Electronic Supplementary Material (ESI) for Nanoscale.

Supporting Information

Nanoscale Visualization of Phase Separation in Binary Supported Lipid Monolayer using Tip-Enhanced Raman Spectroscopy

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Experimental details

1. Preparation of Langmuir-Blodgett films

360 µl of d_{62}-DPPC (10 mg/ml) and 140 µl of DOPC (25 mg/ml) (>99%, Avanti Polar Lipids, USA) were dissolved in chloroform (99.9%, Acros Organics, USA) and used to make a 1:1 stock solution of d_{62}-DPPC:DOPC. The stock solution was sequentially diluted 2× and 7× to get 4.6×10^{-7} M d_{62}-DPPC:DOPC working solution.

Au (111) substrates were prepared by the Clavilier method[1] and annealed in a hydrogen-oxygen flame before every experiment. Au substrates were then placed inside a UV-ozone cleaner (Ossila, UK) for 10 minutes to remove organic contamination from the surface. A Langmuir-Blodgett (LB) trough (KSV, Minitrough, Finland) placed on an antivibration table in a dust-free environment, was thoroughly cleaned using 5% detergent followed by copious amounts of water. The Au substrates were then transferred to the LB trough with the Au surface immersed in the water, which was used as subphase. 20 µl of d_{62}-DPPC:DOPC (1:1) solution was carefully transferred to the water surface inside the LB trough using a glass syringe for subsequent experiments. The chloroform was allowed to evaporate for 30 minutes. The compression rate was set to 10 mm/minute and surface pressure was measured using a Wilhelmy balance with a precision of 0.01 mN/m. Finally, the monolayers were transferred onto Au (111) substrates at 30 mN/m surface pressure and a transfer speed of 2 mm/minute via vertical LB technique. All monolayer transfers were performed at room temperature. The successful transfer of the lipid monolayer on Au(111) was verified using AFM topography imaging performed using peak force tapping mode AFM (Biocatalyst, Bruker, USA) with Scanasyst-Air silicon nitride cantilevers (Bruker, USA), with a scan rate of 0.5 Hz and resolution of 256 pixels per line.

Noteworthy, numerous precautions were taken to ensure that the transfer process itself didn't perturb the phase separation of the lipid monolayer formed at the air/water interface. Firstly, the transfer was performed at a high surface pressure to make the lipid molecules more tightly packed, which made it more difficult for them to diffuse and perturb the equilibrium phase. Secondly, a slow withdrawal speed was used to give the lipid molecules more time to organize themselves in the equilibrium phase. Thirdly, compared to the phase transition temperature (Tm = 36.7 °C) of d_{62}-DPPC, a lower temperature (20-25 °C) was used to slow down the diffusion of the lipid molecules especially at high surface pressure. Fourthly, the transferred monolayers were measured using TERS immediately after preparation. Furthermore, samples were quickly transferred to the STM-TERS measurement chamber where the measurement was performed under a nitrogen environment to prevent oxidation of the domains, which can potentially affect their size. This ensured that the size of the domains transferred from the trough does not change[2]. And finally, a constant surface pressure of 30 mN/m was maintained throughout the transfer process. Notably, no sample degradation was observed during TERS measurements.

2. STM-TERS setup

TERS measurements were performed using a combined top-illumination STM/Raman microscope (NT-MDT, Russia) that combines an STM with a Raman spectrometer and a CCD detector (Andor, Ireland)[3]. A 632.8 nm He–Ne laser (Spectra-Physics, Newport, Germany) with an output power of 17 mW (3 mW on the sample) was used as the excitation source. A 100×, 0.7 numerical aperture objective lens (Mitutoyo, Japan) was used

for excitation as well as collection of TERS signals. TERS spectra were measured with a spectrometer grating of 600 lines/mm. Hyperspectral STM-TERS imaging was performed with a bias voltage of 1V, tunneling current set-point of 0.10 nA, laser power of 100 μW and a spectral integration time of 1 s per pixel. The 40 nm step size was chosen in order to image a large sample area (2×2 μm^2) within a reasonable time to minimize the influence of thermal drift on the TERS imaging. Note that in our instrument, it is technically not possible to simultaneously measure the fingerprint, C-D and C-H stretching regions of a Raman spectrum due to the long focal length of our spectrometer. Moreover, no TERS signals were observed in the fingerprint region, due to the following two factors: (1) A low laser power (100 μW) was used for the TERS measurements to avoid damage to the lipid monolayer and modification of the Ag tip-apex via thermal heating. (2) A low integration time of 1 s per pixel was used for TERS imaging to limit the total measurement time to below 30 minutes, which is necessary to mitigate the effect of sample drift. Therefore, due to the low laser power and low spectrum integration time, no TERS signals could be observed in the fingerprint region and the TERS analysis of the lipid monolayers was performed in the C-D and C-H stretching regions only.

3. Preparation of TERS probes

Electrochemically etched Ag wires (0.25 mm diameter, 99.99% purity, Aldrich, Germany) were used as the STM-TERS probes. A mixture of 1:4 (v/v) perchloric acid:methanol was used as the etchant. Ag wire was first cut to an appropriate length and then immersed into the etching solution with a 1 mm diameter Pt wire loop acting as counter electrode. A voltage of 10 V was applied between the electrodes and switched off using a custom-made circuit. Etching was stopped when a steep drop in current occurred because of the formation of the tip and the loss of the immersed part of the wire. After etching, the tips were rinsed with methanol and water and visually inspected using an optical microscope (Nikon, Japan). The tip etching procedure reproducibly yielded sharp Ag tips with <100 nm diameter that provided a high TERS signal enhancement and good STM imaging. All TERS experiments were performed using freshly etched Ag tips.

4. Data analysis

Data analysis was performed using a custom python script (Python 3.7.5) and Origin software (OriginLab Corporation, USA). Background subtraction of the raw TERS spectra was carried out using the asymmetric least squares method. A Savitzky-Goley smoothening was applied to the spectra measured in the TERS images and the smoothened spectra were used for PCA analysis with SciPy Python library. The first five principal components (PCs) were used for t-SNE analysis, and the obtained t-SNE dimensions were used for Hierarchical Clustering Analysis (HCA). For calculation of the I_{2960}/I_{2850} ratio, pixels with maximum intensity less than 3× the standard deviation of background (signal free region) were removed from the analysis. The area under the band was integrated using Simpson's rule and used for the ratio calculations. Deconvolution of averaged TERS spectra was performed using Origin software. The AFM images were processed using Gwyddion software (Gwyddion 2.54). All TERS images were normalized with laser power and acquisition time. The overlay image in Figures 2e and 4e was prepared using the Addition Layer Mode of the GIMP (GNU Image Manipulation Program) software. In this mode, the RGB color values of the upper and lower layers at each pixel are added to each other. In the resulting image, sometimes the addition equation can result in color values of greater than 255, which are reset to the maximum value of 255.

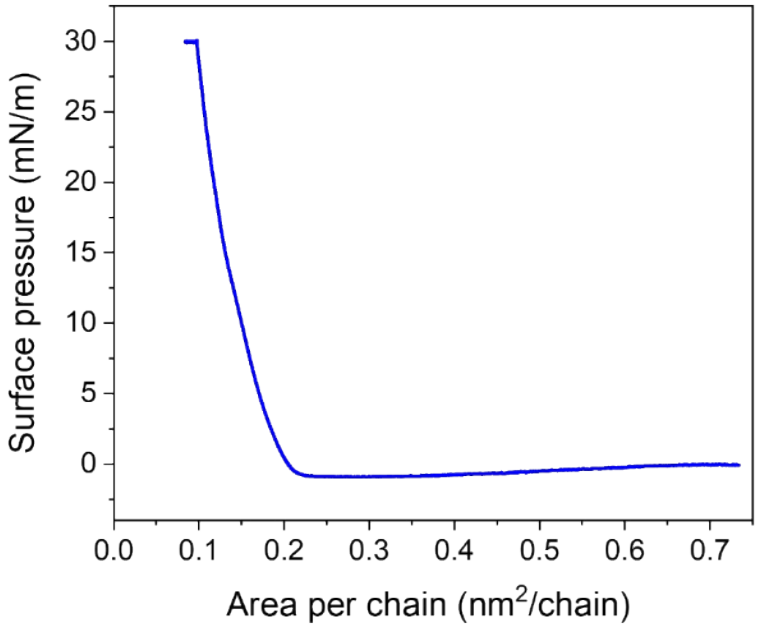

Figure S1. Langmuir isotherm of the d_{62}-DPPC:DOPC (1:1) monolayer transferred at 30 mN/m on Au (111) surface using LB method.

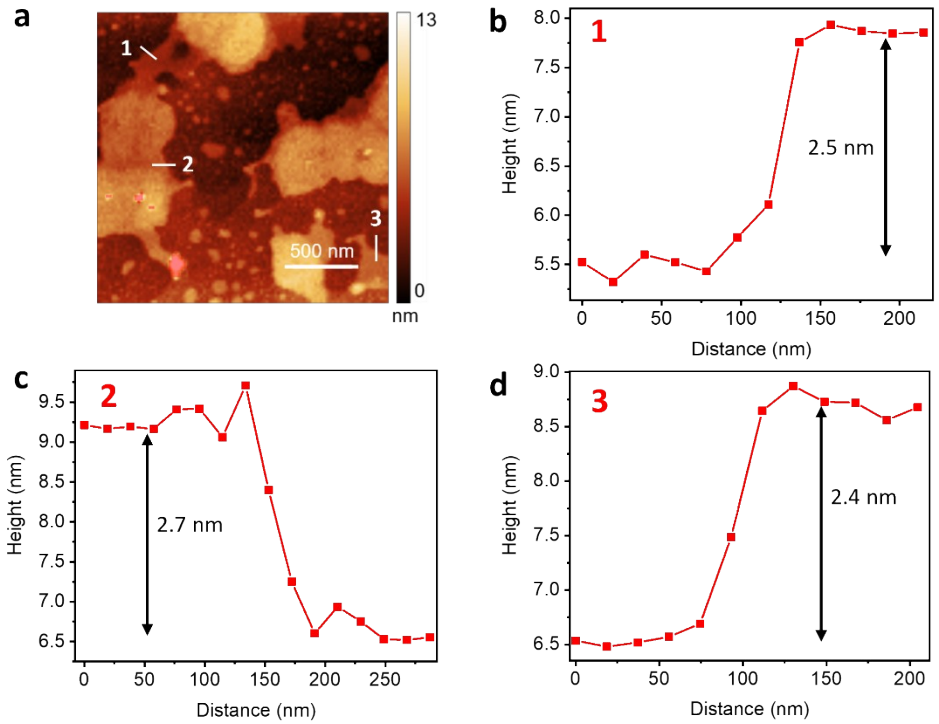

Figure S2. (a) AFM topography image of a d_{62}-DPPC:DOPC monolayer on Au (111) surface transferred using LB method. Step size: 19.5 nm. Scan rate: 0.5 Hz. (c-d) Height profiles along the lines marked as 1 - 3 in Panel a, respectively. The average height of the transferred monolayer is calculated to be 2.5 ± 0.2 nm.

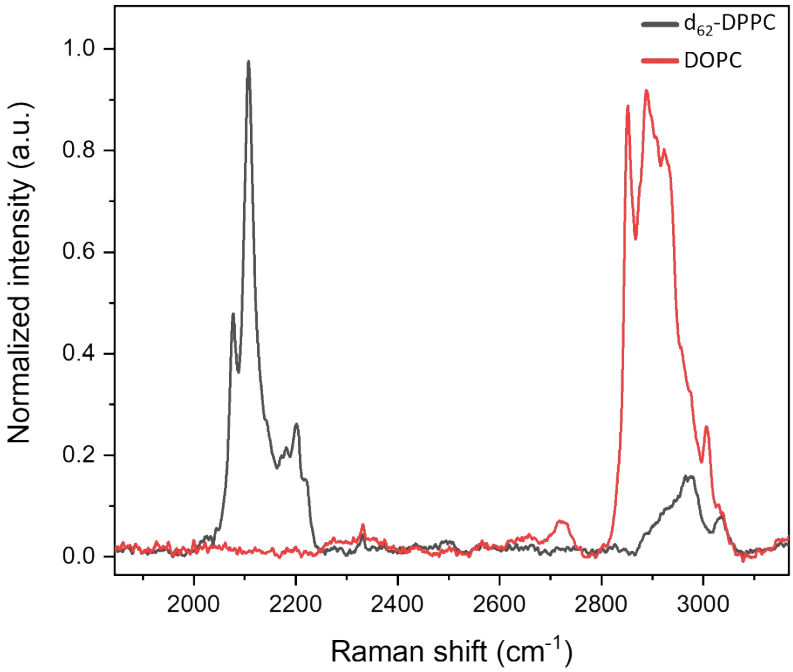

Figure S3. Confocal Raman spectra of d_{62}-DPPC and DOPC thin films normalized to the integration time and laser power. C-D stretching region is dominated by the d_{62}-DPPC signal whereas the C-H stretching region is dominated by the DOPC signal.

Table S1. Assignment of the d_{62}-DPPC and DOPC Raman signals observed in the TERS spectra.

Lipid	Raman peak	Assignment[4-6]
DOPC	2850 cm^{-1}	$\nu_{s(CH_2)}$
DOPC	2920 cm^{-1}	$\nu_{a(CH_2)\,FR}$
DOPC	2930 cm^{-1}	$\nu_{s(CH_3)\,FR}$
DOPC	2960 cm^{-1}	$\nu_{a(CH_3)}$
d_{62}-DPPC	2070 cm^{-1}	$\nu_{s(CD_3)}$
d_{62}-DPPC	2110 cm^{-1}	$\nu_{s(CD_2)}$
DOPC	3050 cm^{-1}	$\nu_{a(CH_3)Choline}$

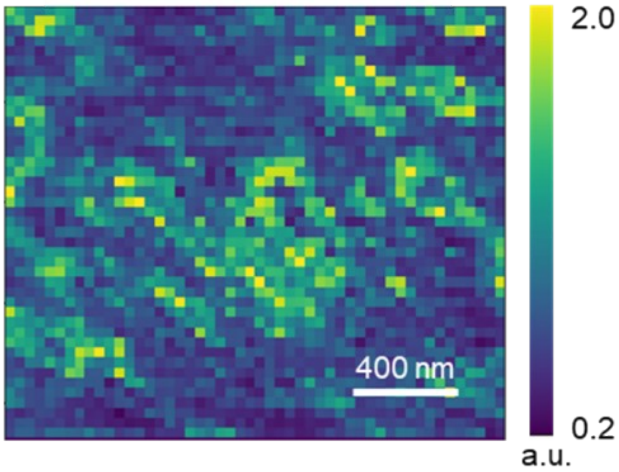

Figure S4. TERS image of the C-D and C-H signals ratio ($I_{C\text{-}D}$ / $I_{C\text{-}H}$) of the d_{62}-DPPC:DOPC monolayer region shown in Figure 2. The high intensity areas of the image represent the d_{62}-DPPC abundant regions.

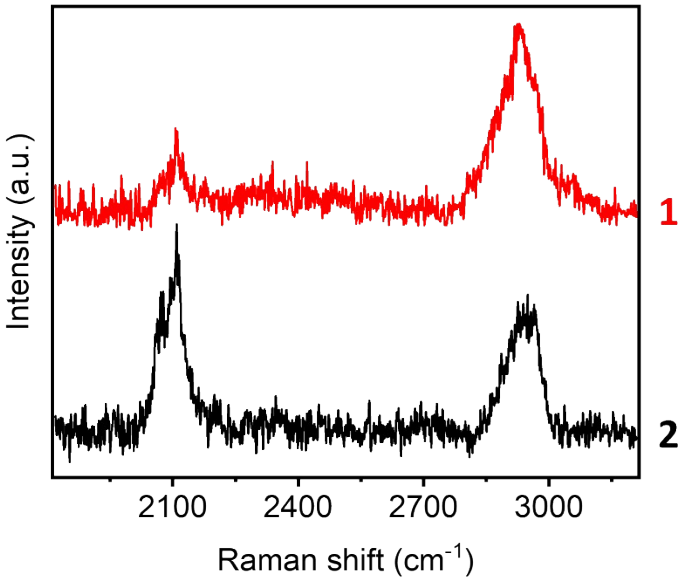

Figure S5. TERS spectra measured at the positions marked as 1 and 2 in Figure 2e. Position 1 shows a relatively higher C-H signal, whereas position 2 shows a relatively higher C-D signal indicating the predominance of DOPC and d_{62}-DPPC at those locations, respectively.

Supplementary references

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[5] Colloids Surf. A Physicochem. Eng. Asp., 2004, 243, 157–167

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