Ion channels play vital roles in biological processes. These channels have been long recognized for their role in controlling electrically excitable tissues such as muscles and nerves. However, there is still debate as to the mechanism with which the ions permeate the channel, specifically if water passes through the channel with the ions or if collisions between the ions are necessary for transduction. Previous work in the Zanni group has utilized two-dimensional infrared (2D IR) spectroscopy to determine the method of ion permeation in a model ion channel, KcsA. 2D IR spectroscopy is an excellent technique for this study because vibrational frequencies and lineshapes are excellent probes of molecular structure and electrostatic environment. Since the publication of the original report, others have questioned our conclusions and have found different ways to average the modeled spectra to match our experimental spectra. Therefore, we need to do more in depth studies of KcsA. We also are working towards a voltage-dependent experiment that will allow us to study the impact of an applied potential on the protein of interest. In order to do these potential experiments, I have been working to develop a surface-sensitive 2D IR method using localized surface plasmon enhancement in order to work with proteins embedded in a single bilayer. This work has yielded promising results in a model peptide and we are working to implement this same technique into larger systems.
Research Plan:

Research Objectives
The objective of my research is to determine the mechanism of ion permeation through ion channels by using two-dimensional infrared (2D IR) spectroscopy, a technique known for its inherent time and structural resolution. We begin by following up on previous work in the Zanni group, studying the potassium (K\(^+\)) channel from Streptomyces lividans (KcsA) in micelles using \(^{13}\)C/\(^{18}\)O isotope labels.\(^{1,2}\) However, since we want to probe the effect of an external stimulus on ion occupancy and general structure, we are moving this work into a single bilayer. This is necessary in order to facilitate a potential drop across the membrane-bound protein of interest. With stacked bilayers, the potential required to trigger protein movement increases additively with each successive layer, leading to unnecessarily dangerous applied potential. With a single bilayer, the applied potential can be as low as 50 mV.\(^{3}\) However, studying a protein in a single bilayer yields a very low signal due to the inherently small amount of material present. This can be remedied by the recent push towards surface-enhanced spectroscopies. Using thin, rough gold as a source of plasmon enhancement, combined with the inherent non-linear scaling of 2D IR, we have been able to take spectra of proteins in a bilayer.\(^{4,5}\)

Determine the ion occupancy of KcsA – Work by the Zanni group that determined the ion occupancy of KcsA to predict the mechanism of permeation has recently come under scrutiny.\(^{6,7}\) In short, it was put forth that our data was inconclusive as to the presence or lack of water in the selectivity filter (a short, highly conserved sequence making up the channel). We need to collect more spectra of KcsA with different isotope labeling schemes in the filter to better determine ion occupancy. Work done by the Skinner Group at the University of Chicago has indicated that in labelling Val76 and Gly77 individually, spectra that are more narrow and red-shifted would be indicative of the water-free (hard-knock) mechanism and broader, blue-shifted spectra will indicate water in the channel during transduction (soft-knock mechanism).

Surface-sensitive techniques – In order to be able to observe a spectrum of a single bilayer, while being able to place a voltage across it, we use a thin, conductive layer of rough gold to function both as an electrode and a source of plasmon enhancement. Furthermore, by taking advantage of the ease of sulfur-gold chemistry, we can easily tether a monolayer of the sample to the gold. The signal enhancement from the gold has been shown to have enhancements of up to 6000x. A further 3x enhancement can be gained by using a reflection geometry for a 3-nm thick thermally evaporated film of gold.\(^{8}\) However, we have found that the 3-nm film is not macroscopically conductive. To overcome this, I am working to find a thickness of gold that is conductive while yielding suitable enhancements in the mid-IR, without lineshape distortions. This could also be accomplished by adding a non-plasmonic conductive layer under the gold (e.g. indium tin oxide (ITO) with a small amount of rough, plasmonic gold coated on top.\(^{9}\) We will investigate this technique by studying the model peptabiol, alamethicin, comparing its static pH and voltage dependences, as it forms pores in the tethered membrane. We will translate these static experiments into transient, voltage-jump experiments to gain insight into the dynamics of voltage-gating.
Background and Significance

Ion Channels and Pores – Nerves and muscles have been known to be electrically excitable for over 200 years. However, the movement of ions across the membrane to trigger this excitation was not discovered until the early twentieth century by Julius Bernstein and built upon by many, such as Nobel Prize winners Hodgkin, Huxley, and MacKinnon.10–13 Ion channels are so central to cell physiology and communication, that study of these channels has expanded from monitoring the ionic flux with voltage- and patch-clamp experiments to determining the crystallographic structure of the membrane-bound proteins themselves.

A notable feature of ion channels, which generally control the flow of Na+, K+, Ca2+, and Cl− ions is that they have extremely high ion specificity while maintaining an ion flux of millions of ions per second, nearly matching the diffusion limit.10 The model potassium channel, KcsA, has been extensively used in order to study the mechanism of ion permeation.14–17 Selection for one ion over another occurs in the selectivity filter, a highly conserved sequence (TVGYG) in K+ channels composed of four binding sites bound by five backbone carbonyls on each of the monomers forming the tetrameric channel.11,17 Currently, there are two competing models for ion transduction: the hard-knock and soft-knock (or knock-on) models as shown in Figure 1. In the hard-knock model, the potassium ions are completely desolvated as they enter the selectivity filter. As a new K+ ion enters, coulombic forces repel the neighboring K+ ion, causing them to move through the channel.5,18 In the soft-knock model, the selectivity filter is filled with alternating K+ ions and water. The new K+ ion enters with an accompanying water, pushing another K+ ion and water out into the extracellular side of the channel.1,19–23 Experiments, such as x-ray crystallography, single-channel patch-clamp experiments, and computational modeling, have generally supported the soft-knock mechanism, though these techniques were not able to give definitive evidence.22–24 However, in recent years, several theoretical groups have proposed the hard-knock model, which they support by its ability to explain the high ion throughput and selectivity of the channel as the energy penalty of desolvation greatly favors K+ over Na+.6

Previous work by the Zanni group resulted in a significant advance in the field, yielding data that could be matched to results from molecular dynamics (MD) simulations. In 2016,
Kratochvil and colleagues took 2D IR spectra of the KcsA channel in micelles.\textsuperscript{1} In order to observe the environment of the selectivity filter, $^{13}$C/$^{18}$O isotope labels at specific residues were used. The additional mass of the labels red-shifts the amide I peak corresponding to the labelled amino acid by approximately 60 cm$^{-1}$, far enough to distinguish it from the rest of the protein. This method provides a vibrational probe of the secondary structure and electrostatic environment of a single residue.\textsuperscript{25–28} The isotope labels are sensitive to the electrostatics of their environment because interactions with the partial negative charge on the $^{18}$O will effectively increase its mass, causing a red-shifted peak in the spectrum. In this case, the inward-facing carbonyls that make up the ion binding sites are sensitive to the electrostatics of the ions and molecules in the selectivity filter. Placing isotope labels in three locations (Val76, Gly 77, and Gly79) allowed Kratochvil et al. to observe the ion positions in the channel. In order to see the isotope labels, which were otherwise masked due to the size of the protein (41 kDa), a non-isotope labeled spectrum was subtracted from the isotope labelled spectrum. Data from the isotope-labelled experiments of KcsA in micelles showed good agreement with spectra derived from MD simulation trajectories of the soft-knock model. A state which included the carbonyl of Val76 flipped out of the selectivity filter was included to create better agreement with experiment. This state has also been observed in NMR structural studies of the channel as well as other MD simulations, though not through X-ray crystallography.\textsuperscript{29–31}

Papers published in the last year have called these results into question.\textsuperscript{6,7} One showed that it was possible to generate a spectrum similar to experiment by using a weighted average of states from the hard-knock model. They argued that the hard-knock model also has a better explanation to the energetics of ion selectivity. They calculated the difference in energy required to desolvate the K$^+$ ion is much less than the Na$^+$ ion. This difference explains the rapid K$^+$ ion flow while Na$^+$ ions are blocked. They also argue against the inclusion of the Val76 flipped state as previous work has indicated that it corresponds to a non-conductive filter. However, in order to fit the hard-knock model to our data, they have a configuration with four successive K$^+$ ions in the channel account for 25 percent of the contributing states, which would involve large repulsive forces in the channel. We take this result to indicate that more experimental data is needed in order to constrain the weights of the individual occupancies and come to a general conclusion. This may include individual carbonyl isotope labels as detailed in future work below. Finally, some of these models take into account the presence of a 50-mV potential drop across the bilayer, which was not used in the previous experiments.\textsuperscript{18}

**Surface Enhanced 2D IR spectroscopy** – Previous work to study ion channels have relied on structural information from X-ray crystallography and NMR. X-ray crystallography has atomic resolution, but is inherently static and the crystallization process can impact structure and ion occupancy.\textsuperscript{11,15,29,32} Furthermore, it is difficult to distinguish between internal waters and ions.\textsuperscript{33} NMR gives both structurally- and time-resolved information but is limited as to the size of protein.\textsuperscript{34} The dynamics of ion channels systems have been studied with time resolved current data from patch-clamp experiments. Patch clamp experiments have good time resolution, but lack overall structural information. In order to get residue-specific structural information from patch-clamp experiments, time-intensive point-mutation must be done.\textsuperscript{32,35} 2D IR has the ability to give residue-specific structural information with inherent femtosecond time resolution and is therefore an excellent method to study these systems without compromising structural or dynamical information.\textsuperscript{4,25,36–38}

Briefly, 2D IR measures the third-order response function of the vibrational transitions of the sample after three light-matter interactions (see the SI for a more details). The vibrations of the molecule are sensitive to which atoms are vibrating and the chemical environment.\textsuperscript{4} In proteins, a commonly probed vibration is the amide I mode, largely composed of the backbone carbonyl...
stretch, which absorbs between 1600 and 1680 cm\(^{-1}\).\(^{39,40}\) In our measurements, we observe the frequencies in this region to determine the secondary structures present in the protein and how they change. The main secondary structures, \(\alpha\)-helix, \(\beta\)-sheet, and random coil, have distinct center frequencies (1650 cm\(^{-1}\), 1620 cm\(^{-1}\), and 1645 cm\(^{-1}\) respectively) and lineshapes (narrower defined structures and broad for random coil).\(^4,39\) Lineshapes also give insights into inhomogeneous and homogenous broadening which are indicative of chemical environment. 2D IR is helpful in distinguishing between these structures because the signal scales with \(\mu^4\), rather than \(\mu^2\) in 1D IR. This increases signal and narrows the lineshape of some peaks while suppressing noise, making it easier to resolve different secondary structures.\(^4,25\)

In order to continue our work with membrane-embedded KcsA, we want to work in a single bilayer. Doing so will allow us to apply external stimuli due to the voltage-divider-like nature of the membrane as well as simulate a more biomimetic set up.\(^3\) 2D IR spectroscopy is an odd-ordered experiment, and is therefore not surface-specific. However, it is possible to generate surface sensitivity, which is necessary to study the small number of molecule in a single bilayer.\(^41\) This is commonly done by utilizing plasmon enhancement, similar to surface-enhanced Raman spectroscopy (SERS) and surface-enhanced infrared spectroscopy (SEIRAS). There are several methods of plasmon enhancement, including nanoarrays of various geometries to create specific wavelengths of plasmonic resonance in a region of interest.\(^42-46\) Our method uses a thin, rough gold film with small islands of gold on the surface. Between the islands are ‘hot spots’ which are created when light is highly confined in the gaps, resulting in a higher concentration of the electric field.\(^41\) The peak plasmon frequency is in the visible range, but extends into the mid-IR (\textit{vide infra}). For our experiments, this non-resonant enhancement leads to smaller enhancements than a resonant enhancement method like SERS, but is still on the order of 1000x.\(^8\) Thicker gold red-shifts the plasmon frequency, thus modulating the enhancement. However, thicker gold leads to lineshape distortions, so a compromise must be struck between enhancement and spectral quality.\(^9\)

Recent work in the Zanni and Fayer groups have further manipulated the spectrometer geometry to yield further surface enhancements.\(^8,47\) In a BOXCARs geometry, which is fully non-collinear, the intensity of the local oscillator (LO), which is overlaid with the signal electric field to produce the heterodyned signal, can be independently modulated in order to reduce the background.\(^4\) Due to the technical challenges of collecting purely absorptive spectra in this geometry, a pump-probe geometry, which is partially collinear and self-heterodyned (i.e. the probe pulse acts as the LO), is popularly employed. However, due to the self-heterodyned nature of the pump-probe geometry, the LO intensity is tied to the probe intensity and directly influences the signal strength. To combat this, we took advantage of the fundamental qualities of different polarizations of light as described in the Fresnel equations. For p-polarized light, there is

![Figure 2. Reflectivity of s- and p- polarized light at a CaF\(_2\)-air interface, where Brewster’s angle is where the p-polarized light has zero reflectivity.](image-url)
an angle (Brewster’s angle) at which when it hits an interface, no light is reflected as shown in Figure 2. At near-Brewster’s angle reflection geometries, therefore, it is possible to excite the sample with the probe beam, but attenuate the LO strength such that the background is suppressed, effectively enhancing the signal in the reflected direction. This was first demonstrated by the Fayer group, and later applied to biological samples in strongly absorbing solvents in my first year. This method itself gives a 4x enhancement that can be combined with localized surface plasmons for enhancements on the scale of $10^4$.8

**Research Progress**

I have developed a method for surface-enhanced 2D IR spectroscopy using a nanometers-thin, thermally-evaporated layer of rough gold on a calcium fluoride window to enhance the signal from a lipid bilayer. I have also demonstrated the ability to not only see signals from a model peptide, but also changes in the peptide caused by changes in its environment.

A single monolayer of 1,2-dipalmitoyl-sn-glycero-3-phosphothioethanol (DPTE) was created by incubating a solution of the DPTE in ethanol with a 2 nm thick gold-coated window in order to form a gold-sulfur bond, tethering the head group of the lipid to the window.48 Vesicles of 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC) were allowed to fuse to the monolayer of DPTE. The window was washed thoroughly to leave a single bilayer behind. Spectra of both the DPTE and the mixed-lipid bilayer show the success of this technique seen in Figure 3. The peaks arise from the ester carbonyls which link the head group to the tails.49 The presence of multiple peaks is likely due to the difference in the two tails of POPC and interactions with the gold. Furthermore, it is important to note that there are no substantial signals arising in the amide I region, which is vital in being able to take peptide spectra without background correction.

![Figure 3. Representative 2D IR spectra of the ester carbonyl region for A) A monolayer of DPTE and B) a single bilayer of DPTE and fused POPC vesicles both tethered to 2-nm of thermally evaporated gold on a CaF₂ window in D₂O.](image)

To determine if we could observe a peptide embedded in the membrane, alamethicin (alm), a 20-residue model peptaibol, was chosen to test our experimental methods. Alm associates with the membrane, and has both $\alpha$- and $3_{10}$-helical character.50,51 A change of voltage or pH causes alm to form bundles and insert itself into a membrane, initiating its antimicrobial activity.48,50,52–55 The alm was deposited onto the membrane at a 10:1 lipid:peptide ratio. The sample was dried then rehydrated with D₂O at a neutral or basic pH. 2D IR spectra of this sample were then taken demonstrating the sensitivity of our methods as seen in figure 4. These spectra are consistent with previous linear IR and SFG spectra taken of alm. We attribute the 1670 cm⁻¹ peak to the $\alpha$-helical half of the peptide. The 1635 cm⁻¹ peak arises from the $3_{10}$-helix, consistent with both SFG spectra of alm and 2D IR spectra of $3_{10}$-helices.52,56
The differences between the pH 6.8 and the pH 12 spectra demonstrate our sensitivity to the change of environment that the amide backbone is experiencing. There are evident shifts between the relative intensities of the 1670 cm\(^{-1}\) and the 1630 cm\(^{-1}\) peaks. This can be explained by a change in angle as the peptide inserts into the membrane or due to peptide-peptide interactions.\(^{48,52,57,58}\) In this system, membrane-peptide interactions that are created can also influence the amide I frequency as the bundles are formed. We can also consider possible changes in secondary structure. For example, the alm is destabilized when it forms a 3\(_{10}\)-helix, causing it to insert into the membrane in order to stabilize the structure. Most importantly, these results gave us the confidence to move beyond small peptides. Being able to detect these structural changes with the small amount of sample is encouraging as we move to larger systems where we will need to detect changes in a single isotope label.

In order to test our abilities to run alm as a voltage-gated experiment, several modifications were needed. First, the thin gold-coated substrates were not macroscopically conductive, despite being thicker than the percolation threshold (thickness needed for conductivity). This required us to determine what thickness of gold yielded a good signal without lineshape distortions previously observed when adding a large amount of plasmonic material to a CaF\(_2\) window.\(^9\) Thicknesses of gold between 2 and 10 nm of gold were tested for both conductivity and signal ‘quality’ with alamethicin. Conductance was determined by measuring the resistance across approximately one centimeter of the coated window with a multimeter. The standard of reasonable conductance of the electrode was taken to be less than 50-k\(\Omega\) of resistance, as previously noted.\(^9\) It was determined that 8 nm of thermally evaporated gold was the minimum thickness that resulted in an appropriate resistance, and spectra were taken using 10 nm and 8 nm windows.

**Figure 4.** Representative 2D IR spectra of the amide I region for A) alamethicin at pH 6.8 B) alamethicin at pH 12 both deposited on a single DPTE/POPC bilayer tethered to 2-nm of thermally evaporated gold on a CaF\(_2\) window in D\(_2\)O.
A new sample cell was also needed to perform these experiments to allow for the connection of electronics to the sample. To this end, I designed and 3D printed several iterations of a spectro-electrochemical cell (Figure 5a). The cell is made of plastic and is therefore safer to use than our conductive metal sample cells. We use conductive aluminum tape to both connect the gold (working electrode) to the potentiometer and act as a counter electrode (Figure 5b). Since we are focused on placing a constant potential over a membrane, we are not currently concerned about having a reference electrode. Due to evaporation issues in losing solution over the course of averaging, a flow cell design was also implemented so that liquid could be replenished over the course of the experiment.

Some promising voltage-dependent data were taken with a 10 nm gold-coated window, but were not replicable. This was due to generally a large negative offset in signal that washed out the peptide signal, perhaps due to the presence of hot carriers. This signal was not seen in a control experiment at a negative waiting time (t2) where the probe pulse arrives before the pump pulse, indicating that it is not scatter. Other experimental set ups attempted included 8 nm of gold in both a transmission and reflection geometry, 10 microns of gold in a reflection geometry, and 10 nm of palladium topped with 2 nm of gold. In the successful preliminary experiment, spectra of the alm on the lipid bilayer in a KCl/D2O buffer were taken at 0 and ±900 mV. The 0-mV spectrum was subtracted from each the -900- and +900-mV spectra in order to both eliminate the non-resonant response of the thick gold and act as a background subtraction shown in figure 6. This yielded difference spectra qualitatively similar to the basic pH spectrum.
Due to aforementioned difficulties, we are attempting to create a more reproducible window as an electrode. Towards this end, our collaborator in the Arnold group is beginning to make substrates with a 5 nm layer of spin-coated then annealed indium tin oxide (ITO) under 3 nm of thermally evaporated gold. This may work better due to the lack of plasmon from the ITO so we are able to use the thickness of the gold we found to be best for the plasmonic enhancement of monolayers. We are still observing a negative response from the material, but have been able to subtract it out to see an alm signal by a peptide-free spectrum from a peptide spectrum.

While working on this new experimental set up, we have also been collecting surface-enhanced spectra of KcsA to clarify the mechanism of ion permeation. As before, we prepared these samples by creating a monolayer of DPTE on a calcium fluoride window coated with 3 nm of gold. Buffered KcsA in vesicles were deposited, allowed to form into a bilayer, then gently washed to eliminate any stacked bilayers. They were then exchanged with D$_2$O 50 times to eliminate any

Figure 6. Spectra of alamethicin in a tethered lipid bilayer on 10 nm thermally evaporated Au at A) 0V B) -900mV and C) the 0V spectrum subtracted from the -900 mV spectrum, yielding qualitative similarities to the basic pH spectrum.

Figure 7. Representative 2D IR spectra of the amide I region for A) unlabeled KcsA B) Val76, Gly 77, and Gly79 labelled KcsA C) a zoom of the unlabeled spectrum subtracted from the labeled spectrum to leave just the isotope labels. The two samples were deposited on the same DPTE coated window with a 3-nm gold thermally evaporated film, hydrated in D$_2$O with a 12 μm spacer.
H$_2$O from appearing in the amide I region as described in previously. The spots are then rehydrated in D$_2$O. Preliminary spectra have shown consistency with the work of previous work in the Zanni group as shown in figure 7. Furthermore, we are able to see isotope labels by using a subtraction scheme where an unlabeled spectrum is subtracted from an isotope labelled spectrum by normalizing to the $\alpha$-helical peak.\textsuperscript{1} This allows the bulk of the amide I region to be eliminated, making the isotope labels visible. We are now prepared to take spectra of the newly labelled KcsA as detailed in my future plans.

**Future Plans**

**Finish work with alamethicin for publication** – In the next few months we will finish work on the alm project. Once we have replicable voltage data, we will write a paper on the static voltage technique. I simply need to finalize the flow cell set up and to find a suitable combination of conductive materials to form the electrode on the CaF$_2$ window. I will then take the voltage data, compare the results both to our own pH-modulated data, and SFG data from other groups. This will pave the way for more complex systems by serving as a strong proof of concept experiment.

**KcsA mechanism validation** – As we finish the work with alamethicin, we will transition to KcsA. We want to add additional constraints to the existing models by adding data from new isotope labelling schemes in the selectivity filter. With our surface-enhanced method, we believe that we will be able to observe single isotope labels, rather than the multi-label scheme employed in previous work. We can then observe Val76 and Gly77 individually in samples prepared with native protein ligation by our collaborators as detailed in the SI. These sites are of significant interest after modeling work done by the group of Jim Skinner at the University of Chicago (private correspondence). They modeled both the hard- and soft-knock models including both the flipped and unflipped states of Val76. They then took the trajectories and used them to generate weighted 2D IR spectra of each individual label as explained in the SI. The models yielded readily distinguishable spectra. In both cases, the soft-knock mechanism yielded an inhomogeneously broadened features between 1570 and 1590 cm$^{-1}$, while the hard knock mechanism had narrower peaks with maxima between 1550 and 1570 cm$^{-1}$. The differences in peak width is especially apparent in the case of Gly77, and examples of the different types of broadening are showing in figure S4. Experiments with the suggested labels will definitively clarify the mechanism for KcsA, which serves as the model for K$^+$ channels. Furthermore, our data benefits computational models that can use this data to further constrain averaged spectra.

Many models of the mechanism of K$^+$ permeation through KcsA has included a 50 mV drop across the bilayer.\textsuperscript{18} Using the static voltage methods I am working to develop, we plan to repeat our KcsA experiments with an applied voltage. This will allow us to rectify any differences between our experimental results and competing models by providing data using the parameters of the simulated data. Again, this is a case of more data contributing both to a better understanding of biological processes and also providing data with which to test simulations and models.

**Transient Voltage-Jump experiment** – Finally, I will work to develop a 2D IR voltage-jump (V-jump) experiment. The end goal is to be able to bridge the transient ion current data currently collected from patch clamp experiments and the static structural data gleaned from
crystallography, taking advantage of the structural and temporal resolution of 2D IR spectroscopy. This will entail creating a new pulsed experiment where we will create a voltage using an arbitrary wave generator (AWG) triggered off of the laser. Using a function generator, we are able to control both the amplitude and duration of the applied potential. We are writing a code to collect 2D IR spectra at different voltage waiting times (T_v) after the laser pulse (Figure 8), similar to temperature-jump experiments. This will allow us to observe the structural dynamics of voltage-gating in real time.

There are two processes that we plan to observe using this method. First, we will be able to observe the ion permeation through the channel by using isotope labels in the selectivity filter of an ion channel and again comparing to models. This will allow us to compare the mechanism we inferred through our static experiments with what is happening in real time. Second, we will be able to observe allosteric processes that occur in the more complex voltage-gated K^+ channel, KvAP. KvAP uses an arginine-rich S4 helix to sense a potential drop.\(^{59-62}\) The change in voltage elicits a yet-to-be-determined physical result in the paddle. There are a few existing theories: the sliding helix (or canonical) model where the S4 helix moves out of the membrane and turns, the paddle model where the angle of the voltage sensing domain changes and the twisted S4 model where a kink in the center of the helix causes the two helical segments to move separately.\(^{63}\) Using the V-jump technique, we will be able to observe the movement of the voltage paddle. By comparing these results with modeled spectra, we will then be able to determine the correct mechanism of gating. We could, also, determine if the movement of the voltage paddle is the rate determining step, or if there is a currently unknown structural rearrangement between the paddle moving and the pore opening that determines the response time of the opening of the ion channel. This can easily be extracted by determining if the movement of the paddle is synchronous with the pore opening (making it the rate determining step) or before (indicating an unknown middle step). This is only the start of what could be asked by the V-jump experiment.

![Figure 8. Proposed pulse sequence for the transient voltage experiments. After zero potential, a potential will quickly be applied. Then at a delay time, t_v a 2D IR spectrum will be taken. After averaging the 2D IR spectrum at the first value of t_v, the delay time will change, allowing for spectra taken at several points after the applied voltage in order to measure the structural response to the potential.](image-url)
Supporting Information:

2D IR spectroscopy: Two-dimensional infrared spectroscopy measures the third-order response function of a sample as a result of three light-matter interactions exciting the vibrational transitions of a molecule. The methods of collecting the spectra vary, but all use an identical pulse sequence (figure S1) where three pulses of light interact with a sample to create a signal. The signal can then be heterodyned by either with the final probe pulse or with a separate local oscillator. The first pulse puts the sample in a coherence state and it stays there during the coherence time, \( t_1 \). The second pulse then brings it into a population state that evolves during the population time, \( t_2 \). Finally, the third pulse will cause a coherence between the ground and first excited state or the first and second excited states, stimulating a combined ground state bleach and stimulated emission or excited state absorption signals respectively.\(^4\)

The direction of this signal depends on the phase matching geometry utilized. The Zanni group uses a pump-probe geometry in which the first two pump pulses are collinear and spatially offset from the third, pump, pulse resulting in a signal that is emitted in the same direction as the probe. This set up is convenient for a few reasons. First, the signal is self-heterodyned in that we use the probe pulse as our local oscillator to extract the phase information from the sample. Secondly, the rephasing and non-rephasing signals are emitted in the same direction, allowing us to cut down on data collection time and yielding purely absorptive data directly.\(^64,65\)

The two pump pulses are created by using a germanium acousto-optical amplifier (Ge-AOM). Briefly, this is used as a pulse shaper to turn our pump pulse into two, phase-controlled pump pulses separated by different time delays. The incoming pulse is first Fourier transformed from the time to frequency domain with a grating. An acoustic wave, or mask, is applied to the Ge crystal, acting as a diffraction grating in the frequency domain for the incoming light. After the Ge-AOM, a second grating is used to return the pulse to the time-domain, yielding the double pump pulse.\(^25,64\) The Ge-AOM can be modulated on a shot-to-shot basis.\(^66\) We generate our spectra in the time-frequency domain such that the pump axis is generated by scanning the delay \( t_1 \) and performing a Fourier transform, while the probe axis is collected by the dispersed frequencies on an MCT array.

The interference between the electric fields of the signal and the local oscillator contains the information in this experimental set up. The signal in the frequency domain is

\[
S \propto \int \left| \left( E_{LO}(\omega) + E_{sig}^{(3)}(\omega) \right)^2 \right| d\omega \approx E_{LO}^2(\omega) + E_{sig}^{(3)}^2(\omega) + 2E_{LO}(\omega)E_{sig}^{(3)}(\omega) \quad \text{Eq. S1}
\]

where \( E_{LO}^2(\omega) \) is the intensity of the pump pulse (local oscillator), \( E_{sig}^{(3)}^2(\omega) \) is the intensity of the signal, and the \( 2E_{LO}(\omega)E_{sig}^{(3)}(\omega) \) cross term is the signal of interest. We are able to isolate this cross term again by using the pulse shaper. Every other pulse takes a spectrum as described, while the intermittent pulse turns “off” the pump by using the Ge-AOM as a chopper. As a result, we have spectra that are just the local oscillator by itself. We can then subtract off the local oscillator as is done is absorption spectroscopy where the signal is approximately

\[
S = \log \left( \frac{\text{pump on}}{\text{pump off}} \right) \approx \log \left( \frac{E_{LO}^2(\omega)E_{sig}^{(3)}^2(\omega) + 2E_{LO}(\omega)E_{sig}^{(3)}(\omega)}{E_{LO}^2(\omega)} \right) \approx \frac{2E_{LO}(\omega)E_{sig}^{(3)}(\omega)}{E_{LO}^2(\omega)} \quad \text{Eq. S2}
\]
The ability to modulate the pump on and off is clearly very useful in data collection. In total, the use of the pulse shaper aids in spectra collection, allows us to reduce pump scatter through phase cycling, and allows for faster data collection.4

Sample Preparation: Alamethicin derived from Trichoderma viride purchased from Sigma-Aldrich was diluted to a concentration of 2 mg/mL in methanol for storage purposes. Lipids, 1-palmitoyl-2-oleoyl-glyero-3-phosphocholine (POPC) and 1,2-Dipalmitoyl-sn-Glycero-3-Phosphothioethanol (DPTE) (Figure S2) both in chloroform at a concentration of 10 mg/mL were purchased from Avanti Polar Lipids Inc. Calcium fluoride (CaF2) windows (25 mm x 2 mm) were purchased from Crystran.

Gold films (Au% > 99.99%) were deposited via thermal evaporation to varying thicknesses at the rate of 0.1 Å/s at a pressure of 8 x 10^{-7} torr onto the CaF2 window. Films of indium tin oxide (ITO) and gold were created first by sputter-coating the window with 5 nm of ITO and annealing at 350 °C for 1.5 hours. The gold was then deposited to the desired thickness as described. Th ey layered metal (Pd/Au) samples were created by first thermally evaporating the palladium, then adding the gold on top separately.

A 100-μL aliquot of DPTE in chloroform (10 mg/mL) and 250-μL aliquot of POPC in chloroform (10 mg/mL) were dried down under N2 and lyophilized overnight to remove remaining solvent. DPTE was rehydrated in 10 mL of spectroscopic-grade ethanol. The coated window was then submerged in the DPTE/ethanol solution overnight. The window was then rinsed with ethanol and allowed to dry. For alamethicin samples, POPC vesicles were prepared by rehydrating the POPC in 100 mM NaCl buffer and applying five freeze-thaw cycles in isopropanol and dry ice and lukewarm water respectively. The solution was then placed in a bath sonicator for 10 minutes to create approximately 50-nm diameter vesicles. Window was then submerged in the POPC vesicle solution overnight to allow for vesicle rupture. The window was then washed with deionized water. Alamethicin was prepared by diluting 5.2 μL of the stock solution in 50 μL of deionized water. This was then distributed over half of the lipid-coated CaF2 window. Assuming total surface coverage of lipid, this should create a 20:1 lipid:peptide ratio. The window was dried under N2 and then rehydrated with D2O or phosphate buffer (pH 6, 12) in D2O and a second window was added separated with a 56 μm spacer.

For the KcsA samples, the DPTE monolayer was created in the same fashion. KcsA (1.5 mg/mL) in 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) (0.25% w/v) and 50 mM Tris, 150 mM KCl, and 1 mM DTT buffer (pH 7.5), both those that were unlabeled and labelled, were deposited in separated 0.2-μL spots on the same window, and gently rinsed to remove excess sample. The spots were then exchanged 50 times with D2O by adding 0.2 μL at a time and allowing it to dry under N2. The spots were then rehydrated with 0.2 μL of D2O and placed between two CaF2 windows with a 12-μm spacer.

2D IR data acquisition: A Coherent Libra regenerative amplifier centered at 800 nm (3.4 W, 50 fs) pump an optical parametric amplifier (OPA) (Light Conversion TOPAS). The output signal and idler pulses are then mixed in a AgGaS2 difference frequency generation (DFG) crystal to generate the mid-IR light centered at 6 μm with pulses of <100 fs with energies around 18 μJ. The mid-IR

![Figure S2. The structures of the lipids](image-url)
is then split with a 90/10 CaF$_2$ beam splitter into the pump and probe lines. The pump line is directed through an acousto-optical modulator (AOM) which shapes the pulses as previously described. The polarization (s- or p-) of both the pump and probe lines are modulated by successive half-wave plates and polarizers. A 7.5-cm focal length parabolic mirror is used to focus the spatially and temporally overlapped pulses at the sample. Due to the pump-probe geometry used, the probe/signal beam is then directed into a monochromator to be dispersed onto a 32-pixel MCT array.

**MD Simulations:**
The route from MD simulation to spectra is outlined briefly here.$^{4,67}$ Spectra are derived from MD simulations by taking the frequency trajectory of the vibration of interest, in our case the isotope-labelled backbone carbonyl, and from the trajectory determine the distribution of frequencies. This distribution will yield a frequency fluctuation correlation function, which can be used to determine the lineshape function. The lineshape function is key in creating the response function. Then, the response functions for the rephasing and non-rephasing signals can be treated like collected time-domain data, and Fourier transformed and summed to create the purely absorptive spectra. Because 2D IR spectra take an ensemble average of the sample of interest, it is necessary to take into account the different ion occupancies of selectivity filter. This was done by taking the trajectories of each of the possible ion occupancies for both models individually and determine the resulting spectra. These spectra were then combined in a weighted average to create the final modeled spectra.

![Models S4](image)

**Figure S4.** Modeled spectra with A) homogenous broadening B) partially inhomogeneous broadening C) inhomogeneous broadening.
References:


