Label-Free Phase Change Detection of Lipid Bilayers Using Nanoscale Diamond Magnetometry

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The nitrogen-vacancy (NV) center in diamond is a quantum sensor with exceptional quality for highly sensitive nanoscale analysis of nuclear magnetic resonance (NMR) spectra and thermometry. In this study, nanoscale phase change detection of lipid bilayers is investigated utilizing ensemble-averaged nuclear spin detection from small volume ≈(6 nm)$^3$, which is determined by the depth of the NV center. Analysis of nanoscale NMR signal confirms thickness of lipid bilayer to be 6.2 nm ± 3.4 nm with proton density of 65 protons per nm$^3$ on top of diamond sample. The result of the correlation spectroscopy from nanoscale volume is compared with the 2D molecular diffusion model constructed by Monte Carlo simulation combined with results from molecular dynamics (MD) simulation. There is a change in diffusion constant from 1.5 ± 0.25 nm$^2$ µs$^{-1}$ to 3.0 ± 0.5 nm$^2$ µs$^{-1}$ when the temperature changes from 26.5 to 36.0 °C. The results demonstrate that the multi-parameter detection of changes in translational diffusion and temperature is possible in label-free measurements using nanoscale diamond magnetometry. The method paves the way for label-free imaging of cell membranes for understanding their phase composition and dynamics.

1. Introduction

The cell membrane is a nanoscale 2D fluid crystalline assembly with sub-compartment domains that are critical for cellular functions, including transport of molecules, communications, and metabolic properties with its external medium.[1–3] These domains are distinguished by different phases of lipid membranes, and extensive research has focused on understanding the structure and dynamic properties of such domains.[4] The fluidity of the lipid bilayer, described by the 2D translational diffusion of lipid molecules,[5] determines the most fundamental property of lipids in different phases and therefore domains. Fluorescence microscopy has been most effective for measuring fluidity.[6,7] Most advanced example includes stimulated emission depletion-fluorescence correlation spectroscopy (STED-FCS) utilized for detection of nanoscale diffusion and identification of nanoscale domain.[8] However, the use of fluorescent probes in such a technique changes the mass and structure of target molecules and deteriorates the observed dynamics.[9,10] For direct measurement of the diffusion constant without additional perturbation in a biological environment, a label-free technique with nanoscale detection volume is necessary. Nanoscale nuclear magnetic resonance (NMR) and correlation spectroscopy using a nitrogen-vacancy (NV) center has emerged as a quantum measurement platform that allows for label-free diffusion measurement with nuclear spin from a small detection volume of ≈(6 nm)$^3$.[11] The detection volume is determined by the location of the NV center from the surface of the diamond.[12,13]

The NV center also allows the detection of local temperature with sub-degree precision,[14,15] enabling the multi-parameter detection of temperature and diffusion in nanoscale samples in a biological environment, which is optimal for the phase change detection of biological samples. Our measurement technique enables direct measurement of cell membrane by simply placing cells on top of diamond. This measurement technique makes it possible to achieve imaging down to a diffraction-limited spot.

In this study, the NV center was formed as a perfectly aligned delta doped layer[16] within 10 nm from the surface as a highly sensitive detection probe for the ensemble-averaged detection of nanoscale diffusion in the lipid bilayer. Correlation spectroscopy observed from the NV center revealed a change in relaxation time as a function of change in temperature. Monte Carlo 2D translational diffusion simulation was combined with molecular dynamics (MD) simulation to depict dynamics that are observed...
in our data. Our simulation demonstrated that the translational diffusion constant changed from $1.5 \pm 0.25 \text{ nm}^2 \text{ µs}^{-1}$ to $3.0 \pm 0.5 \text{ nm}^2 \text{ µs}^{-1}$ by changing the temperature from 26.5 to 36.0 °C. Our simulation demonstrates the phase change from an ordered phase to a rippled or disordered phase.

We report the first direct observation of phase change in the lipid bilayer; this was achieved using the nanoscale detection volume of the NV center. Direct observation of changes in the diffusion constant paves the way for the label-free identification of domains that are formed in the cell membrane to understand the relationship between cell membrane dynamics and cell function.[17]

2. Results

2.1. Experiment Setup

Figure 1a presents the overall setup of our experiment. A perfectly aligned shallow ensemble NV center was formed 10 nm from the surface through CVD growth.[16] NV center measurements were performed using a home-built optical microscope based on Olympus IX73 for confocal and wide-field measurements. Details are discussed in Section 5. Supported lipid bilayer (SLB) was formed using dipalmitoylphosphatidylcholine (DPPC) molecules on top of the shallow NV center using the vesicle fusion method.[18] DPPC was used as the model in this study for the closeness of properties to sphingomyelin (SM), a major constituent of membrane rafts.[19] Phases in lipids are defined by fluidity of the lipid molecules at a given temperature, and therefore change in diffusion constant by change in temperature corresponds to change in the phase of lipids.[20] As shown in Figure 1b, DPPC exhibits a solid ordered (So) phase at ≈25 °C and as the temperature is raised close to Tm (transition temperature) of DPPC ≈41 °C, a phase change occurs in the DPPC toward the liquid disordered (Ld) phase.[21] To measure the change in diffusion rates of the lipid bilayer, an optically defined averaged readout from a shallow ensemble NV center with a detection volume ≈(6 nm)$^3$ was used for nanoscale NMR and correlation spectroscopy as shown in the Supporting Information. Application of correlation spectroscopy using pulse sequence shown in Figure 1a allows comparison of the detected phase accumulated in the NV center between two XY8-N measurements spaced by $\tau$. Correlation spectroscopy has been shown to detect 3D nanoscale diffusion of protons in oil.[11] In this study, we applied this technique to high density (≈60–70 nm$^{-3}$) proton nuclear spins in a lipid bilayer to study the diffusion characteristics, as shown in Figure 1b.

The sample was placed in an incubator, as shown in Figure 1a, to control temperature and maintain a steady temperature. For precise interpretation of phase transition, high-precision measurement of temperature is extremely important. Detection of the splitting between spin state $|0\rangle$ and $|\pm 1\rangle$ states enables readout of the resonance frequency $D(T)$ that depends on the local temperature $T$. The temperature dependence is $dD(T)/dT = -74 \text{ kHz K}^{-1}$.[22] We applied the pulse sequence shown in Figure 1a known as thermal echo (T-Echo)[23,24] to determine $D(T)$ of the NV center by changing the applied frequency and measuring the observed oscillation frequency.

2.2. Measurement Confirmation of the Lipid Bilayer on the Diamond Surface

The formation of the lipid bilayer on the diamond sample was confirmed through florescent recovery after photobleaching (FRAP) performed with DPPC sample mixed with 1% mol Rhodamine B. The sample was excited with a 532 nm laser with a dichroic mirror (LPF at 600 nm) using a 40× objective lens (Olympus LCACHN40XIPC) and detected with a color
camera (V230CFL or DP53). Figure 2a depicts a typical image of a diamond sample with excitation with a 532 nm laser. Emission above 600 nm from Rhodamine B was confirmed on top of the diamond. Laser excitation was applied at a high intensity of $\approx 10^5$ mW cm$^{-2}$ for more than 5 min to perform photo-bleaching, where emission from Rhodamine B was depleted. Figure 2b presents a typical image obtained after bleaching of the sample. Depletion of emission was confirmed on the spot defined by iris used to block the laser. As shown in Figure 2c, the DPPC/Rhodamine-PE sample showed recovery of emission after 5 min. FRAP measurement demonstrates recovery of emission on laser damaged spot of lipid bilayer, which confirms two things; Rhodamine B is bleached in the area of high laser exposure and lipid bilayer stayed intact as a bilayer on top of diamond substrate. Continuity of lipid bilayer provides fluidity that allows recovery of florescence over 5 min. In the case of randomly deposited lipids, the laser damaged spot shows no recovery because no diffusion is observed between damaged spot and non-damaged spot due to absence of continuous lipid bilayer. FRAP measurement proved the existence of SLB composed of DPPC molecules on top of the diamond sample.

Nanoscale NMR measurements were performed using a confocal setup for protons in the DPPC sample without Rhodamine B to confirm the existence of DPPC on top of the shallow NV center. Sample preparation was applied in the same manner as DPPC/Rhodamine-PE described above, except that Rhodamine-PE was not introduced, and only DPPC (1,2-Dipalmitoyl-sn-PC, Larodan) was used as the molecule for deposition. Deionized (DI) water was used instead of phosphate-buffered saline (PBS) solution to avoid uncertainty caused by interaction between PBS contents and microwave excitation that is used for NV measurement. DI water is also commonly used for the preparation of lipid bilayer and no effect on change in phase transition for change in pH or sodium chloride concentration has been reported in previous study. For nanoscale NMR measurements, a magnetic field of 71.8 mT was applied and confirmed from the ODMR spectrum of the NV center. The proton signal was observed by application of the XY8-64 sequence. A schematic representation of the estimation of lipid bilayer thickness $t_{LB}$ is presented in Figure 2d. The proton density and proton thickness of the lipid bilayer were calculated using the equation below with $\alpha = 0$ for NV centers oriented toward [111] direction as stated in

\[
\alpha = 0
\]
Section 5.\textsuperscript{12} \(C(\tau)\) in Equation (1) represents normalized contrast observed in nanoscale NMR measurement.

\[
C(\tau) \approx \exp\left[-\frac{2}{\pi^2} \gamma_r^2 B_{\text{RMS}}^2 K(N\tau)\right].
\]

\[
B_{\text{RMS \ calib.}}^2 = \rho \left(\frac{\mu_0 H_{\text{rot}}}{4\pi}\right)^2 \left(\frac{\pi [8 - 3\sin^2(a)]}{128 d_{\text{NV}}}\right)
\]

\[
B_{\text{RMS \ lipid \ bilayer}}^2 = \rho \left(\frac{\mu_0 H_{\text{rot}}}{4\pi}\right)^2 \left(\frac{\pi [8 - 3\sin^2(a)]}{128}\right) \times \left[\frac{1}{(d_{\text{NV}})^3} - \frac{1}{(d_{\text{NV}} + t_{\text{LB}})^3}\right]
\]

Initially, the depth of the NV center was calibrated by measuring the protons in oil and using Equation (1) to evaluate the depth of the NV center to be 6.6 ± 0.5 nm. Proton density inside lipid bilayer has been calculated by fitting data obtained from radially distributed function to be 65 [proton nm\(^{-3}\)]. Details on the calculation of uniform proton density are shown in Figure S2, Supporting Information. Using the calibrated and calculated value, the thickness of the lipid bilayer and the density of protons in the lipid bilayer were calculated using Equation (2). The calculated thickness of the lipid bilayer was estimated to be 6.2 ± 3.4 nm with a proton density of 65 [proton nm\(^{-3}\)]. The lipid packing density estimated from proton density = 0.49 [nm\(^2\) per lipid] is comparable to the reported lipid packing density 0.47 [nm\(^2\) per lipid] for DPPC.\textsuperscript{26} Our measurement demonstrates observation of proton density and thickness that are comparable to reported values from DPPC on top of the diamond sample. Details of the calculation of lipid packing density is shown in “Calculation of proton density in lipid bilayer and conversion to lipid packing density” section in the Supporting Information. Measurement of nanoscale NMR was performed using confocal microscope with diffraction limited spot size of ≈ 300 nm. And measurement was performed on terrace section of diamond with flatness of less than ≈ 2 nm.

### 2.3. Modeling of Lipid Bilayer and Observation of Phase Change

Calculation of diffusion constant from correlation spectroscopy requires detailed modeling of dynamics for observed nuclear spin. A previous study demonstrated a 3D diffusion model for proton nuclear spin in oil.\textsuperscript{[11]} The relaxation rate observed in correlation spectroscopy is determined by two factors, \(T_2\) of observed nuclear spin and probability of detecting nuclear spin within the detection volume determined by the depth of the NV center. Because we observe proton spins with the \(S = 1/2\) system, the contribution to \(T_2\) is determined by dipolar coupling between protons.\textsuperscript{[27,28]} As shown in Figure 3a, the molecular dynamics of the lipid bilayer were divided into intramolecular and intermolecular parts, where the intramolecular part was used to model the rotation and wobble effect, and the intermolecular part was used to model the effect of diffusion. Equations used for calculation of \(T_2\) are given below,\textsuperscript{[28]} where \(r\) is the intramolecular distance between two protons in the lipid molecule, \(\omega_{\text{Larmor}}\) is the proton gyromagnetic ratio, \(\omega_{\text{Larmor}}\) is the proton Larmor frequency, \(\hbar\) is the Dirac constant, \(\tau_{\text{rot}}\) is the correlation time for rotation, \(N\) is the density of proton in the lipid bilayer, \(d\) is the intermolecular distance between two protons, and \(\tau_{\text{trans}}\) is the correlation time for translation.

\[
\frac{1}{T_2 \text{ intras}} = \frac{3}{20} \frac{\gamma_r^4 \hbar^2}{\omega_{\text{Larmor}}^2} \left[\frac{3}{d_{\text{NV}}} + \frac{5}{N^2 \omega_{\text{Larmor}}^2} \frac{\tau_{\text{rot}}}{1 + \omega_{\text{Larmor}}^2 \tau_{\text{rot}}^2}\right]
\]

\[
\frac{1}{T_2 \text{ inters}} = \frac{2\pi}{10} \frac{N \gamma_r^4 \hbar^2}{d^3} \left[\frac{3}{d_{\text{NV}}} + \frac{5}{N^2 \omega_{\text{Larmor}}^2} \frac{\tau_{\text{trans}}}{1 + \omega_{\text{Larmor}}^2 \tau_{\text{trans}}^2}\right]
\]

As shown in Figure 3b, molecular dynamics (MD) simulation was used for dynamics in a small time scale of ≈ 50 ns to calculate \(r\), \(d\), \(N\), and \(\tau_{\text{rot}}\). The values of \(r\), \(d\), and \(N\) were all estimated from the integrated radially distributed function (RDF) (Figure S3b,c, Supporting Information). \(\tau_{\text{rot}}\) was determined by estimating the correlation time for vector defined along the acid chain of DPPC (Figure S3d, Supporting Information). The results from MD simulation were combined with Monte Carlo simulation to calculate the change in the probability of detection in nanoscale magnetometry with a diffusion constant as the only free parameter for fitting to our model. The change in the probability of detection was calculated using a 2D diffusion model, where the overlap between the diffusion area and detection area was calculated to extract the change in the probability of diffusion (Figure S4c,d, Supporting Information). The detection area was calculated in detail using a model from a previous report\textsuperscript{[13]} (Figure S1a,b, Supporting Information). In the Monte Carlo simulation, ≈ 1 nm proton layer observed on acid cleaned diamond surface\textsuperscript{[11,16,29]} has been included as an immobile proton layer on the bottom of lipid bilayer.\textsuperscript{[11]} This assumption is consistent with neutron scattering results on existence of proton layer on the bottom of lipid bilayer.\textsuperscript{[30,31]}

For the measurement of the phase transition in DPPC molecules, the temperature setting in the incubation chamber was used to change the temperature, and the NV center was used as a local probe for quantum thermometry using the thermal-echo (T-Echo) method. Pulse sequence was applied in a confocal setup at dual frequency to remove the effect of the magnetic field during measurement for precise measurement of \(D(T)\) values below 1 °C precision. The results are shown in Figure 3c,d, where the energy difference between the bright |0\rangle and dark |±1\rangle states were observed as oscillations in the T-Echo signal. The T-Echo signal without heat supply from the incubator resulted in a \(D(T)\) value of +0.10 MHz ± 24 kHz with respect to theoretical value of 2.87 GHz. The temperature at this measurement setting was calibrated with a K class thermocouple (WT 100) to be 26.5 °C. T-Echo measurement with a temperature setting of 45 °C in an incubator resulted in a \(D(T)\) value of -0.59 MHz ± 44 kHz with respect to theoretical value of 2.87 GHz. The difference in the value of \(D(T)\) between the two temperature settings provides a temperature difference of 9.45 °C with a 0.47 °C temperature precision determined by the distribution of the value for different
Figure 3.  
a) Schematic for modeling of lipid bilayer. Lateral diffusion and rotation were separated for different time scales. Dipolar interaction was conceived for each dynamic by considering intermolecular and intramolecular proton–proton distance. b) Typical example of molecular dynamics simulation on DPPC at 25 °C. Simulation was performed for 50 ns with 1 ps resolution. c) T-Echo measurement performed at 26.5 °C for different set frequency relative to $D(T)$ value. d) T-Echo measurement performed at 36.0 °C for different set frequency relative to $D(T)$ value. e) Typical example of correlation spectroscopy performed at 26.5 °C. f) Absolute value of correlation spectrum at 26.5 °C (1st and 2nd) and 36.0 °C are shown and compared with results of 2D molecular diffusion simulation.

frequencies of the applied pulse on T-Echo. All measurements at different temperature were carried after at least 12 hours of waiting time to stabilize temperature.

For both 26.5 °C and 36.0 °C, correlation spectroscopy was performed using an EMCCD camera as the detector for diffusion analysis of protons in DPPC molecules. The ODMR spectrum obtained from the NV center was used to determine the applied magnetic field of 71.8 mT. Example of result obtained from correlation spectrum for 1.6–20 µs is shown in Figure 3e. As shown in Figure 3e, oscillation at 3.06 MHz corresponding to Larmor frequency of proton was observed at 26.5 and 36.0 °C. The obtained data are compared with Monte Carlo simulation results in Figure 3e. As shown in Figure 3e, relaxation characteristic of the correlation spectrum obtained at 26.5 °C shows a comparable
character to the simulation with a diffusion constant $D_t$ of 1.5 nm$^2$ µs$^{-1}$. For detailed comparison of obtained data and Monte Carlo simulation, absolute value of correlation spectrum at 26.5 and 36.0 °C is plotted in Figure 3f and compared with results of 2D molecular diffusion simulation. Figure 3f shows as the temperature is increased to 36.0 °C, the relaxation characteristic of correlation spectrum shows result similar to the simulation with a diffusion constant of 3.0 nm$^2$ µs$^{-1}$. And correlation spectrum obtained at 26.5 °C shows relaxation characteristic similar to the simulation with a diffusion constant of 1.5 nm$^2$ µs$^{-1}$.

Difference in observed diffusion constant at different temperature is clearly demonstrated by comparison with simulation. And relaxation characteristics of simulation with different diffusion constant are clearly distinguished. Accuracy of this measurement was tested by measuring diffusion constant of DPPC molecules when temperature of sample was once again lowered to 26.5 °C. The result is shown in Figure 3f as 26.5 °C 2nd which shows complete overlap on relaxation characteristic with 26.5 °C 1st measurement demonstrating reversibility of phase transition with measured diffusion constant of 1.5 nm$^2$ µs$^{-1}$. This result is consistent with previous reports on measurement of reversibility of phase transition in lipid bilayer.[32] From correlation spectroscopy and comparison with 2D Monte Carlo simulation, diffusion constant of DPPC molecules have been estimated to be 1.5 ± 0.25 nm$^2$ µs$^{-1}$ at 26.5 °C and 3.0 ± 0.5 nm$^2$ µs$^{-1}$ at 36.0 °C with an error bar included for uncertainties (e.g., exact thickness of proton layer and exact location) caused by proton layer on the bottom of lipid bilayer. All measurements were performed on same location, calibrated at diffraction limited resolution (∼300 nm) by confocal scan of step-edge structure[36] observed on shallow ensemble NV centers. These measurements confirmed observation of phase change through change in the diffusion constant of DPPC molecules.

4. Conclusion

In this paper, we report determination of the diffusion constant of a lipid bilayer, a biological parameter that determines the dynamics of the lipid bilayer, by making use of extremely small detection volume defined by the depth of the NV center, allows label-free measurement of diffusion constants from cell membranes without complication imposed by sample preparation and measurement system. This technique paves way for diagnostics of cell membranes, where imaging of fluidity on each individual cells are necessary.

5. Experimental Section

**NV Measurement System:** The breadboard was placed inside Olympus IX-73 to guide the laser into the objective lens through a dichroic mirror. The detector side could be switched to an EM-CCD camera (iXonUltra) or a pinhole with an APD detector (SPCM-AQRH-14-FC-NIR) or a color CCD camera (N230CFL or DP53) through the adjustable placed on the lower deck of IX-73. The incubator was placed inside the piezo stage (P-545.3CS) to control the temperature with a thermostatted heater in order to change the temperature of the system. MW was delivered to the NV center through a 20 µm diameter copper wire with a sputtered Ti/Cu/Au electrode on a cover slide. Microwaves were delivered from the SG (Anritsu MG3700A and SynthHD PRO Dual RF Signal Generator) combined with an amplifier (R&K CGA701M62-444R or Amplifier Research Model 50W1000A). The pulse sequence was controlled by DTGS274 and the MW pulse was truncated by a switch (Mini circuit ZASWA-2-50DRA+). A high-power laser (Verdi G5) was pulsed through an AOM (Gooch Housedge Model:3250-220) with an RF driver (3910-XX).

**NV Center Sample:** Shallow NV ensemble was formed within 10 nm from the surface by the CVD.
growth technique on [111]-oriented diamond with step flow growth.16
NV centers that are used in this experiment are oriented toward [111] di-
rection. Diamond surface could be categorized into two parts, step edge
structure and terrace section.16 Overall surface roughness of 2–6 nm in-
duced by step edge structures and surface roughness of less than 2 nm is
achieved on terrace section of sample surface.
In the case of shallow ensemble NV center, performing nanoscale NMR
or correlation spectroscopy results in ensemble averaged detection of
each individual single NV center with detection volume defined by the
depth of NV center. In the optical readout process, each phase detected
at individually independent (ensemble) NV centers were readout as aver-
age from optically defined detection area. This type of detection is gener-
alized as an ensemble average detection of NV center which has
age from optically defined detection area. This type of detection is gener-
at individually independent (ensemble) NV centers were readout as aver-
aged over 20 pixels spectroscopy for better signal-to-noise ratio observed by obtaining data
ternal resolution on each pixel. EMCCD camera was used for all correlation
scope.

Laser Spot Size and Number of NV Center Used for Each Measurement:
In this paper, optically defined area was ≈300 nm diameter defined by optical
diffraction-limited FWHM of Gaussian laser profile. Density of NV centers used in this experiment are ≈1016 cm−3 corresponding to ≈10 NV
centers per optically defined detection spot for the case of confocal micro-
scope.
Spot size of laser used for EMCCD camera detection was 120 µm diam-
eter and fluorescence was projected to EMCCD camera with ≈300 nm spa-
tial resolution on each pixel. EMCCD camera was used for all correlation
spectroscopy for better signal-to-noise ratio observed by obtaining data
averaged over 20 pixels × 20 pixels on EMCCD camera. Number of NV
center used to detect data for correlation spectrum using EMCCD camera
was ≈4000 NV centers.

Preparation Method of DPPC Supported Lipid Bilayer: Lissamine
Rhodamine B 1,2-dihexadecanoyl-sn-Glycero-3-Phosphoethanolamine
(Rhodamine-PE, Thermo Fisher Scientific) was mixed at 1% mol with 1,2-
dipalmitoyl-sn-glycero-3-phosphocholine (DPPC, Avanti Polar Lipids) in PBS
buffer. DPPC was dissolved in PBS at 0.5 mg mL−1 concentration and sonicated in a warm water bath at 60 °C for 5–15 min. After sonication,
the sample was incubated for at least 1 h on a hot plate at 70 °C. After
incubation, the sample was sonicated in a warm water bath at 60 °C until a transparent sample was obtained. CVD-grown diamond samples with a shallow NV center were exposed to an acid treatment with a 1:3 mixture of HNO3 and H2SO4 at a hotplate temperature of 400 °C for 45 min,
followed by rinsing with DI water to obtain an oxygen-terminated surface.
XPS measurements of the acid-treated diamond samples confirmed
increased oxygen coverage for confirmation of the oxygen termination
of the surface. 100 µL of DPPC/Rhodamine-PE solution was deposited on
top of diamond sample and incubated for 20 min. After incubation,
1 mL of PBS solution was injected onto diamond, and sample wash was
performed ten times.

MD Simulation: The MD simulations were conducted using the Fuj-
itsu PRIMEGY CX600M1/CX1640M1 (Oakforest-PACS) and SGI Rackable
C1102-GP8 (Redbush).

Supporting Information
Supporting Information is available from the Wiley Online Library or from the
author.

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Author Contributions
H.I. conceived the concept, built the experimental setup for nanoscale
NMR, correlation spectroscopy, and T-echo, fabricated shallow ensemble
NV center, fabricated lipid bilayers, performed the measurements, built
Python code for Monte Carlo 2D molecular diffusion analysis, analyzed the
data, and wrote the manuscript. H.C.W. built and performed the MD simu-
lation. S.H. advised lipid bilayer discussion and preparation. H.I., H.C.W.,
S.H., T.I., and M.H. discussed the study.

Data Availability Statement
Data available on request from the authors.

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