Published in final edited form as:

J Phys Chem B. 2015 February 19; 119(7): 2869–2876. doi:10.1021/jp511677j.

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

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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 head-group region of the lipid (α LAX(25)) and one buried in the interior of the bilayer (α LAX(16)), were used to examine the structure of the nitrile with respect to the membrane normal, the assumed direction 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 towards the membrane interior; for several different concentrations of peptide, the absorption energy of the nitrile located in the middle of the bilayer was $\sim 3\text{ cm}^{-1}$ 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 a value 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.

Keywords

Lipid bilayer; vesicles; vibrational Stark effect spectroscopy; nitrile chromophore; electrostatic fields in membranes

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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_{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.^{1–3} 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.^{1–3} Despite this, several indirect measurement techniques such as ratiometric fluorescence^{4,5}, cryogenic electron microscopy⁶, atomic force microscopy⁷, and ion-transport rates⁸, as well as molecular dynamics simulations^{9–12}, have been used to estimate the magnitude of this field as ranging from 1–10 MV/cm, depending on the thickness of the membrane under investigation. This field is thus significantly larger than the field generated from V_{trans} (~0.25 MV/cm) or V_s (0.1 MV/cm)⁸, 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 structure, dynamics and function of biological macromolecules including proteins, DNA fragments, and peptides.^{13–18} 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.^{13–20} Nitriles have recently been used to characterize biomolecular structure and interactions in membranes. For example, Londergan and coworkers have characterized the interaction of a potent synthetic antimicrobial peptide, CM15, with the membrane-water interface by using cyanalated cysteine as a site-specific vibrational reporter of perturbations at the local membrane peptide interfaces.²¹ Gai and coworkers have successfully shown the sensitivity of vibrational mode of the nitrile group in 5-cyanotryptophan to the extent of hydration around the side chain.²² The same group has also established other two nitrile-derivatized amino acids, β -cyanoalanine and *p*-cyanophenylalanine as local environment sensors in protein dynamics.^{23,24} *p*-CN-Phe is particularly interesting because it is also a useful fluorescent probe that can be selectively excited in presence of other aromatic amino acids;^{25,26} this makes it an attractive probe to study membrane proteins, which are often tryptophan-abundant.²⁷ Blasie and coworkers 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}.²⁸ Likewise, Tucker and coworkers have also studied the nitrile vibrational stretching of several *p*-cyanophenylalanine derivatized mutated mastoparan x, a well studied membrane binding peptide, to determine the hydration state of specific sites of membrane interactive peptides.²⁹

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).³⁰ VSE spectroscopy employs the sensitivity of a vibrational probe towards its local surrounding and correlates the change in IR frequencies (ν_{obs}) with the local electrostatic field ($\vec{F}_d$)

$$\Delta E = hc\Delta\nu_{obs} = -\Delta\vec{\mu} \bullet \vec{F}_d \quad (1)$$

where h is the Planck's constant and c is the speed of light. The extent of the frequency shift depends on strength and direction of the difference dipole moment, $\Delta\vec{\mu}$, also known as the Stark tuning rate of vibrational probe.^{19–20, 31} This work concluded that the dipole field was -6 MV/cm, and decreased rapidly at the membrane-water interface and more slowly at low dielectric alkyl interior of the bilayer.³⁰ While this was an exciting first step towards 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 it is not a useful model system for measuring the consequences of chemical and structural diversity 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 α -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 head group region to the membrane interior, by inserting four α -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 *in vivo* and through molecular dynamics simulation.^{30, 32–33} The GGPG sequence anchors the peptide at the lipid head group 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 to the depth of a bilayer moving from the C- to N-terminus of the peptide such that α LAX(25) probes the lipid-water interface, α LAX(16) probes the hydrophobic interior, and α LAX(23) and α 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, α LAX(25) and α 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 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.

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. Hepes, NaN₃ and D₂O were purchased from Sigma Aldrich (St. Louis, MO). PrCl₃·6H₂O was purchased from Alfa Aesar (Ward Hill, MA) and used in ³¹P NMR spectroscopy to check for lamellarity. HPLC grade water and chloroform were purchased from Fisher Scientific (Pittsburg, 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.³⁴ Briefly, approximately 30 mg of DMPC powder and 2.0 mg (0.5 mM) or 4.0 mg (1 mM) peptide were dissolved in approximately 1 mL of chloroform, dried under vacuum for at least 2 hours and then placed in a N₂ (g)-purged glove box overnight. The dried sample was then hydrated in 1.5 mL of 10 mM Hepes buffer with 0.02% (w/v) NaN₃ 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 (the transition temperature of DMPC) for further usage. A quick 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. ³¹P NMR spectroscopy, dynamic light scattering and atomic force microscopy were used to characterize the sample as unilamellar vesicles with fairly small diameters of about 60 ± 10 nm (data not shown).^{35–38}

Circular dichroic (CD) Spectroscopy

To collect CD spectra, vesicle solutions had to be further diluted to avoid saturating the detector. 1 mM 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 mM 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 range of 190 – 250 nm wavelength 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 μm thick PETE spacers in a Bruker Vertex 70 FTIR instrument. The sample cell was illuminated with light in the range of 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 antimonide (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, ν_{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 $\alpha\text{LAX}(16)$ and $\alpha\text{LAX}(25)$ (i.e. the inner- and outer-most 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 field⁴¹ for the acyl chain atoms of the lipid molecules and the OPLS force field⁴² for their head group region and the peptide atoms. The CHARMM-GUI membrane builder facility^{43, 44} was used to setup the initial configurations of the membrane-intercalated peptide system. The simulated bilayer membrane contained 128 DMPC molecules (64 in each leaflet), 5630 TIP3P water molecules⁴⁵ and one of the two simulated helical peptides ($\alpha\text{LAX}(16)$ and $\alpha\text{LAX}(25)$). The initial configurations of the peptides were taken from a previous study, in which model α -helices were constructed with Avogadro 1.0, an open-source molecular modeling tool.³⁰ Initially, the peptides were embedded vertically inside the bilayer systems with the helical axis parallel to the normal to the membrane surface. There were 23025 total atoms for the $\alpha\text{LAX}(16)$ simulation and 23022 atoms for the $\alpha\text{LAX}(25)$ simulation. The dimensions of the simulation boxes were $63.4 \times 63.4 \times 80.0 \text{ \AA}^3$. Periodic boundary conditions were imposed in the three spatial directions and the particle mesh Ewald method⁴⁶ was used for long-range electrostatic interactions with a grid of $64 \times 64 \times 80$. The cutoffs for van der Waals interactions and for the real space component of the electrostatics forces were set to 9.8 $\text{\AA}$. In all the simulations we constrained water bond length and angles with a matrix implementation of the SHAKE algorithm.^{47,48} The simulations were done with multiple time steps according to r-RESPA⁴⁹,

with 1 fs time step to integrate the bonding, van der Waals and real-space component of the Ewald sum, and a 4 fs time step to evaluate the reciprocal-space component of the Ewald sum. After 50 ns equilibration period, production runs of 100 ns for α LAX(16) and 200 ns for α LAX(25) were carried out at 298 K and constant volume. Configurations were saved every 1 ps for determination of the distribution of tilt and nitrile probe angles. Convergence of the sampled configurations was assessed by estimating block averages of the tilted angle of the helix vector with respect to the membrane axis. The atomic positions of the C α of H1 and H31 were used to estimate the helix vector. The sampled configurations from the molecular dynamics configurations were also used to compute the electric field at the position of the nitrile probe. For the calculation of the electric field we place a unit charge at the position of C α 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.⁵⁰

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 nm 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.⁵¹ 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 inner-most placed nitrile groups, α LAX(25) and α LAX(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 α LAX(16) and 200 ns for α LAX(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.^{53,54} 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 bilayer, but does introduce error on the orientation of the nitrile bond vector within the membrane.⁵⁵ 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 helical axis (defined by a line through the center of peptide backbone stretching from C α 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 tilt angle of α LAX(25) (black) and α LAX(16) (red), with respect to the bilayer normal calculated over the course of the simulation. Both peptides show distribution of tilt angles at slightly different average values: $21 \pm 7^\circ$ for α LAX(25) and $25 \pm 4^\circ$ for α LAX(16). Although the estimated tilt angle of 24.1° obtained from a simple molecular modeling argument indeed gave a correct value of tilt angle, the MD simulations provide a distribution width (i.e. standard deviation) for this important property.

Along with perturbations in tilt angle, the position of nitrile probe along the bilayer normal was also affected by rotation of the side chain about the χ_1 torsion angle (defined by N-C α -C β -C γ), 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 snap shot 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, α LAX(25) (black) and α LAX(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 snap shots of each peptide. In the case of α LAX(25), the vibrational probe remained fixed at an angle of $\sim 67^\circ$ for the first 50 ns of the simulation, and then abruptly switched its position to 122° and stayed there for rest of 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 towards the membrane-water interface interacting closely with the zwitterionic head group 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 $122 \pm 15^\circ$. In the case of α LAX(16), the nitrile molecule remained relatively stable at 110° (i.e. approaching an orientation perpendicular to the plane of the membrane) 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 α LAX(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 nitrile orientation that remained relatively fixed at 110° with occasional excursions to other stable conformations. We determined an average nitrile angle of $112 \pm 23^\circ$ for the nitrile probe on the peptide α LAX(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 χ_1 torsion angle.

Dipole Field

Figure 5 shows a representative normalized vibrational absorption spectra of the nitrile probes on peptides, α LAX(25) (black), α LAX(23) (blue), α LAX(21) (green) and α LAX(16) (red), in DMPC vesicles at 0.5 mM concentration. In Table 2, we present the average mean peak frequencies and full width half maximum (fwhm) of each of the nitrile stretching bands at both peptide concentrations, 0.5 mM 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 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, 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 $+2.9 \text{ cm}^{-1}$ and $+2.6 \text{ cm}^{-1}$ upon moving into the bilayer interior for samples containing 0.5 mM and 1 mM peptide, respectively. With these measured values of ν_{obs} , $0.67 \text{ cm}^{-1}/(\text{MV}/\text{cm})$ for Stark tuning rate of nitrile, and an angle of $122 \pm 15^\circ$ between nitrile bond and bilayer normal computed for α LAX(25) in equation 1, we calculate the magnitude of the dipole electric field to be $8.1 \pm 4 \text{ MV}/\text{cm}$ for 0.5 mM peptide and $7.3 \pm 3 \text{ MV}/\text{cm}$ for 1 mM peptide. We also calculate the field values based on α LAX(16)'s nitrile-membrane normal angle of $112 \pm 23^\circ$ and the results are $11.4 \pm 11 \text{ MV}/\text{cm}$ and $10.3 \pm 10 \text{ MV}/\text{cm}$ for 0.5 mM and 1 mM peptide respectively, essentially within error of the value calculated at the larger angle. The magnitude 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 MV/cm 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 local molecular environment surrounding around an oscillator.^{13,16,30} For example, Gai and coworkers¹⁶ have shown that the nitrile stretching vibration in *p*-CN-Phe has 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 α LAX(25) to α LAX(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 head group, 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 C α of CN-Phe on α LAX(25) and α LAX(16) for every snapshot in our MD trajectories. The normalized distribution of fields calculated on both positions are shown in Figure 6, where the distributions α LAX(25) and α LAX(16) are shown in black and red, respectively. When the nitrile is near the middle of the membrane, small changes in 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 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 7 and inferred from the small fwhm in Table 2. However, when the nitrile is near the lipid head group, 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 head group, 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 bicelle 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 experiment 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 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.

Acknowledgements

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. are gratefully acknowledged.

References

1. Honig BH, Hubbell WL, Flewelling RF. Electrostatic Interactions in Membranes and Proteins. *Annu. Rev. Biophys. Biophys. Chem.* 1986; 15:163–193. [PubMed: 2424473]
2. McLaughlin S. The Electrostatic Properties of Membranes. *Annu. Rev. Biophys. Biophys. Chem.* 1989; 18:113–136. [PubMed: 2660821]
3. Cevc G. Membrane Electrostatics. *Biochim. Biophys. Acta.* 1990; 1031:311–382. [PubMed: 2223819]
4. Gross E, Bedlack RS, Loew LM. Dual-Wavelength Ratiometric Fluorescence Measurement of the Membrane Dipole Potential. *Biophys. J.* 1994; 67:208–216. [PubMed: 7918989]
5. Franklin JC, Cafiso DS. Internal Electrostatic Potentials in Bilayers: Measuring and Controlling Dipole Potentials in Lipid Vesicles. *Biophys. J.* 1993; 65:289–299. [PubMed: 8396456]
6. Wang L, Bose PS, Sigworth FJ. Using Cryo-EM to Measure the Dipole Potential of a Lipid Membrane. *PNAS.* 2006; 103:18528–18533. [PubMed: 17116859]
7. Yang Y, Mayer KM, Wickremasignhs NS, Hafner JH. Probing the Lipid Membrane Dipole Potential by Atomic Force Microscopy. *Biophys. J.* 2008; 95:5193–5199. [PubMed: 18805919]
8. Clarke RJ. The Dipole Potential of Phospholipid Membranes and Methods for Its Detection. *Adv. Colloid Interface Sci.* 2001; 89–90:263–281.
9. Lairion F, Disalvo EA. Effect of Dipole Potential Variations on the Surface Charge Potential of Lipid Membranes. *J. Phys. Chem. B.* 2009; 113:1607–1614. [PubMed: 19193165]
10. Schamberger J, Clarke RJ. Hydrophobic Ion Hydration and the Magnitude of the Dipole Potential. *Biophys. J.* 2002; 82:3081–3088. [PubMed: 12023231]
11. Sheu SY, Schlag EW, Selzle HL, Yang DY. Hydrogen Bonds in Membrane Proteins. *J. Phys. Chem. B.* 2009; 113:5318–5326. [PubMed: 19354309]
12. Krishtalik LI, Tae GS, Cherepanov DA, Cramer WA. The Redox Properties of Cytochromes b Imposed by the Membrane Electrostatic Environment. *Biophys. J.* 1993; 65:184–195. [PubMed: 8396453]
13. Waegle MM, Culik RM, Gai F. Site-Specific Spectroscopic Reporters of the Local Electric Field, Hydration, Structure, and Dynamics of Biomolecules. *J. Phys. Chem. Lett.* 2011; 2:2598–2609. [PubMed: 22003429]

14. Fafarman AT, Boxer SG. Nitrile Bonds as Infrared Probes of Electrostatics in Ribonuclease S. *J. Phys. Chem. B.* 2010; 114:13536–13544. [PubMed: 20883003]
15. Silverman LN, Pitzer ME, Ankomah PO, Boxer SG, Fenlon EE. Vibrational Stark Effect Probes for Nucleic Acids. *J. Phys. Chem. B.* 2007; 111:11611–11613. [PubMed: 17877390]
16. Getahun Z, Huang CY, Wang T, DeLeon B, DeGrado WF, Gai F. Using Nitrile-Derivatized Amino Acids as Infrared Probes of Local Environment. *J. Am. Chem. Soc.* 2003; 125:405–411. [PubMed: 12517152]
17. Stafford AJ, Ensign DL, Webb LJ. Vibrational Stark Effect Spectroscopy at the Interface of Ras and Rap1A Bound to the Ras Binding Domain of RalGDS Reveals an Electrostatic Mechanism for Protein-Protein Interaction. *J. Phys. Chem. B.* 2010; 114:15331–15344. [PubMed: 20964430]
18. Webb LJ, Boxer SG. Electrostatic Fields Near the Active Site of Human Aldose Reductase: 1. New Inhibitors and Vibrational Stark Effect Measurements. *Biochem.* 2008; 47:1588–1598. [PubMed: 18205401]
19. Andrews SS, Boxer SG. Vibrational Stark Effects of Nitriles I. Methods and Experimental Results. *J. Phys. Chem. A.* 2000; 104:11853–11863.
20. Andrews SS, Boxer SG. Vibrational Stark Effects of Nitriles II. Physical Origins of Stark Effects from Experiment and Perturbation Models. *J. Phys. Chem. A.* 2002; 106:469–477.
21. Alfieri KN, Vienneau AR, Londergan CH. Using Infrared Spectroscopy of Cyanylated Cysteine To Map Membrane Binding Structure and Orientation of the Hybrid Antimicrobial Peptide CM15. *Biochemistry.* 2011; 50:11097–11108. [PubMed: 22103476]
22. Waegle MM, Tucker MJ, Gai F. 5-Cyanotryptophan as an Infrared Probe of Local Hydration Status of Proteins. *Chem. Phys. Lett.* 2009; 478:249–253. [PubMed: 20161057]
23. Getahun Z, Huang CY, Wang T, DeLeon B, DeGrado WF, Gai F. Using Nitrile-Derivatized Amino Acids as Infrared Probes of Local Environment. *J. Am. Chem. Soc.* 2003; 125:405–411. [PubMed: 12517152]
24. Mukherjee S, Chowdhury P, DeGrado WF, Gai F. Site- Specific Hydration Status of an Amphipathic Peptide in AOT Reverse Micelles. *Langmuir.* 2007; 23:11174–11179. [PubMed: 17910485]
25. Tucker MJ, Oyola R, Gai F. A Novel Fluorescent Probe for Protein Binding and Folding Studies: p-Cyano-Phenylalanine. *Biopolymers.* 2006; 83:571–576. [PubMed: 16917881]
26. Serrano AL, Troxler T, Tucker MJ, Gai F. Photophysics of a Fluorescent Non-Natural Amino Acid: p-Cyanophenylalanine. *Chem. Phys. Lett.* 2010; 487:303–306. [PubMed: 20419080]
27. Aprilakis KN, Taskent H, Raleigh DP. Use of the Novel Fluorescent Amino Acid p-Cyanophenylalanine Offers a Direct Probe of Hydrophobic Core Formation during the Folding of the N-terminal Domain of the Ribosomal Protein L9 and Provides Evidence for Two- State Folding. *Biochemistry.* 2007; 46:12308–12313. [PubMed: 17924662]
28. Liu J, Strzalka J, Tronin A, Johansson JS, Blasie JK. Mechanism of Interaction between the General Anesthetic Halothane and a Model Ion Channel Protein, II: Fluorescence and Vibrational Spectroscopy Using a Cyanophenylalanine Probe. *Biophys. J.* 2009; 96:4176–4187. [PubMed: 19450488]
29. Tucker MJ, Getahun Z, Nanda V, DeGrado WF, Gai F. A New Method for Determining the Local Environment and Orientation of Individual Side Chains of Membrane-Binding Peptides. *J. Am. Chem. Soc.* 2004; 126:5078–5079. [PubMed: 15099085]
30. Hu W, Webb LJ. Direct Measurement of the Membrane Dipole Field in Bicelles Using Vibrational Stark Effect Spectroscopy. *J. Phys. Chem. Lett.* 2011; 2:1925–1930.
31. Chattopadhyay A, Boxer SG. Vibrational Stark Effect Spectroscopy. *J. Am. Chem. Soc.* 1995; 117:1449–1450.
32. Hessa T, Kim H, Bihlmaier K, Lundin C, Boekel J, Andersson H, Hilsson I, White SH, Heijne G. von. Recognition of Transmembrane Helices by the Endoplasmic Reticulum Translocon. *Nature.* 2005; 433:377–381. [PubMed: 15674282]
33. Johansson ACV, Lindahl E. Amino-Acid Solvation Structure in Transmembrane Helices from Molecular Dynamics Simulations. *Biophys. J.* 2006; 91:4450–4463. [PubMed: 17012325]
34. Morrissey, JH. [accessed March 15, 2012] Morrissey Lab Protocol for Preparing Phospholipid Vesicles (SUV) by Sonication. Trigger Website. <http://tf7.org/suv.pdf>

35. Szoka F Jr, Papahadjopoulos D. Comparative Properties and Methods of Preparation of Lipid Vesicles (Liposomes). *Ann. Rev. Biophys. Bioeng.* 1980; 9:467–508. [PubMed: 6994593]
36. Huang C. Studies on Phosphatidylcholine Vesicles. Formation and Physical Characteristics. *Biochemistry.* 1969; 8:344–352. [PubMed: 5777332]
37. Fröhlich M, Brecht V, Peschka-Süss R. Parameters influencing the determination of liposome lamellarity by ^{31}P -NMR. *Chem. Phys. Lipids.* 2001; 109:103–112. [PubMed: 11163348]
38. Xue-feng L, Gao-Yong Z, Jin-Feng D, Xiao-Hai Z, Xin-Lin H. An Atomic Force Microscopy Study on Small Unilamellar Vesicle Structures on Mica. *Chin. J. Chem.* 2006; 24:311–315.
39. Elber R, Roitberg A, Simmerling C, Goldstein R, Li HY, Verkhivker G, Keasar C, Zhang J, Ulitsky A. Moil a program for simulations of macromolecules. *Comput. Phys. Commun.* 1995; 91:159–189.
40. Ruymgaart AP, Cardenas AE, Elber R. MOIL-opt: Energy-Conserving Molecular Dynamics on a GPU/CPU System. *J. Chem. Theory Comput.* 2011; 7:3072–3082. [PubMed: 22328867]
41. Berger O, Edholm O, Jähnig F. Molecular dynamics simulations of a fluid bilayer of dipalmitoylphosphatidylcholine at full hydration, constant pressure, and constant temperature. *Biophys. J.* 1997; 72:2002–2013. [PubMed: 9129804]
42. Jorgensen WL, Tiradorives J. The OPLS Potential Functions for Proteins. Energy Minimization for Crystals of Cyclic Peptides and Crambin. *J. Am. Chem. Soc.* 1988; 110:1657–1666.
43. Jo S, Kim T, Iyer VG, Im W. CHARMM-GUI: A web-based graphical user interface for CHARMM. *J. Comput. Chem.* 2008; 29:1859–1865. [PubMed: 18351591]
44. Jo S, Lim JB, Klauda JB, Im W. CHARMM-GUI Membrane Builder for Mixed Bilayers and Its Application to Yeast Membranes. *Biophys. J.* 2009; 97:50–58. [PubMed: 19580743]
45. Jorgensen WL, Chandrasekhar J, Madura JD, Impey RW, Klein ML. Comparison of simple potential functions for simulating liquid water. *J. Chem. Phys.* 1983; 79:926–935.
46. Essmann U, Perera L, Berkowitz ML, Darden T, Lee H, Pedersen LG. A smooth particle mesh Ewald method. *J. Chem. Phys.* 1995; 103:8577–8593.
47. Weinbach Y, Elber R. Revisiting and parallelizing SHAKE. *J. Comput. Phys.* 2005; 209:193–206.
48. Ryckaert JP, Ciccotti G, Berendsen HJ. C Numerical integration of the cartesian equations of motion of a system with constraints: molecular dynamics of n-alkanes. *J. Comput. Phys.* 1977; 23:327–341.
49. Tuckerman M, Berne BJ, Martyna GJ. Reversible multiple time scale molecular dynamics. *J. Chem. Phys.* 1992; 97:1990–2001.
50. Patra M, Karttunen M, Hyvonen MT, Falck E, Lindqvist P, Vattulainen I. Molecular dynamics simulations of lipid bilayers: Major artifacts due to truncating electrostatics interactions. *Biophys. J.* 2003; 84:3636–3645. [PubMed: 12770872]
51. Litman BJ. Effect of Light Scattering on the Circular Dichroism of Biological Membranes. *Biochemistry.* 1972; 11:3243–3247. [PubMed: 4261766]
52. Humphrey W, Dalke A, Schulten K. VMD - Visual Molecular Dynamics. *J. Molec. Graphics.* 1996; 14:33–38.
53. Ku erka N, Nieh MP, Katsaras J. Fluid Phase Lipid Areas and Bilayer Thicknesses of Commonly Used Phosphatidylcholines as a Function of Temperature. *Biochim. Biophys. Acta.* 2011; 1808:2761–2771. [PubMed: 21819968]
54. Ku erka N, Liu Y, Chu N, Petrache HI, Tristram-Nagle S, Nagle JF. Structure of Fully Hydrated Fluid Phase DMPC and DLPC Lipid Bilayers Using X-Ray Scattering from Oriented Multilamellar Arrays and from Unilamellar Vesicles. *Biophys. J.* 2005; 88:2626–2637. [PubMed: 15665131]
55. Webb RJ, East JM, Sharma RP, Lee AG. Hydrophobic Mismatch and the Incorporation of Peptides into Lipid Bilayers: A Possible Mechanism for Retention in the Golgi. *Biochemistry.* 1998; 37:673–679. [PubMed: 9425090]

Figure 1. Representative CD spectra of 1 mM $\alpha\text{LAX}(25)$, $\alpha\text{LAX}(23)$, $\alpha\text{LAX}(21)$, and $\alpha\text{LAX}(16)$ dissolved in TFE (black) and intercalated in DMPC vesicles (blue). Spectral intensity is presented in units of mdeg.

Figure 2.

Snapshots of molecular dynamic simulations of α LAX(25) (A) and α LAX(16) (B) embedded vertically into a bilayer composed of 128 DMPC molecules (green), 5630 TIP3P water molecules (red and gray) and a molecule of each peptides (with the *p*-CN-Phe probe in space-filling representation). Molecular snapshots were prepared with the program VMD.⁵²

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.

Figure 4. Normalized histogram of the angle between the nitrile probe on transmembrane peptide α LAX(25) (black) and α LAX(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.

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 “x”.

Figure 6. Normalized histogram of the absolute value of the electric field on Ca of the nitrile probe on transmembrane peptide αLAX(25) (black) and αLAX(16) (red) embedded into bilayer composed of 128 DMPC lipid molecules computed by molecular dynamics simulation.

Table 1

Amino acid sequences of the four polypeptides described in this work. X = *p*-CN-Phe.

Peptide	Sequence
α LAX(25)	HHGGPGLALALALALALALALAXGPGGHH
α LAX(23)	HHGGPGLALALALALALALALAXALGPGGHH
α LAX(21)	HHGGPGLALALALALALALAXALALGPGGHH
α LAX(16)	HHGGPGLALALALALXALALALALGPGGHH

Table 2

Average mean peak frequencies (ν_{obs}) and full width half maximum (fwhm) of the nitrile stretching band for 0.5 mM and 1 mM peptides intercalated into DMPC vesicles.

Peptide	0.5 mM peptide		1 mM peptide	
	ν_{obs}	fwhm	ν_{obs}	fwhm
α LAX(25)	2228.18 ± 0.07	7.9 ± 0.1	2227.98 ± 0.06	7.0 ± 0.2
α LAX(23)	2228.51 ± 0.04	7.8 ± 0.4	2228.43 ± 0.05	6.9 ± 0.8
α LAX(21)	2229.86 ± 0.06	7.5 ± 0.1	2229.75 ± 0.06	6.9 ± 0.3
α LAX(16)	2231.05 ± 0.02	6.2 ± 0.1	2230.57 ± 0.05	6.2 ± 0.2