Measurement of the Membrane Dipole Electric Field in DMPC Vesicles Using Vibrational Shifts of p-Cyanophenylalanine and Molecular Dynamics Simulations

Rebika Shrestha, Alfredo E. Cardenas, Ron Elber, and Lauren J. Webb

ABSTRACT: The magnitude of the membrane dipole field was measured using vibrational Stark effect (VSE) shifts of nitrile oscillators placed on the unnatural amino acid p-cyanophenylalanine (p-CN-Phe) added to a peptide sequence at four unique positions. These peptides, which were based on a repeating alanine-leucine motif, intercalated into small unilamellar DMPC vesicles which formed an α-helix as confirmed by circular dichroic (CD) spectroscopy. Molecular dynamics simulations of the membrane-intercalated helix containing two of the nitrile probes, one near the headgroup region of the lipid (αLAX(2S)) and one buried in the interior of the bilayer (αLAX(16)), were used to examine the structure of the nitrile with respect to the membrane normal, the assumed direction of the dipole field, by quantifying both a small tilt of the helix in the bilayer and conformational rotation of the p-CN-Phe side chain at steady state. Vibrational absorption energies of the nitrile oscillator at each position showed a systematic blue shift as the nitrile was stepped toward the membrane interior; for several different concentrations of peptide, the absorption energy of the nitrile located in the middle of the bilayer was ∼3 cm⁻¹ greater than that of the nitrile closest to the surface of the membrane. Taken together, the measured VSE shifts and nitrile orientations within the membrane resulted in an absolute magnitude of 8–11 MV/cm for the dipole field, at the high end of the range of possible values that have been accumulated from a variety of indirect measurements. Implications for this are discussed.

INTRODUCTION

The complex electrostatic environment around and within biological lipid bilayer membranes is composed of three distinct potentials distributed heterogeneously throughout the membrane structure: the transmembrane potential ($V_{\text{trans}}$) resulting from differences in ionic strength and/or pH on either side of the membrane; the surface potential ($V_s$) resulting from the charged lipid head groups, ions, and biomolecules aligned at the membrane–water interface; and the dipole potential ($V_d$) resulting from the alignment of molecular dipole moments in lipids and lipid-associated water molecules. Although the dipole potential has long been thought to be significant to membrane structure, organization, and function, it is entirely located within the low dielectric, hydrophobic interior of the membrane bilayer, and thus has been difficult to measure directly. Despite this, several indirect measurement techniques such as ratiometric fluorescence, cryogenic electron microscopy, atomic force microscopy, and ion-transport rates, as well as molecular dynamics simulations, have been used to estimate the magnitude of this field as ranging from 1 to 10 MV/cm, depending on the thickness of the membrane under investigation. This field is thus significantly larger than the field generated from $V_{\text{trans}}$ (∼0.25 MV/cm) or $V_s$ (0.1 MV/cm), and therefore demands direct measurement and manipulation for greater understanding of its origin and function in the living membrane.

In this paper, we report the application of p-cyanophenylalanine (p-CN-Phe) as a vibrational Stark effect (VSE) probe in directly measuring the dipole electric field across a vesicle bilayer, along with molecular dynamics simulations that clearly identify a narrow distribution of orientations of the nitrile with respect to the membrane interior structure. Recently, nitrile-derivatized amino acids have gained popularity as site-specific spectroscopic probes in investigating the structure, dynamics, and function of biological macromolecules including proteins, DNA fragments, and peptides. This has been because of the nitrile oscillator’s sensitivity to its local environment, including solvent polarity, hydrogen bonding, and electrostatic field; its absorption frequency in a clear region of the biomolecular infrared spectrum (∼2200 cm⁻¹); its ease of inserting into biological molecules without significant disruption of structure; and its relative stability in aqueous and membrane environments. Nitriles have recently been used to characterize biomolecular structure and interactions in membranes. For example, Londergan and co-workers have characterized the interaction of a potent synthetic antimicrobial peptide, CM15, with the membrane–water interface by using cyanlated cysteine as a site-specific vibrational reporter of perturbations at the local membrane–peptide interfaces.

Received: November 21, 2014
Revised: January 13, 2015
Published: January 20, 2015

DOI: 10.1021/jpc511677j
and co-workers have successfully shown the sensitivity of the vibrational mode of the nitrile group in S-cyanotryptophan to the extent of hydration around the side chain. The same group has also established two other nitrile-derivatized amino acids, \(\alpha\)-cyanoalanine and \(\beta\)-cyanophenylalanine, as local environment sensors in protein dynamics.\(^{22,23}\) \(p\)-CN-Phe is particularly interesting because it is also a useful fluorescent probe that can be selectively excited in the presence of other aromatic amino acids;\(^{24,25}\) this makes it an attractive probe to study membrane proteins, which are often tryptophan-abundant.\(^{26}\) Blasie and co-workers have taken advantage of both fluorescence and vibrational spectroscopy of \(p\)-CN-Phe to investigate the interaction between the inhalation anesthetic halothane and an anesthetic-binding model membrane protein hbAP-Phe\(_{CN}\).\(^{27}\) Likewise, Tucker and co-workers have also studied the nitrile vibrational stretching of several \(p\)-cyanophenylalanine derivatized mutated mastoparan \(\alpha\), a well studied membrane binding peptide, to determine the hydration state of specific sites of membrane interactive peptides.\(^{28}\)

Earlier work from our laboratory demonstrated the utility of membrane-bound peptides labeled with \(p\)-CN-Phe while using the vibrational Stark effect (VSE) spectroscopy to directly measure the membrane dipole field in the interior of small lipid bicelles (5 and 15 nm in diameter) composed of a long-chain lipid of 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and a short-chain lipid of 1,2-dihexanoyl-sn-glycero-3-phosphocholine (DHPC).\(^{29}\) VSE spectroscopy employs the sensitivity of a vibrational probe toward its local surrounding and correlates the change in IR frequencies (\(\Delta\nu_{\text{obs}}\)) with the local electrostatic field (\(F_{\text{r}}\)):

\[
\Delta E = \hbar \cdot \Delta \nu_{\text{obs}} = -\Delta \mu \cdot F_{\text{r}}
\]

where \(\hbar\) is the Planck constant and \(\nu\) is the speed of light. The extent of the frequency shift depends on the strength and direction of the difference dipole moment, \(\Delta \mu\), also known as the Stark tuning rate of the vibrational probe.\(^{19,20,31}\) This work concluded that the dipole field was \(-6\ \text{MV/cm}\), and decreased rapidly at the membrane–water interface and more slowly at the low dielectric alkyl interior of the bilayer.\(^ {30}\) While this was an exciting first step toward the goal of measuring the magnitude of the dipole field directly, this study suffered from significant limitations. First, while bicelles are a useful model system for small and simple lipid bilayers, it is difficult to increase their chemical diversity by adding, for example, cholesterol, and therefore, they are not a useful model system for measuring the consequences of chemical and structural complexity on a real lipid bilayer system. Second, our understanding of the position and orientation of the nitrile probe within the monolayer was based solely on molecular modeling by matching the hydrophobic length of the energy-minimized structure of the folded \(\alpha\)-helix with the thickness of the DMPC membrane. Uncertainty in this model could contribute significantly to uncertainty in the magnitude and even direction of the measured field.

In the work reported here, we apply VSE spectroscopy of a nitrile probe carried by the unnatural amino acid \(p\)-CN-Phe for a direct measurement of dipole electric field spanning from the membrane–water interface into the middle of a bilayer in unilamellar vesicles. Furthermore, we quantify the structure of the transmembrane peptide and the orientational distribution of the nitrile probe with respect to the plane of the membrane through extensive molecular dynamics sampling. We exposed nitrile infrared probes located at four different positions along the axis normal to the bilayer, beginning from the headgroup region to the membrane interior, by inserting four \(\alpha\)-helical peptides containing \(p\)-CN-Phe. The polypeptide sequences of these peptides are given in Table 1. This transmembrane helix is known to have stable secondary structure \textit{in vivo} and through molecular dynamics simulation.\(^{30,32,33}\) The GGPQ sequence anchors the peptide at the lipid headgroup region and ensures its orientation is approximately parallel to the bilayer normal, and the two histidines at the ends increase the peptide’s solubility in aqueous buffer. The positions of the nitrile probes were chosen in correspondence with the depth of a bilayer moving from the C- to N-terminus of the peptide such that \(\alpha\)LAX(25) probes the lipid–water interface, \(\alpha\)LAX(16) probes the hydrophobic interior, and \(\alpha\)LAX(23) and \(\alpha\)LAX(21) are intermediate between these two extremes. Circular dichroic (CD) spectroscopy was used to verify the helical structure of the transmembrane peptides inside the bilayer. Finally, molecular dynamics simulations were used to determine the orientation of the helical axis and the orientation of the nitrile on \(p\)-CN-Phe with respect to the plane of the membrane. These simulations indeed showed that the slight hydrophobic mismatch between the hydrophobic helix length and hydrophobic bilayer thickness was compensated for by tilting of the helix by approximately 21–25°. In addition, these simulations clearly showed that the two most distant probes, \(\alpha\)LAX(25) and \(\alpha\)LAX(16), respond differently to their different local electrostatic environments, resulting in different average angles between the nitrile bond and membrane normal and different distributions of observed stable structures. This was used to explain the observation that the full width at half-maximum (fwhm) of the nitrile-stretching band decreased as the infrared probe was systematically moved from the heterogeneous membrane–water interface to the more homogeneous hydrophobic membrane interior. Despite variations in physical orientation of probes at different locations, we measured the magnitude of the dipole field to be 8–11 MV/cm, near the 1–10 MV/cm range that has long been estimated. The results of this work clearly generalize our findings about the membrane dipole potential to more complex and robust biological membranes.

### Table 1. Amino Acid Sequences of the Four Polypeptides Described in This Work

<table>
<thead>
<tr>
<th>peptide</th>
<th>sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\alpha)LAX(25)</td>
<td>HHHGPGGLALALALALALALAXGPGGHH</td>
</tr>
<tr>
<td>(\alpha)LAX(23)</td>
<td>HHHGPGGLALALALALALALAXGPGGHH</td>
</tr>
<tr>
<td>(\alpha)LAX(21)</td>
<td>HHHGPGGLALALALALALALALAXGPGGHH</td>
</tr>
<tr>
<td>(\alpha)LAX(16)</td>
<td>HHHGPGGLALALALALXALALALALGPGGHH</td>
</tr>
</tbody>
</table>

\(X = p\)-CN-Phe.

### MATERIALS AND METHODS

#### Preparation and Characterization of Vesicles.

1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC) powder was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL) and was used as received. Heps, Na\(\text{NO}_3\), and \(\text{D}_2\)O were purchased from Sigma-Aldrich (St. Louis, MO). \(\text{PrCl}_3, \text{H}_2\)O was purchased from Alfa Aesar (Ward Hill, MA) and used in \(\text{\textsuperscript{31P}}\) NMR spectroscopy to check for lamellarity. HPLC grade water and chloroform were purchased from Fisher Scientific (Pittsburgh, PA). All peptides were synthesized using standard Fmoc solid-state peptide synthesis and obtained from Abgent.
Technologies (San Diego, CA) and InnoPep (San Diego, CA). Vesicles were prepared by sonication as described previously. Briefly, approximately 30 mg of DMPC powder and 2.0 mg (0.5 mM) or 4.0 mg (1 mM) of peptide were dissolved in approximately 1 mL of chloroform, dried under a vacuum for at least 2 h and then placed in a N\(_2\) (g)-purged glovebox overnight. The dried sample was then hydrated in 1.5 mL of 10 mM Hepes buffer with 0.02% (w/v) NaN\(_3\) pH 7.2, mixed through vortexing, and sonicated until a clear suspension was achieved. The sample was further centrifuged to remove any residue and then stored above 23 °C (transition temperature of DMPC) for further usage. A visual check for precipitation was done before experiments to ensure stability and solubility of vesicles. All experiments were performed at 27 °C or higher temperature.

Circular Dichroic (CD) Spectroscopy. To collect CD spectra, vesicle solutions had to be further diluted to avoid saturating the detector. 1 and 0.5 mM solutions were diluted by factors of 1/10 and 1/5, respectively, resulting in solutions that were 0.1 mM overall in peptide. CD spectra were collected in both buffer and TFE solvent. The initial concentrations of 1 and 0.5 mM corresponded to 900 and 450 peptides per vesicle, respectively. CD spectra were recorded using a 1 mm path length quartz cell on a Jasco J-815 CD spectrometer over the wavelength range 190–250 nm at 0.2 nm resolution, a scanning rate of 50 nm/min, and a response time of 4 s. The spectra were background subtracted using 10 mM Hepes buffer as the background with Spectra Manager for Windows 95/NT Spectra Analysis software.

VSE Spectroscopy. Fourier transform infrared (FTIR) measurements of the nitrile absorption energy were recorded at room temperature in a sample cell comprised of two sapphire windows separated by 125 \(\mu\)m thick PETE spacers in a Bruker Vertex 70 FTIR instrument. The sample cell was illuminated with light in the range 2000–2500 cm\(^{-1}\) selected by a broad bandpass filter (Spectrogon, Parsippany, NJ) placed in front of the instrument’s IR source. Spectra were collected with a liquid-nitrogen-cooled indium antimide (InSb) detector and were composed of 3000 scans at 2.0 cm\(^{-1}\) spectral resolution. Background-subtracted spectra were fitted to a single Gaussian line shape with a custom least-squares fitting program to determine the peak center, \(\nu_{\text{obs}}\), and the full width at half-maximum (fwhm). Uncertainty in absorption energy is reported as the standard deviation of at least three measurements.

Molecular Dynamics Simulations. We performed MD simulations of two of the helical systems probed in the experiments, \(\alpha\)LAX(16) and \(\alpha\)LAX(25) (i.e., the inner- and outermost nitrile positions), in a bilayer of DMPC molecules. All simulations were performed with the program MOIL\(^{39,40}\) using a combination of the united-atom Berger force field\(^{46}\) for the acyl chain atoms of the lipid molecules and the OPLS force field\(^{42}\) for their headgroup region and the peptide atoms. The CHARMM-GUI membrane builder facility\(^{43,44}\) was used to set up the initial configurations of the membrane-intercalated peptide system. The simulated bilayer membrane contained 128 DMPC molecules (64 in each leaflet), 5630 TIP3P water molecules,\(^{45}\) and one of the two simulated helical peptides.

![CD spectra](https://example.com/cdspectra.png)

**Figure 1.** Representative CD spectra of 1 mM \(\alpha\)LAX(25), \(\alpha\)LAX(23), \(\alpha\)LAX(21), and \(\alpha\)LAX(16) dissolved in TFE (black) and intercalated in DMPC vesicles (blue). Spectral intensity is presented in units of mdeg.
truncation errors for the estimate of the electric field at the position of C
in the nitrile probe. For the calculation of the electric field, we placed a unit charge at the position of Cα in the nitrile probe and use Coulomb’s law with a larger cutoff of 18 Å to minimize truncation errors for the estimate of the electric field.30

RESULTS AND DISCUSSION

Peptide Secondary Structure. Figure 1 shows representative CD spectra for all four peptides incorporated inside vesicles (in blue) and, as a control, when dissolved in hydrophobic TFE solvent (in black). Because the hydrophobic amino acids leucine and alanine comprise most of the core of the amino acid sequence, these polypeptides were extremely hydrophobic and were insoluble in buffer. They dissolved only in hydrophobic solvents such as TFE or when incorporated into the lipid mixture. When dissolved in TFE, peptides showed strong α-helical characteristics with two minima at 208 and 222 nm and a high maximum at 195 nm. CD spectra were nearly superimposable for all peptide sequences and conditions. This confirmed that changing the position of the unnatural amino acid along the polypeptide chain did not appear to have any effect on the secondary structure of the helix. The CD spectra of the peptides intercalated inside DMPC vesicles, shown in blue in Figure 1, also exhibit signatures of helicity including minima at 208 and 222 nm, although with decreases in the magnitude of the spectral intensity compared to the control solutions, likely caused by the increased turbidity of the vesicle solution.31 The spectra in Figure 1 demonstrate that these peptide sequences exhibit strong α-helical characteristics when intercalated into our DMPC vesicles, as expected.

Peptide Tilt and Orientation. CD spectra, while clearly confirming a stable helical secondary structure for each peptide studied here, do not provide any information on the position or orientation of that helix within the membrane. We therefore performed extensive molecular dynamics simulations on the two peptides containing the outer- and innermost placed nitrile groups, αLAX(25) and αLAX(16), respectively, embedded in a slab of DMPC lipids representing the bilayer. Figure 2 shows representative snapshots of both peptides surrounded by DMPC lipids and explicit water molecules.

The α-helical secondary structure of both of the transmembrane peptides remained intact for the entire simulation, 100 ns for αLAX(16) and 200 ns for αLAX(25), as expected from the steady-state CD spectroscopy. Simulations were initiated with both peptides inserted vertically into the bilayer (i.e., helical axis parallel to the bilayer normal), but in both cases, a slight tilting of the helical backbone of the peptide was soon observed in the trajectories. When each peptide studied here folds into its energy minimized helical structure, the length of the hydrophobic repeating LA motif is 28.5 Å.30 This is longer than the hydrophobic thickness of a DMPC bilayer, ~26 Å, based on the length of adjacent hydrocarbon tails.33,54 This hydrophobic mismatch between the lipid’s hydrophobic bilayer thickness and the hydrophobic stretch of the helix predicts that the helix will tilt within the membrane to ensure stable transmembrane insertion and avoid distortion of the lipid...
bender but does introduce error on the orientation of the nitrile bond vector within the membrane. Because VSE spectroscopy measures the projection of the electric field vector on the nitrile bond axis, this in turn introduces uncertainty into the meaning of vibrational energy measurements and interpreting the magnitude of electric fields that they imply. It is possible to estimate a tilt angle of 24.1° by simply minimizing the difference in hydrophobic length of the peptide versus the membrane. However, this would not provide any information on the range of stable conformations that would be expected during a steady-state experiment. To address this issue, MD simulations were used to quantify the magnitude of this tilt by measuring the angle of the helical axis (defined by a line through the center of the peptide backbone stretching from Cα of histidine residues at both termini of the peptide) with respect to the membrane normal for every snapshot collected in our 100–200 ns simulations. Figure 3 shows the normalized histogram of the tilt angle distribution of transmembrane helical peptides, αLAX(25) (black) and αLAX(16) (red), with the normal of a lipid bilayer composed of 128 DMPC lipid molecules computed by molecular dynamics simulation.

![Figure 3. Normalized histogram of tilt angle distribution of transmembrane helical peptides, αLAX(25) (black) and αLAX(16) (red), with the normal of a lipid bilayer composed of 128 DMPC lipid molecules computed by molecular dynamics simulation.](image)

Along with perturbations in tilt angle, the position of the nitrile probe along the bilayer normal was also affected by rotation of the side chain about the χ₁ torsion angle (defined by N–Cα–Cβ–Cγ), and therefore fluctuated greatly. Because the angle of the nitrile with respect to the membrane normal is the relevant physical quantity in determining the electric field measured through VSE, we also determined this angle for every snapshot of our simulations. Figure 4 shows the normalized histogram of the angle the nitrile bond makes with respect to the membrane normal for both probe locations, αLAX(25) (black) and αLAX(16) (red). Unlike the helix tilt angle shown in Figure 3, these nitrile angle distribution plots are bimodal and show a heterogeneous population of at least two stable conformations. To determine the cause of this observation, we investigated individual representative snapshots of each peptide. In the case of αLAX(25), the vibrational probe remained fixed at an angle of ~67° for the first 50 ns of the simulation, and then abruptly switched its position to 122° and stayed there for the rest of the 150 ns simulation period. Upon further inspection, structures observed during the first 50 ns of the simulation appeared to be perturbed, with the hydrophobic CN-Phe side chain pointed toward the membrane–water interface interacting closely with the zwitterionic headgroup of the lipid and TIP3P water. When the angle switched to 122°, the CN-Phe side chain was now completely immersed in the hydrophobic membrane interior, and remained within a small range of angles around this value. We therefore used the equilibrated conformations from the last 150 ns of simulation data to estimate the nitrile angle. This gave a value of 122 ± 15°. In the case of αLAX(16), the nitrile molecule remained relatively stable at 110° with occasional perturbations to angles as large as 140°, i.e., approaching an orientation perpendicular to the plane of the membrane. These observations are consistent with the different environments in which the nitriles are placed by virtue of their different amino acid sequences. Because of its position near the center of the lipid bilayer, the nitrile probe on αLAX(16) was located in a low dielectric hydrocarbon membrane interior and experienced essentially identical intermolecular interactions no matter how it was oriented about the helical axis. This resulted in a nitrite orientation that remained relatively fixed at 110° with occasional excursions to other stable conformations. We determined an average nitrite angle of 112 ± 23° for the nitrite probe on the peptide αLAX(16). The larger distribution of observed angles at this position was due to the more homogeneous hydrophobic environment found at the core of the lipid bilayer membrane, with a smaller penalty for orientation sampling of the CN-Phe side chain around the χ₁ torsion angle.

![Figure 4. Normalized histogram of the angle between the nitrile probe on transmembrane peptide αLAX(25) (black) and αLAX(16) (red) and the normal of the bilayer composed of 128 DMPC lipid molecules computed by molecular dynamics simulation. The computed angle is the angle between the nitrile bond and the membrane axis.](image)

DOI: 10.1021/jp511677j

Dipole Field. Figure 5 shows representative normalized vibrational absorption spectra of the nitrile probes on peptides, dissolved in water (2237.5 cm$^{-1}$ and 1 mM Peptides Intercalated into DMPC Vesicles Measured data points are shown by “×”).

![Normalized Absorbance vs. Absorption Energy](image)

Figure 5. Normalized representative FTIR spectra of 0.5 mM peptides αLAX(25) (black), αLAX(23) (blue), αLAX(21) (green), and αLAX(16) (red) inserted in vesicles composed of 30 mM DMPC. Measured data points are shown by “×”.

αLAX(25) (black), αLAX(23) (blue), αLAX(21) (green), and αLAX(16) (red), in DMPC vesicles at 0.5 mM concentration. In Table 2, we present the average mean peak frequencies and full width at half-maximum (fwhm) of each of the nitrile stretching bands at both peptide concentrations, 0.5 and 1 mM, along with their associated standard deviation from at least three experimental replicates. The mean peak frequencies for the absorption bands of nitrile in all four peptides are lower than the absorption frequency measured for p-CN-Phe dissolved in water (2237.5 cm$^{-1}$).23 The red shift exhibited by all positions of the nitrile probe in a lipid bilayer environment suggests that, no matter where in the sequence the probe is placed, intercalation of the helix into the membrane places each nitrile probe into an environment significantly less hydrophilic than water. This is clear evidence that both the molecular model and the MD sampling are accurately describing the position of the nitrile within the bilayer interior.

In Table 2, we see that for both concentrations of peptides, the absorption energy increased as the oscillator was systematically moved from position 25, near the hydrophilic zwitterionic headgroups, to position 16, located in the hydrophobic hydrocarbon tail region of the bilayer. The nitrile was exposed to two significantly different electrostatic environments at these positions, and displayed a Stark shift of +2.9 and +2.6 cm$^{-1}$ upon moving into the bilayer interior for samples containing 0.5 and 1 mM peptide, respectively. With these measured values of $\Delta\nu_{\text{obs}}$ (0.67 cm$^{-1}$/(MV/cm)) for the Stark tuning rate of nitrile, and an angle of 122 ± 15° between the nitrile bond and bilayer normal computed for αLAX(25) in eq 1, we calculate the magnitude of the dipole electric field to be 8.1 ± 4 MV/cm for 0.5 mM peptide and 7.3 ± 3 MV/cm for 1 mM peptide. We also calculate the absolute field values based on αLAX(16)'s nitrile-membrane normal angle of 112 ± 23°, and the results are 11.4 ± 11 and 10.3 ± 10 MV/cm for 0.5 and 1 mM peptide, respectively, essentially within error of the value calculated at the larger angle. The magnitudes of the calculated dipole field based on the distribution of orientations simulated for both peptides fall within the error range of each other and are larger than the dipole field value of ~6 MV/cm determined previously for lipid bicelles.30 The absolute error of ~3 and ~11 MV/cm is a direct consequence of the large standard deviation associated with the angle between the nitrile probe and bilayer normal, not with uncertainty in the vibrational absorption energy of the nitrile.

In a steady-state FTIR experiment such as those described here, the line width of the absorption peak can be used to assess the homogeneity of the local molecular environment surrounding around an oscillator.15,16,30 For example, Gai and co-workers16 have shown that the nitrile stretching vibration in p-CN-Phe has a wider spectrum in polar solvents like water compared to apolar solvents like THF because of a greater number of microenvironments around the nitrile as it forms hydrogen bonds with water. The data in Table 2 clearly show that the fwhm of nitrile's vibrational peak systematically decreased as the probe was moved deeper into the membrane (i.e., from αLAX(25) to αLAX(16)) by about 1–1.5 cm$^{-1}$. This translocation moves the nitrile probe from the heterogeneous lipid–solution interface containing water, buffer ions, the zwitterionic headgroup, and the acyl linkage to the fatty acid chain, all near position 25, into a significantly more homogeneous environment dominated by the hydrocarbon tail of the lipid at position 16. To investigate the role of the electric field in this result, we calculated the absolute value of the electric field experienced by the Cα of CN-Phe on αLAX(25) and αLAX(16) for every snapshot in our MD trajectories. The normalized distributions of fields calculated on both positions are shown in Figure 6, where the distributions for αLAX(25) and αLAX(16) are shown in black and red, respectively. When the nitrile is near the middle of the membrane, small changes in the structure of the system do not result in large differences in the immediate chemical environment around the nitrile, since it is fully immersed in the middle of the bilayer. At position 16, even if the nitrile experiences large conformational changes relative to the helical backbone, because it is in the interior of the membrane, it will experience a

<table>
<thead>
<tr>
<th>peptide</th>
<th>0.5 mM peptide</th>
<th>1 mM peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\nu_{\text{obs}}$</td>
<td>fwhm</td>
</tr>
<tr>
<td>αLAX(25)</td>
<td>2228.18 ± 0.07</td>
<td>7.9 ± 0.1</td>
</tr>
<tr>
<td>αLAX(23)</td>
<td>2228.51 ± 0.04</td>
<td>7.8 ± 0.4</td>
</tr>
<tr>
<td>αLAX(21)</td>
<td>2229.86 ± 0.06</td>
<td>7.5 ± 0.1</td>
</tr>
<tr>
<td>αLAX(16)</td>
<td>2231.05 ± 0.02</td>
<td>6.2 ± 0.1</td>
</tr>
</tbody>
</table>

Table 2. Average Mean Peak Frequencies ($\nu_{\text{obs}}$) and Full Width at Half-Maximum (fwhm) of the Nitrile Stretching Band for 0.5 and 1 mM Peptides Intercalated into DMPC Vesicles.
constant hydrophobic hydrocarbon environment. This chemical homogeneity of the functional groups around the probe results in a tighter distribution of electric fields, seen clearly in Figure 6 and inferred from the small fwhm in Table 2. However, when the nitrile is near the lipid headgroup, it is in a heterogeneous chemical environment where small changes in the position of the nitrile can make large changes in its nearest chemical neighbors, including the lipid tail region, the headgroup, or water and ions in the near-surface region. This heterogeneous chemical environment near the lipid–solvent interface means that even small changes in orientation can place the nitrile in dramatically different environments. This in turn leads to a wider distribution of electric fields experienced by the nitrile in the course of a steady state experiment, as calculated in Figure 6 and seen clearly in the vibrational absorption spectrum described in Table 2. While this intuitive result is not surprising, the close connection of the measured fwhm to calculated electrostatic fields from the MD simulations demonstrates the importance of good, long-time-scale MD simulations in interpreting spectral data of complex and heterogeneous biological systems.

In conclusion, we have demonstrated the use of VSE spectroscopy on site-specific nitrile chromophores coupled with molecular dynamic simulations in a model vesicle membrane to directly measure the dipole electrostatic field contained within the low dielectric interior of the bilayer. Previous VSE spectroscopy of nitriles introduced into model bicontinuous membranes assumed an orientation of the nitrile chromophore from simplistic models and lacked details on the distribution of orientations the nitrile might assume within the bilayer. We have addressed these shortcomings by using extensive molecular dynamic sampling to determine the distribution of angles the nitrile probe assumes in our experiments both from the tilt of the helix in the bilayer and the torsional rotation of the CN-Phe side chain. While our experimental results agree with previous measurements in bicelles, as well as other measurements in the literature, we now have included a range of values for the magnitude of the dipole field. Vesicles are a more chemically diverse and robust model membrane system than bicelles, and we have demonstrated a simple spectroscopic methodology to probe the membrane electrostatic environment while maintaining the membrane integrity. Ongoing work in our laboratory is investigating the molecular origin of the dipole field and its role in membrane organization and function.

AUTHOR INFORMATION

Corresponding Author

*E-mail: lwebb@cm.utexas.edu.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by the Welch Foundation (Grant No. F-1722) and the Burroughs Wellcome Fund (Grant No. 1007207.01) to L.J.W. Support from the Welch Foundation (F-1785) and the NIH (GM059796) to R.E. is gratefully acknowledged.

REFERENCES
