Supporting Information

Adsorption Kinetics Dictate Monolayer Self-Assembly for Both Lipid-in and Lipid-out Approaches to Droplet Interface Bilayer Formation

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Materials and Methods

Lipid-in solution preparation:

To prepare lipid-in aqueous liposome solution, phospholipid powder is suspended at a concentration of 2 mg/ml (2.4 mM) in an aqueous buffer (referred to simply as buffer) of 10 mM MOPS, 100mM NaCl, pH 7 in deionized water (DI). This mixture is subjected to 4-5 freeze-thaw cycles to obtain multilamellar vesicles, and then extruded 11 times through a 100 nm-pore polycarbonate membrane (Avanti Polar Lipids) using an Avanti Mini-extruder to form unilamellar vesicles. This solution is then diluted appropriately to achieve the required concentration.

Lipid-out solution preparation:

To prepare lipid-out solutions of inverse micelles solutions in nonpolar solvent, lipid powder is dissolved in hexadecane at 10mg/ml concentration simply by mixing appropriate amount of lipid

powder in hexadecane followed by gentle vortexing.[2] This solution is then diluted accordingly for further use. All the solutions are stored at room temperature. "Hydrated" lipid-out solutions are prepared by adding 1 µl of DI water into a vial containing 1 ml fresh lipid-out solution; this mixture is periodically vortexed. "Aged" lipid-out solutions are prepared by storing freshly prepared solutions at room temperature for 10 days prior to use.

Interfacial Tension Measurement:

A pendant drop tensiometer (Ramé-Hart Instrument Co. Model 590) and DROPimage Advanced software are used to measure the interfacial tension of an OW interface as described elsewhere. In this study, two very similar setups are used to measure interfacial tension for lipid-in and lipid-out cases at an OW interface. For lipid-out IFT measurements, a rectangular glass cuvette is filled with lipid-out solution and an aqueous pendant droplet is formed with buffer solution at the tip of a vertically oriented stainless steel, blunt needle. For lipid-in measurements, the cuvette is filled with lipid-in solution and an inverted pendant droplet of oil is formed with pure hexadecane at the tip of a J-shaped, blunt needle (Figure S1.a). This inverted configuration is incorporated because a hanging lipid-in pendant droplet consistently falls from the needle when the interfacial tension reduces below approximately 5 mN/m, making measurement of equilibrium tension impossible. Dynamic measurements of IFTs are found to be closely

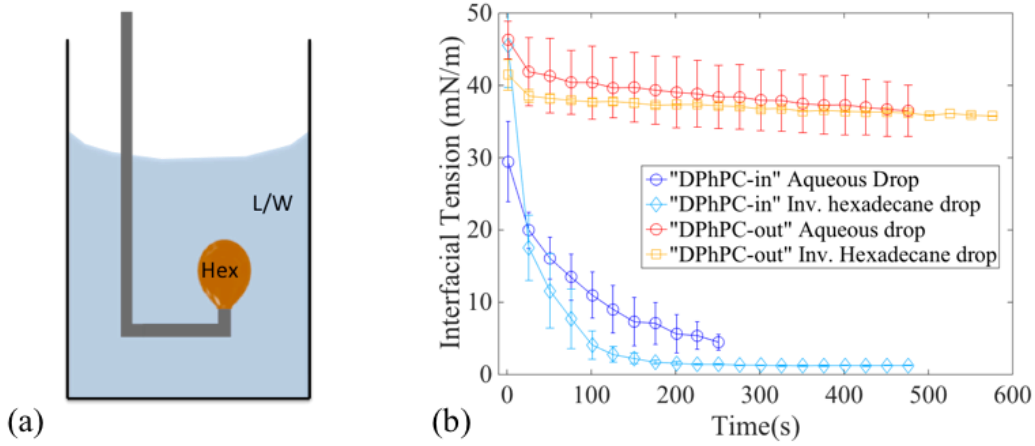

Figure S1. (a) Inverse pendant drop IFT measurement setup with lipid-in-buffer solution in the cuvette and hexadecane drop formed at the tip of the needle. (b) IFT data measured with pendant drop and inverse pendant drop approach for both DPhPC-in and DPhPC-out cases.

comparable between these two setups (Figure S1.b).

In order to measure the equilibrium tension in both lipid-out and lipid-in cases, the droplet volume is maintained at 1µl (unless mentioned otherwise) so that the droplet does not detach from the needle. Consistent droplet size also enables direct comparison of the kinetics. All measurements are performed in constant-volume mode by using the dispenser's feedback control feature. Measurements are taken at a rate of 60 samples per minute (1Hz), and the IFT measurement is begun a few seconds before dispensing the droplet to enable recording of the complete dynamic change in interfacial tension. Prior to each measurement, the glass cuvettes are rinsed successively with isopropyl alcohol IPA and DI water and then dried in an oven at 80°C. About 3ml of the bulk solution is then dispensed into the cuvette for a given test. A clean 23-gauge (1-1/2" length) (Ramé-hart) blunt tip stainless needle is then attached to the end of the dispenser tubing, and the tip of the needle is positioned in such a way that the pendant droplet remains completely submerged in the bulk solution and away from any other surfaces. A minimum of 5 measurements is taken for all cases, and all experiments are performed in a class (1000) clean room with an ambient temperature of 22 ± 0.5°C and relative humidity of 38.5 ± 2.5%.

Stirring of the bulk solution is performed in some tests using a magnetic stir bar (Sigma Aldrich) and a custom magnetic stirrer positioned below the glass cuvette. A 9 mm (dia.) cylindrical magnetic stir bar is placed in the cuvette before adding bulk solution. Because stirring affects the IFT measurement, the solution is stirred intermittently during tests and data points collected during stirring are disregarded. The stir speed is limited such that the droplet does not detach from the needle due to agitation or excessive flow.

DIB formation and contact angle measurement:

DIB contact angle is measured by connecting droplets within a PMMA substrate with multiple divots (height: 5mm, width: 4 mm, depth: 8 mm each). After thorough cleaning and drying of the PMMA substrate, about 250 µl of nonpolar solvent is dispensed into the well. 500 nl (or 200 nl when specified) aqueous droplets are then placed in two separate wells for incubation. After incubation, droplets are pushed into a common well using a pipette tip to gently bring the droplets into contact. When brought into contact, the droplets spontaneously form a planar bilayer or coalesce into one droplet. This event is recorded using a digital camera (QImaging)

fitted to an inverted microscope (Olympus IX51). Images of successful DIBs are analyzed using MATLAB to extract the contact angle between the droplets as explained elsewhere. The bilayer tension and the energy of droplet adhesion is calculated based on the equations described elsewhere.[3, 4] A minimum of 20 trials is performed for each lipid condition.

Electrical characterization of lipid bilayers:

Electrical characterization of DIBs is performed in open PMMA substrates (described above) with droplets hanging on wire-type silver-silver chloride (Ag/AgCl) electrodes that are mounted on micromanipulators (WPI). The electrodes are connected to an Axopatch 200B patch clamp amplifier (Molecular Devices) that allows the user to apply voltage and measure the induced pico-ampere range current. 500nl droplets are placed on the two agarose-coated electrode tips. After the incubation time, the electrodes are then brought closer to each other to allow the droplets come into contact with each other. Due to the capacitive nature of the bilayer, applying a triangular waveform voltage (10 mV at 10 Hz frequency) across the bilayer induces a square wave current whose amplitude is proportional to the capacitance and area of the bilayer. For all cases tested, the square wave response is recorded during bilayer thinning, and the area of the bilayer is adjusted by manipulating the relative electrode positions to read an equivalent square wave current of about 100 pA (4.17×10^{-4} cm^2). To estimate the electrical resistance of the bilayer, DC voltage is applied across the bilayer in incremental steps of 25 mV, for about 30 seconds in each step, in alternating bias starting from 0 mV until the bilayer ruptures. The resultant current is digitized with a Digidata 1440A (Molecular Devices) at a sampling rate of 2 kHz and analyzed using MATLAB to determine the electrical resistance of the bilayer, extracted as the inverse of the slope of current-voltage data obtained between -75 mV and +75 mV. Finally, the voltage at which the bilayer ruptures is recorded as the rupture potential. A minimum of 5 measurements is performed for every case discussed. Specific capacitance measurements are performed by a method described elsewhere.[1]

Example current-voltage relationships for DIBs

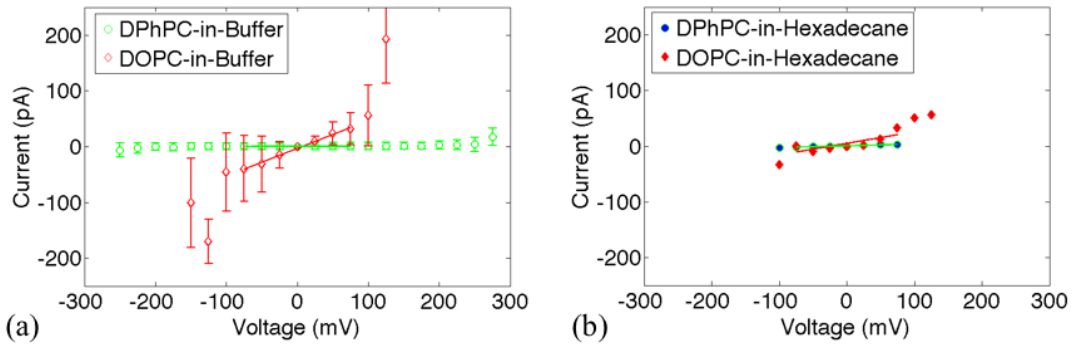

Figure S2. Current-voltage curves for DPhPC and DOPC (a) lipid-in; (b) lipid-out. Slopes are plotted between -75mV and 75mV to calculate resistance of the bilayers (inverse of slopes).

Calculation of Bilayer Tension and Adhesion Energy for DIB

Bilayer tension and energy of adhesion are calculated by the formulas described elsewhere.[3, 4] Bilayer tension is calculated from equation give below,

$$2\cos(q_b) = \frac{g_b}{g_m},$$

where, γ_m is the monolayer tension, γ_b is bilayer tension, and θ_b is the external half angle between droplets (as marked in Figure S3). The energy of adhesion is calculated by $2\gamma_m - \gamma_b$. By definition, both bilayer tension and energy of adhesion have units of energy (i.e. force x length) per unit area (i.e. length squared), which is equivalent to units of force per unit length.

Specific Capacitance Measurements:

Specific capacitance measurements are performed by a method described elsewhere.[5] Briefly, the nominal capacitance, *C*, of a bilayer (calculated from electrical current measurement in response to an applied triangular voltage waveform) for different bilayer areas, *A* (estimated from optical images) are used to calculate the specific capacitance, C_m, of a DIB as given by

$$\frac{C}{A} = C_m = \frac{e_0 e}{d},$$

where ε_0 is the dielectric permittivity in vacuum (8.854×10^{-12} F/m), ε is the relative permittivity of the hydrophobic region of the membrane, and d is the thickness of the hydrophobic region.

Specifically, two droplets hanging from agarose-coated electrode tips are connected to form a DIB under/on an inverted microscope. Triangular wave is applied to the bilayer continuously and the resultant square wave is recorded. Length of the bilayer is varied in discrete steps by moving one droplet with respect to another by using a micromanipulator, which in turn alters the measured square wave. At every step, after the measured square wave reaches a steady state, an

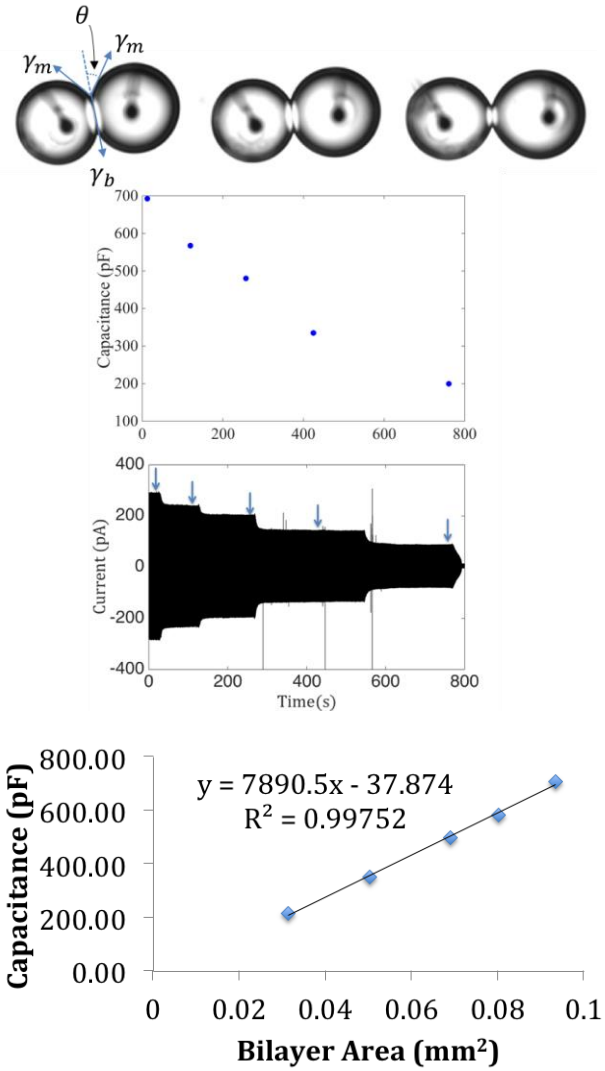

Figure S3. Specific capacitance measurement procedure. [1]

image of the interface is taken. These images are then processed to estimate the area of the bilayer with an assumption of circular interface. Figure S3 is plotted using the calculated capacitance values of the bilayers at the exact moments of image captures and the area of the bilayer estimated from the images.

MD Simulation Method

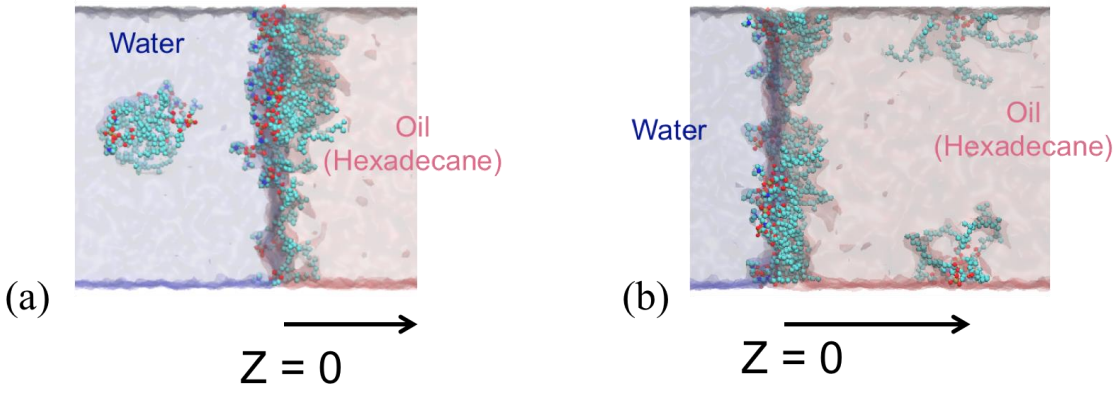

Figure S4. Molecular dynamics system setup (DPhPC): (a) the micelle (lipid-in), and (b) the inverse micelle (lipid-out) are pulled toward the OW interface.

Table S1. Measured physical properties of DIBs

	DPhPC-in/Hexadecane	DOPC-in/Hexadecane	DPhPC-out/Buffer	DOPC-out/Buffer
Contact Angle (°)	20 ± 2	27 ± 5	18 ± 5	21 ± 5
Bilayer Tension ($mN.m^{-1}$)	2.2 ± 0.4	3.5 ± 0.9	3.9 ± 0.3	3.36 ± 0.6
Energy of Adhesion ($mN.m^{-1}$)	0.14 ± 0.001	0.43 ± 0.004	0.20 ± 0.01	0.24 ± 0.002
Specific Capacitance ($\mu F.cm^{-2}$)	0.685 ± 0.068	0.914 ± 0.274	0.638 ± 0.068	0.968 ± 0.072
Calculated Thickness (Å)	2.8 ± 0.3	2.1 ± 0.7	3.1 ± 0.3	2.0 ± 0.3

Dynamic Light Scattering Measurements of Liposomes and Inverse Micelles:

Dynamic light scattering (DLS) was performed using Malvern (Model no.). A glass cuvette was filled with the sample solution and placed in the DLS machine. Appropriate refractive index (Water: 1.333; Hexadecane: 1.434) was used. All measurements were performed at room temperature.

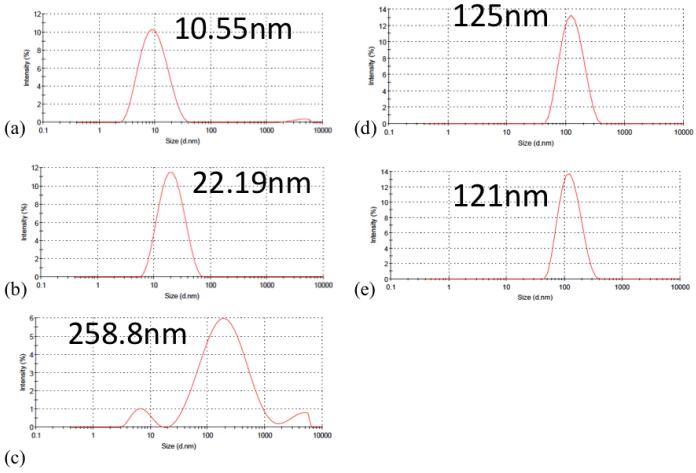

Figure S5. DLS measurements showing size distribution in terms of scattering intensity for a a) fresh DPhPC-out sample, b) 10-day old DPhPC-out sample, and c) hydrated "swollen" DPhPC-out sample, d) fresh DPhPC-in liposome sample, and e) 10-day old DPhPC-in sample.

Additional IFT Data for Lipid-in and Lipid-out Conditions:

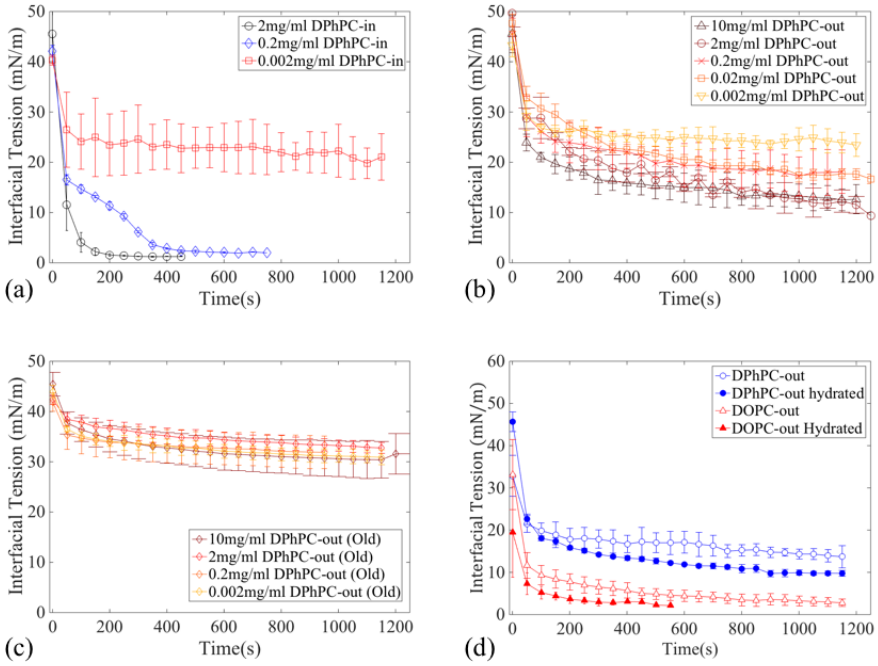

Figure S6. Interfacial tension versus time for: (a) 2, 0.2, and 0.002mg/ml DPhPC-in concentrations; and (b) 10, 2, 0.2, 0.02 and 0.002mg/ml DPhPC-out concentrations; (c) 4 different old DPhPC-out concentrations; (d) fresh DPhPC-out, hydrated DPhPC-out, fresh DOPC-out, and hydrated DOPC-out.

Interfacial Tension Measurement Analysis

Fitting of Model to Short Segments of IFT Data:

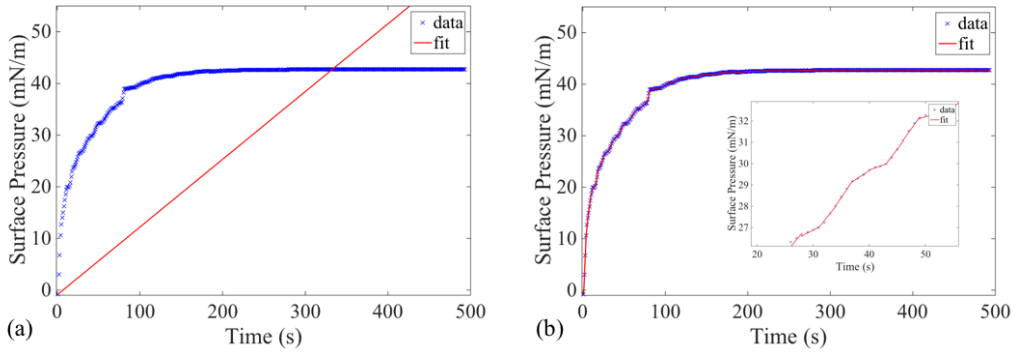

Figure S7. DPhPC-in surface pressure data and calculated fit using equation 5 for, a) the entire length of data with a single mka, and b) estimating mka values for 4s segments. **Inset:** A zoomed-in view of the same plot to show the short 4s data segments and its fit.

Note for Figures 5 & 6:

As V_0 is multiplied with mk_a to obtain the values on y-axis, even the smallest gradient in surface tension will lead to a high value of lipid adsorption flux. Hence, the V_0mk_a values do not gradually decrease down to zero in Figures 5 & 6.

Validity of Model:

Consider DPhPC-in. Integrating Equation 3, an approximate estimate of total number of

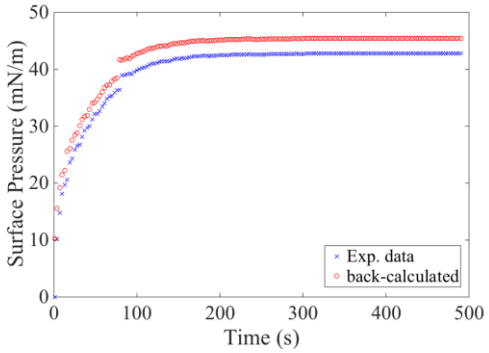

Figure S8. Comparison of experimental surface pressure and back-calculated surface pressure from the model for DPhPC-in at 2mg/ml.

adsorbed lipids at equilibrium state, Γ^{eq} is obtained: 1.67 x 10^{-5} moles/m^2. Using the equilibrium surface pressure value of 42.7mN/m in equation 4 leads to a Γ^{eq} value of 1.75 x 10^{-5} moles/m^2, which is roughly equal to Γ^{eq} obtained from integrating equation 3. Figure S8 compares the experimental surface pressure to the back-calculated surface pressure from the model. The agreement found between these two curves validates the modeling approach.

Limitations of Model:

The concentration range in which C_0 can be assumed to be constant must be determined (see Table S2) to determine the applicability of the proposed model. C_0 is assumed to be constant where the diffusion coefficient of lipid particles is high enough such that adsorbed lipids from the subsurface are constantly replenished by lipids originating from the bulk. For lipid-in cases, at high concentrations such as 0.2mg/ml, just 4% of the total lipid content is required to completely cover the droplet surface. Thus the concentration gradient between the subsurface and the bulk is maintained, allowing C_0 to remain constant. However, at 0.02mg/ml or below, C_0 may not be assumed to be constant as a significant portion (40%) of lipids are required to form a fully-packed monolayer. The calculation shown for a concentration of 0.002mg/ml shows that an insufficient number of lipids are present to fully pack the interface, which explains why IFT only reduces to ~22 mN/m (Fig. S5.a).

Aside from the number of lipids present, the concentration gradient established between the interface and the bulk reduces for low lipid concentrations, which also prevents C_0 from remaining constant.

The bottom half of the table shows that even for a small lipid-out reservoir volume of 10µL, there exists an ample amount of lipids required for complete droplet coverage. Therefore, the calculation justifies that at reasonable concentrations, especially those higher than 0.02mg/ml, depletion of lipids as a result of adsorption is minimal and our assumption that C_0 is constant is valid.

Table S2. Qualitative determination of suitable minimum lipid concentration.

	Number of lipids required to cover a 1µl droplet surface: **$6x10^{12}$ molecules** (approx.)				
	Concentration	2mg/ml	0.2mg/ml	0.02mg/ml	0.002mg/ml
Lipid-in	No. of lipid molecules present in 1µl aqueous droplet (approx.)	1.4×10^{15}	1.4×10^{14}	1.4×10^{13}	1.4×10^{12}
	% required for complete coverage (approx.)	0.4%	4%	40%	419% (More lipids required than available)
Lipid-out	No. of lipid molecules present in 10µl hexadecane solution (approx.)	1.4×10^{16}	1.4×10^{15}	1.4×10^{14}	1.4×10^{13}
	% required for complete coverage (approx.)	0.04%	0.4%	4%	40%

DOPC Self-assembly Free Energy Curves

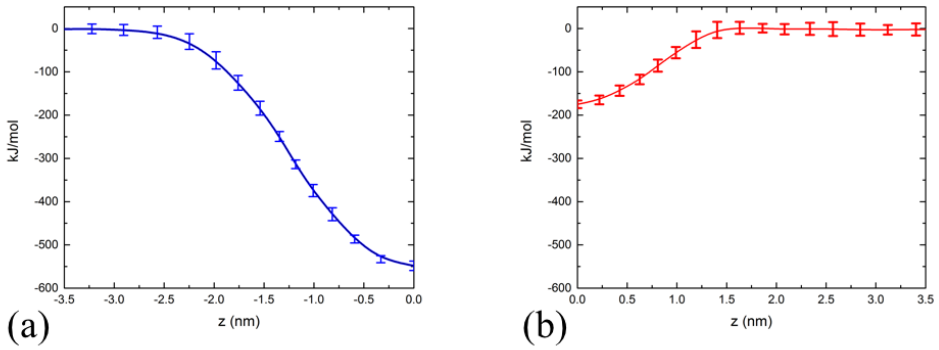

Figure S9. Free energy curves of (a) the micelle (lipid-in) for DOPC and (b) the inverse micelle (lipid-out) for DOPC as a function of the distance from the pristine OW interface.

References:

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