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ABSTRACT

Unsaturated lipids with C=C groups in their alkyl chains are widely present in the cell membrane and food. The C=C groups alter the lipid packing density, membrane stability, and persistence against lipid oxidation. Yet, molecular-level insights into the structure of the unsaturated lipids remain scarce. Here, we probe the molecular structure and organization of monolayers of unsaturated lipids on the water surface using heterodyne-detected sum-frequency generation (HD-SFG) spectroscopy. We vary the location of the C=C in the alkyl chain and find that at high lipid density, the location of the C=C group affects neither the interfacial water organization nor the tail of the alkyl chain. Based on this observation, we use the C=C stretch HD-SFG response to depth-profile the alkyl chain conformation of the unsaturated lipid. We find that the first 1/3 of carbon atoms from the headgroup are relatively rigid, oriented perpendicular to the surface. In contrast, the remaining carbon atoms can be approximated as free rotators, introducing the disordering of the alkyl chains.

INTRODUCTION

The lipid or surfactant mono-/bi-layer appears in the biological environment as well as in food.[1,2] The lipid/surfactant mono-/bi-layer consists of saturated and unsaturated lipid/surfactant, where "unsaturated" means the presence of one or more C=C groups within the alkyl chain of the lipid/surfactant. This C=C group of the *cis*-structure causes the kink of the alkyl chain, affecting the packing density, structural stability, and fluidity of the membrane,[3–5] persistence against oxidation of the lipid/surfactant monolayer,[6–8] and interaction of membranes with small and large molecules, including cholesterol and β-amyloid peptide.[9–11] Despite the importance of unsaturation, details of the molecular organization of unsaturated lipids, such as the organization of the alkyl chains, are largely unknown.[12] Here, we employ advanced vibrational spectroscopy to probe the unsaturated 18:1 sn-glycero-3-phosphocholine (18:1 PC) lipids with different C=C group positions and explore the alkyl chain organization of the 18:1 PC lipid monolayer (6-*cis*, 8-*cis*, 9-*cis*, and 11-*cis*) [see the chemical structure in Fig. 1(a)].

We employ heterodyne-detected sum-frequency generation (HD-SFG) spectroscopy as a label-free, vibrational probe. In HD-SFG, a second-order optical technique using infrared (IR) and visible laser beams, the sum-frequency of those two beams is generated upon reflection from the surface. Due to the selection rule of SFG, the signal from the centrosymmetric bulk region is prohibited, allowing us to obtain the signal arising solely from the interface. The HD-SFG signal is enhanced when the IR beam frequency is resonant with the molecular vibration of the system, providing molecule-specific information. Furthermore, HD-SFG allows us to identify the absolute orientation of the molecules, e.g., pointing *up* or *down* with respect to the interface,[13] by enabling the determination of the orientation of the specific molecular moiety. As such, HD-SFG is an ideal probe for the lipid/surfactant–water interfaces. Indeed, HD-SFG has been used frequently for probing such interfaces.[14–23] However, the probe for the alkyl chain has been limited to the vibrations of the

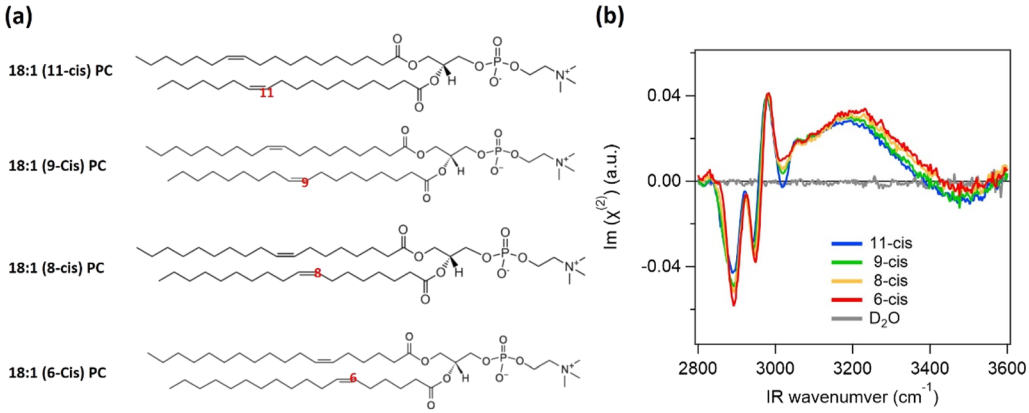

FIG. 1. (a) Unsaturated 18:1 PC lipids studied in this work. (b) Measured Im $\chi^{(2)}_{ssp}$ spectra at the lipid-H_2O interface in the C—H stretch and O–H stretch modes frequency region. The Im $\chi^{(2)}_{ssp}$ spectrum of the air-D_2O interface is also shown to ensure that the phase of the HD-SFG is accurate.

terminal $-CH_3$ group or $-CH_2-$ groups. Here, by probing the C=C stretch mode of the unsaturated part as well as the various C—H stretch modes of the alkyl chains and the C=O stretch mode of the carbonyl group, we aim to uncover the orientation of the alkyl chains for the unsaturated lipids.

Here, we probe the 18:1 PC lipid monolayer with the four different C=C group positions on the water surface in the disordered liquid crystalline phase (at room temperature) at a saturated concentration of lipids. Our C—H stretch HD-SFG data of the terminal $-CH_3$ groups as well as the surface tension data indicate that at the saturated concentration of lipid, the alkyl chain tail conformation of 18:1 PC and interfacial water conformation are indistinguishable among the studied 18:1 PC monolayers. In contrast, we find that the C=C stretch mode HD-SFG spectra differ significantly with different C=C group positions. By analyzing the C=C stretch mode spectra, we find that not all alkyl chains behave as free rotators. Up to the position of the sixth carbon atom from the headgroup, the alkyl chains are almost completely ordered, while beyond the eighth carbon atom, it behaves approximately as a free rotator.

EXPERIMENTAL PROCEDURE

Sample preparation

The 18:1 sn-glycero-3-phosphocholine (18:1 PC) lipids with different C=C bond positions (6-*cis*, 8-*cis*, 9-*cis*, and 11-*cis*) were purchased from Avanti Polar Lipids and used as received. We dissolved these 18:1 PC lipids in a mixture of 90% chloroform (Fischer Scientific, stabilized with amylene, >99%) and 10% methanol (VWR Chemicals, 99.8%), at a concentration of 4.3×10^{-4} mol/l. For probing the C—H stretch mode, we used H_2O, which was obtained from a Milli-Q system (resistivity ≥ 18.2 MΩ cm and TOC ≤ 4 ppb). For probing the C=C stretch mode, to avoid the overlap between the C=C stretch mode (~1645 cm^{-1}) and the H—O—H bending mode (~1650 to 1750 cm^{-1}), we used D_2O (>99.9%), which was purchased from Sigma-Aldrich.

The 20 ml of H_2O or D_2O were poured into a PTFE trough with a diameter of 8 cm. Subsequently, we added ~50 μl of lipid solution to the water surface using a click syringe. Assuming all the deposited lipids end up in the monolayer, we calculate the surface area per lipid molecule to be ~39 $Å^2$, where the 18:1 PC membrane is assumed to be tightly packed.[24,25] The prepared samples were equilibrated for at least 15 min. The trough was rotated to avoid lipid monolayer distortion due to heat accumulation.[20] The speed of the sample at the laser irradiation spot was ~1.0 cm/s.

Heterodyne-detected SFG measurement

For probing the C—H stretch mode, we used a visible pulse centered at ~800 nm and a tunable IR pulse with incident angles of 64° and 50°, respectively. The IR and visible beams were first focused onto a 200 nm-thick ZnO thin film deposited on a 1 mm-thick CaF_2 window to generate the local oscillator (LO) signal.[26,27] A 1.5 mm-thick fused silica plate was inserted in the optical path of the LO beam. The IR, visible, and LO beams were then focused onto the sample surface. The SFG signal from the sample interfered with the LO signal, generating the SFG interferogram. The SFG interferogram was decomposed into the frequency domain through a spectrometer (Shamrock 303i, Andor Technology) and, subsequently, detected by an EMCCD camera (Newton, Andor Technology).

For probing the C=C stretch mode, we used a visible pulse centered at ~800 nm and a tunable IR pulse, which were combined collinearly. They were first focused onto a 20 μm-thick *y*-cut quartz plate to generate the LO signal. Subsequently, they passed through an 8 mm-thick CaF_2 plate for the phase modulation and were again focused onto the sample surface with their incident angles of 45°. The SFG signal from the sample interfered with the LO beam, generating the SFG interferogram. The SFG interferogram was decomposed into the frequency domain through a spectrometer (SpectraPro HRS-300, Princeton Instruments) and detected by a liquid N_2-cooled CCD (PyLoN®, Princeton Instruments). The measurement was done at room temperature.

We normalized the sample's SFG signals with those obtained from a *z*-cut quartz crystal.[13] All the measurements were performed

with the *ssp* polarization combination (denoting the *s*-, *s*-, and *p*-polarizations of SFG, visible, and IR beams, respectively). To avoid the effect of IR absorption by water vapor, the optical path within the SFG setup was purged with N_2. Further details of these setups have been presented in Refs. 20 and 25.

RESULTS AND DISCUSSION

First, we examine whether the location of the C=C bond of the alkyl chain in the unsaturated lipids alters the organization of the lipid monolayer. In principle, the precise location of the C=C bond in the alkyl chain is important. For instance, that location determines the lipid phase transition temperature, at which the lipid physical state changes from the ordered gel phase, with fully extended and tightly packed hydrocarbon chains, to the disordered liquid crystalline phase. In particular, for 18:1 PC lipids with one C=C bond studied here, the transition temperature is 1 °C when the double bond is located at the sixth carbon atom from the headgroup, whereas it is −17 °C when it is located at position 9.[28,29] Extrapolating these transition temperatures, it appears apparent that in our room temperature experiments, the studied 18:1 PC lipids are in the disordered liquid crystalline phase. In the disordered liquid crystalline phase with saturated lipid concentration, the measured surface tensions were 26.5, 26.8, 26.5, and 27.6 (±0.5) mN/m for the 6-*cis*, 8-*cis*, 9-*cis*, and 11-*cis* PC samples, respectively, and were very similar.[25] The similarity of the surface tensions for these lipids, independent of the location of the C=C group of the studied 18:1 PC lipids, implies that the conformations of the studied 18:1 PC lipids are similar.

This notion is further confirmed by the SFG spectra of the C−H stretch mode of lipids. Figure 1(b) shows the variation of the C−H stretch modes. The negative 2880 and positive 2970 cm^{-1} peaks arise from the symmetric and antisymmetric C−H modes of the terminal $-CH_3$ group, respectively, while the negative 2950 cm^{-1} peak arises from the Fermi resonance of the C−H stretch and bend overtone.[21,30] The signs of these peaks indicate that the terminal $-CH_3$ group points *up* to the air.[16,31] The data show that the three C−H stretch peaks associated with the terminal $-CH_3$ group are similar, indicating that the organization of the alkyl chain's tail is similar among the studied unsaturated 18:1 PC lipids. In addition, the SFG data show a broad 3050–3400 cm^{-1} band arising from water's O–H stretch mode. The similar band feature of the water-18:1 PC lipid interface with different locations of the C=C group indicates that the interfacial water organization near the 18:1 PC is also insensitive to the location of the C=C group. As such, the location of the C=C group is insensitive to the structure of the unsaturated lipid monolayer and water. Note that we observed no clear signature at 2850 cm^{-1} in contrast with Ref. 8. This 2850 cm^{-1} peak arises from the C−H stretch mode of the $-CH_2-$ group and is associated with the gauche defect in the acyl chains.[8,21,32] Its amplitude depends highly on the surface area per lipid;[32] the decrease in the surface area leads to a drastic increase in the 2900 cm^{-1} symmetric C−H stretch mode peak of the $-CH_3$ group, masking the 2850 cm^{-1} peak (see the supplementary material). Because we performed the measurements at a saturated concentration of lipids, the C−H stretch peak of the $-CH_2-$ group is almost negligible. This might imply that the net orientation of the transition dipole moment of the CH_2 group is parallel to the surface.

The insensitivity of the location of the C=C group to the organization of the water-18:1 PC lipid interface suggests that the C=C stretch mode probe can be used as a marker of a specific part of the alkyl chain. Based on this motivation, we probed the C=C stretch mode via the HD-SFG technique. Before measuring the C=C stretch mode, we explored the sign of the $\mathrm{Im}\,\chi^{(2)}_{ssp}$ with the orientation of the *cis* $-CH_2-CH=CH-CH_2-$ moiety. The C=C stretch mode does not create the transition dipole moment along the C=C bond direction, while the *cis*-conformation of the $-CH_2-CH=CH-CH_2-$ moiety creates the transition dipole moment perpendicular to the C=C bond on the $-CH=CH-$ plane because the C=C stretch migrates the charge from the carbon atom to the hydrogen atom. The density functional theory calculation at the B3LYP/aug-cc-pVTZ level of theory for the CH_3–$CH=CH-CH_3$ molecule indicates that when the vector pointing from the middle of two carbon atoms to the middle of the two hydrogen atoms for the HC=CH part is *up*- (*down*-) oriented, the sign of the $\mathrm{Im}\,\chi^{(2)}_{ssp}$ peak is negative (positive). The molecular orientations of the $-CH=CH-$ group and the corresponding sign of the $\mathrm{Im}\,\chi^{(2)}_{ssp}$ peak are schematically depicted in Figs. 2(a) and 2(b), respectively.

With the information on the C=C stretch mode, we measured the HD-SFG spectra of the C=C stretch mode for the unsaturated 18:1 PC lipids. The spectra are displayed in Figs. 2(c)–2(f). The data show positive 1710 cm^{-1} and negative 1740 cm^{-1} peaks, together with a negative 1645 cm^{-1} peak. The positive 1710 and negative 1740 cm^{-1} peaks are assigned to the C=O stretch modes of the *trans*- and *cis*-conformations of the C−CO−O−C group.[33] Similar $\mathrm{Im}\,\chi^{(2)}_{ssp}$ amplitude of the C=O stretch modes further illustrates that the lipid conformation C=O is insensitive to the location of the C=C group. In contrast, the 1645 cm^{-1} negative peak is strongly affected by the location of the C=C group. This 1645 cm^{-1} peak arises from the C=C stretch mode. The negative sign of the 1645 cm^{-1} peak signifies that the C−H vector of the $-CH=CH-$ part points out of the surface toward the air. Although the $\mathrm{Im}\,\chi^{(2)}_{ssp}$ peak amplitude depends on the surface density and orientations of vibrational chromophores; the surface density of the lipid is unchanged, as is evidenced by the surface tension data and C−H/C=O stretch SFG data. Thus, the drastic changes in the 1645 cm^{-1} peak amplitude can be attributed to the change in the orientation of the C=C group, considering that the surface density of the 18:1 PC lipids is unchanged among the studied 18:1 PC lipids at saturated concentrations of lipids.

The $\mathrm{Im}\,\chi^{(2)}_{ssp}$ spectra commonly show the positive offset. This is not because of the phase error of our HD-SFG measurement, as our $\mathrm{Im}\,\chi^{(2)}_{ssp}$ spectra at the air-D_2O interface do not show any offset (see the supplementary material). In fact, such an offset has previously been reported at the charged lipid–water interface,[19,34,35] and our result illustrates that such an offset occurs even at the zwitterionic lipid–water interface.

To quantitatively evaluate the ordering degree of the lipid alkyl chains, we plot the peak areas vs the location of the C=C group. This data is displayed in Fig. 3. Notably, the peak area is nearly zero for the 6-*cis* sample, while it clearly becomes nonzero for the 9-*cis* sample. The negligibly small peak area for the 6-*cis* sample is somewhat surprising because it indicates that at the sixth position, the C=C group (the C=C stretch transition dipole moment) is almost parallel

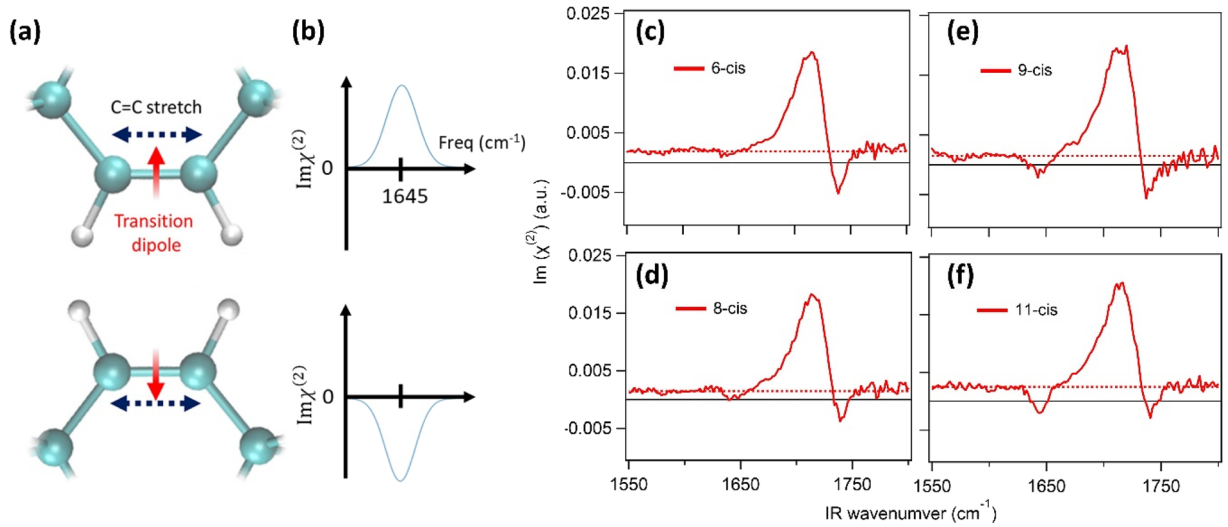

FIG. 2. (a) Schematic of the different conformations of the C=C group and the corresponding orientations of the transition dipole moment with respect to the C=C stretch. (b) The sign of the Im $\chi^{(2)}_{ssp}$ peak for corresponding geometries. (c)–(f) Measured Im $\chi^{(2)}_{ssp}$ spectra at the 18:1 PC-D_2O interfaces in the C=C stretch mode and C=O stretch mode frequency regions. The dotted lines represent the offset, determined from the average value between 1550 and 1600 cm^{-1}.

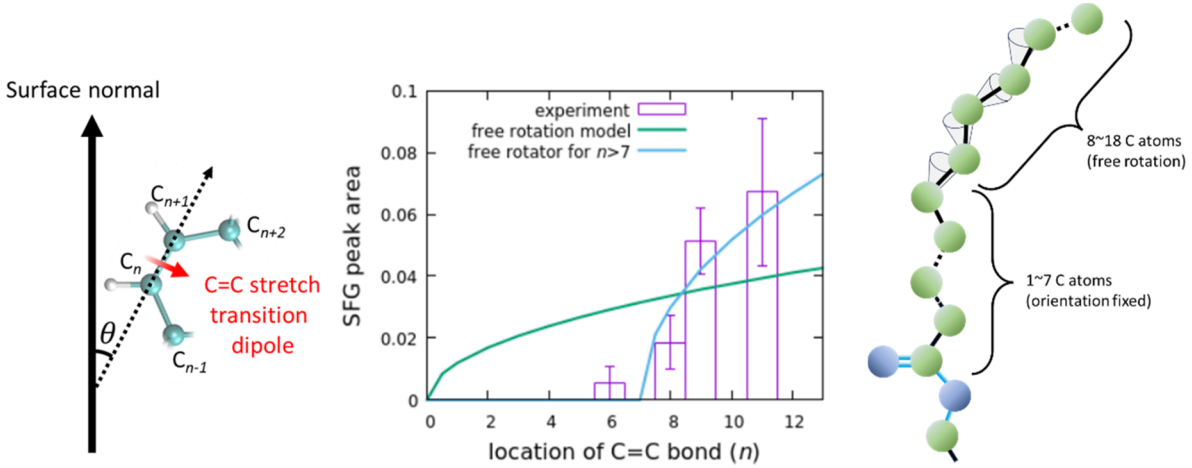

FIG. 3. (Left) Schematic of the C_n=C_{n+1} bond orientation and its corresponding transition dipole direction. (Middle) Comparison of the experimental SFG peak area vs the modeled peak area using Eqs. (1)–(3). (Right) Schematic of the alkyl chain part and carbonyl part of the PC lipid. Blue and green balls represent oxygen and carbon atoms, respectively.

(perpendicular) to the surface normal. In contrast, the C=C group at the ninth position is tilted out of the surface plane.

Subsequently, we model the SFG amplitude by using the free-rotating alkyl chains model in one-dimensional space. We assume that the orientational distribution of the C_n=C_{n+1} group with respect to the surface normal can be described by

$$f_n(\theta) = N \exp\left(-\theta^2/2n\sigma^2\right), \quad (1)$$

where C_n denotes the carbon atom at position n, N is the normalization coefficient, θ is the angle formed by the C=C group and the surface normal, and σ is the standard deviation of the orientational distribution of a $-CH_2-CH_2-$ group in the one-dimensional space. The SFG peak area of the C_n=C_{n+1} stretch mode, A_n, is then proportional to

$$A_n \propto \int f_n(\theta) \sin\theta d\theta. \quad (2)$$

Here, we assumed that the C=C bond and C=C stretch bond transition dipole moment are orthogonal [see Fig. 2(a)]. By assuming σ = 0.1 rad, we plot the variation of the SFG peak area vs C=C bond location and compare it with the experimental data. The trend predicted by Eq. (2) cannot reproduce the trend of the experimental SFG peak area. Apparently, the whole alkyl chain of the lipid does not behave as a free rotator. We note that the value of σ does not affect the trend predicted by Eq. (2).

The depth distribution of the alkyl chain density at the lipid–water interface computed from the molecular dynamics simulation shows that the alkyl chain near the headgroup is more crowded than the tail of the alkyl chain.[36] This occurs because the headgroup positions are not the same. In a crowded alkyl chain region, the alkyl chain is expected to be less disordered, against the assumption used above that the whole alkyl chain behaves as a free rotator. The restricted rotation of the alkyl chain close to the headgroup part is consistent with our observation that the C=C stretch mode Im $\chi^{(2)}_{ssp}$ peak is negligibly small. Based on these facts, we consider the modified model that a free rotation starts at the C=C bond position of m. The corresponding distribution function is given by

$$f_n(\theta) = \begin{cases} \delta(\theta) & \text{for} \quad n \le m, \\ N \exp\left(-\theta^2/2(n-m)\sigma^2\right) & \text{for} \quad n > m. \end{cases} \tag{3}$$

The SFG peak area predicted from the modified free rotation model with m = 7 is also shown in Fig. 3. Despite the simplicity of the model combining fixed orientation for $n \le m$ and free rotation for $n > m$, the SFG peak area trend is well reproduced. This indicates that, effectively, the disorder pattern can be described by a combination of the fixed orientation at a position below 7 and free rotation at at a position above 7 of the 18 carbon atoms of the alkyl chains, which promotes the disordering of the alkyl chains.

Finally, we compare the vinyl group symmetric C—H stretch mode at 3110 cm^{-1}[25,37] with the C=C stretch mode at 1645 cm^{-1}. Essentially, both provide information on the transition dipole moment perpendicular to the C=C group. In fact, Fig. 1(b) reveals a similar trend of the 3110 cm^{-1} vinyl C—H stretch mode to the C=C stretch data in Fig. 3(b); the 6-*cis* sample shows a very tiny vinyl peak feature, while the 11-*cis* shows a large negative peak. However, quantifying the peak area of the vinyl C—H stretch peak is rather challenging, even for the HD-SFG data, because the 2980 cm^{-1} positive peak arising from the antisymmetric C—H stretch mode of the terminal $-CH_3$ group is nearby, prohibiting reliable quantification of the vinyl group contribution. As such, the C=C stretch mode provides a more direct means to connect the morphology of the C=C bond conformation than the vinyl C—H stretch mode.

CONCLUSION

We performed the HD-SFG measurement for the unsaturated 18:1 PC lipid–water interface with different locations of the C=C bond. The data show that the orientation of the terminal CH_3 group, interfacial water organization, and the surface tension value are insensitive to the location of the C=C bond at the saturated concentration of lipid. By utilizing the C=C stretch mode as a marker of the local orientation of the alkyl chain, we examined how the disordering evolves along the length of the alkyl chain. We found that, coming from the headgroup, the first 1/3 of the alkyl chain of the 18:1 PC lipid is almost perpendicular to the surface due to the steric effect caused by the crowded alkyl chain environment. In contrast, the remaining 2/3 of the alkyl chain can be described very well as a free rotator.

SUPPLEMENTARY MATERIAL

The supplementary material includes (1) the Fresnel factor correction on HD-SFG spectra, (2) the surface density dependence of the methylene C—H stretch band amplitude, (3) variations of the vinyl C—H stretch amplitude of the C=C groups, (4) the surface pressure-area (P-A) isotherm of the lipid monolayers, and (5) the phase accuracy of the HD-SFG measurements.

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AUTHOR DECLARATIONS

Conflict of Interest

The authors have no conflicts to disclose.

Author Contributions

Chun-Chieh Yu: Data curation (equal); Validation (equal); Writing – review & editing (equal). **Takakazu Seki**: Data curation (equal); Validation (equal); Writing – review & editing (equal). **Kuo-Yang Chiang**: Data curation (equal); Writing – review & editing (equal). **Yongkang Wang**: Data curation (equal); Writing – review & editing (equal). **Mischa Bonn**: Supervision (equal); Writing – review & editing (equal). **Yuki Nagata**: Conceptualization (equal); Formal analysis (equal); Supervision (equal); Writing – original draft (equal); Writing – review & editing (equal).

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

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