results suggest that it is possible to measure the oligomerization process from the observed changes in the ^{14}N quadrupole splitting of phosphocholine lipid bilayers. While prior knowledge on the secondary structure and charge distribution for the peptide in a membrane environment would be helpful in applying this technique, we believe that this approach is likely to be useful even when peptides are present in higher oligomeric structures such as trimers and tetramers.

Conclusions

In this study we have demonstrated the capabilities of a simple 1D ^{14}N solid-state NMR experiment to study the lipid−peptide and peptide−peptide interactions in phosphocholine lipid bilayers at ambient temperatures. Data presented in this paper suggest that the membrane orientation of peptides can be

determined. In addition, it is inferred that the oligomerization process for a cationic peptide can be investigated by measuring the ^{14}N quadrupole splitting as a function of the concentration of the peptide. While POPC lipid bilayers were used in this study, these experiments can also be extended to other choline-containing lipids such as DMPC. Therefore, this methodology will find applications in the study of antimicrobial, toxic, fusogenic, amyloidogenic, and channel-forming membrane-associated peptides. We believe that the use of ^{14}N static NMR experiments on aligned samples at a lower temperature will be useful to measure residual ^{14}N−X (where X could be ^{31}P, ^{13}C, ^{2}H, ^{19}F, or ^{15}N) dipolar couplings between the lipid (POPC or DMPC) and other molecules like peptides, proteins, or drugs. ^{14}N MAS experiments to measure the electrostatic potential near the lipid headgroup regions and REDOR (rotational echo double resonance)[51] experiments to measure the intermolecular dipolar couplings from MLVs at a lower temperature would also be valuable in studying the oligomerization of membrane-associated peptides.[52–55]

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