

Spontaneous Formation of Lipid Structures at Oil/Water/Lipid Interfaces

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We report the spontaneous formation of emulsion droplets and multilamellar concentric onions when a water drop is immersed into dodecane containing phospholipids. We show that the origin of the spontaneous emulsification is the formation of a semicrystalline multilamellar film at the dodecane–water interface, which swells with water, shedding the emulsion and onion droplets. We use coherent anti-Stokes Raman scattering microscopy to determine that the shell of the onion structures is composed of partially hydrated concentric bilayers, and the core is composed of lipids, water, and dodecane.

Introduction

The production of emulsion droplets and of concentric multilamellar structures called onions usually requires the addition of energy to the system.^{1–3} In some instances, however, the initial composition of the system can be chosen so that emulsion droplets or onions can form spontaneously. This is of great interest for potential applications such as the delivery of agricultural chemicals and drugs and for oil recovery processes and thus has stimulated extensive studies of the phenomenon.^{4–6}

Spontaneous emulsification of direct (water-in-oil) emulsions has been reported for two surfactant systems: AOT (Aerosol OT, sodium bis(2-ethylhexyl) sulfosuccinate),⁷ an anionic surfactant also known to stabilize microemulsions, and C₁₂E₆ (dodecylhexaglycol polyoxyethylene 6 lauryl ether),⁸ a nonionic surfactant that can promote spontaneous emulsification when a cosolvent soluble in both oil and water is present. By contrast, to our knowledge, no observations of spontaneously formed inverted (oil-in-water) emulsions have been reported. Spontaneous emulsification is not a thermodynamic equilibrium process but instead is highly dependent on initial conditions such as the initial composition of the oil and water phases.

Four possible mechanisms^{4–6} have been proposed to explain the spontaneous formation of emulsion droplets. Diffusion of water into the oil can result in nucleation and swelling of water–surfactant aggregates. Alternatively,

spontaneous emulsification may be caused by a reduction of the surface tension,^{4,9} although this mechanism is still debated.^{10–12} Adsorption of the surfactant can drive the surface tension negative; as a result, the area of the interface can spontaneously increase and vibrations or thermal fluctuations can break the interface thereby shedding droplets. Local convection currents in the fluid adjacent to the interface can drive surface tension gradients that can cause deformation of the surface and can tear droplets away from the interface. Finally, spontaneous emulsification may be caused by the self-assembly at the interface of surfactant into an ordered multilayer film similar to a lyotropic liquid-crystalline phase,^{13,14} which can swell by diffusion of water or oil and form droplets which are then shed from the interface. These mechanisms can also be applied to spontaneous formation of inverted emulsions, where the droplets are water, and the surfactant is in the oil phase.

A second important class of surface active materials is lipids, which can also stabilize an oil–water interface. The most common of these is lecithin, which is widely used as a natural emulsifying agent in food, cosmetics, pharmaceuticals, and drug delivery systems. Its effectiveness as an emulsion stabilizer is attributed to interactions between tail groups of lecithin molecules self-assembled at the interface between oil and water.^{15,16} When decane containing lecithin is placed in contact with water, a macroscopic semicrystalline film can develop at the interface¹³ and interfacial waves can form, with wavelengths of the order of millimeters; however, it is unclear whether spontaneous formation of emulsion droplets or onions occurs. Thus, spontaneous emulsification with lipids is still an unexplored phenomenon.

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In this paper, we study the interfacial phenomena occurring at a dodecane–water interface when lipids are initially dispersed in dodecane. We report the first observation of the spontaneous formation of an inverted emulsion consisting of water droplets in oil, stabilized by both anionic and nonionic lipids. We also demonstrate that multilamellar onions and unilamellar vesicles are spontaneously produced. This is the first reported observation of the spontaneous formation of onions in the oil phase; the only previous report of spontaneously formed multilamellar particles was for onions formed in water from the swelling of a pure surfactant.^{17,18} We describe several experiments designed to investigate the mechanisms responsible for these observations. We show that spontaneous emulsification occurs in both water-saturated and dry dodecane in the presence of lipid, indicating that water diffusion into dodecane is not responsible for the spontaneous emulsification. We use optical microscopy to directly visualize the oil–water interface and show that it does not fluctuate, precluding fluctuation-based mechanisms. Instead, we find that lipid assemblies are present at the interface.^{15,16,19} Lipids adsorb at the interface, but unlike most surfactants, they form a nonuniform multilamellar liquid-crystalline phase. Hydration of this film is most likely responsible for the spontaneous formation of emulsion droplets by analogy with the hydration of a dry lipid film or a pure surfactant system in contact with water. Using coherent anti-Stokes Raman scattering (CARS) microscopy, we determine that these onions are composed of lipid, water, and oil in proportions that depend on the initial conditions. We show that the microscopic organization of these onions is highly dependent on the type of lipid used.

Materials and Methods

Materials. The aqueous solution used in these experiments was a 5 mM Tris buffer containing 100 mM sodium chloride at pH = 7.4. Anhydrous dodecane (Sigma, Saint Louis, MO) was used to prepare two different dodecane solutions. A dodecane solution saturated with water was prepared by stirring 100 mL of buffer into 1 L of dodecane for 24 h; subsequently excess buffer was removed from the dodecane. Dry dodecane solution was prepared by adding 1 vol % of heavier silicone oil to the anhydrous dodecane; the addition of silicone prevents the diffusion of water into dodecane. Finally, fully deuterated dodecane (Cambridge Isotope Laboratories; Cambridge, MA) was used for the CARS experiments. The phospholipids (Avanti Polar Lipids, Alabaster, AL) POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) and POPS (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine) were used, without further purification, within 2 months of purchase. These lipids were stored in chloroform and kept in a freezer at $-20\text{ }^{\circ}\text{C}$ to prevent degradation.

To prepare the dodecane–lipid suspension used for these experiments, we first formed a dry lipid film of known mass by evaporating the chloroform from the stock solution under nitrogen. After the film was formed, dodecane was added to resuspend the lipids at the desired concentration, and the dispersion of the film was achieved by sonicating the dodecane–lipid suspension for 30 min. Finally, the solution was left to stabilize overnight at $30\text{ }^{\circ}\text{C}$.

Surface Tension Measurement. The surface tension of the dodecane–water interface was measured using a pendant drop method⁶ where a drop of the denser fluid, water, is suspended from a capillary tube in the lighter fluid, dodecane, containing the surfactant molecules. Gravity acts to elongate the drop, while interfacial tension opposes elongation because the surface energy increases with the interfacial area. When surfactant diffuses

from the bulk to the interface, its adsorption reduces the surface tension and the drop deforms more. Changes in drop shape are monitored with a CCD camera, and the contour of the drop is extracted and fit to determine the interfacial tension⁶ as a function of time.

The time evolution of the surface tension depends on the material transport of the surfactant from the bulk to the surface. When the drop radius is large and the transport is diffusion limited, a solution to Fick's law can be found²⁰ for short times and low concentrations; then the surface tension should decrease from its initial value with the square root of time,²¹

$$\gamma(t \rightarrow 0) \approx \gamma_0 - 4RTC_{\text{bulk}}(Dt/\pi)^{1/2} \quad (1)$$

Here, γ_0 is the surface tension of the pure dodecane–water interface, R is the ideal gas constant, T is the temperature, C_{bulk} is the initial concentration of the surfactant in the lighter phase, and D is the diffusion coefficient of the surfactant in the lighter phase. For systems where these assumptions are valid, eq 1 can be fit to the measured time dependence of the surface tension to determine the diffusion coefficient of the surfactant. This method has been used for systems such as surfactants and polymers to determine the time evolution of the surface tension, the diffusion coefficient of the surfactant molecule, its area at equilibrium, its affinity for the interface, the interaction between molecules²² at the interface,²² and the time scale for the rearrangement of polymer brushes at the interface.²³

In the experiments reported here, the time evolution of the surface tension was measured by rapidly forming a $10\text{ }\mu\text{L}$ pendant drop at the tip of a vertical stainless steel needle (0.8 mm inner diameter) in a 10 mL rectangular glass cuvette containing lipid-saturated dodecane. The shape evolution of the drop was monitored and imaged every 5 s with a CCD camera. The contour of the drop was determined and the surface tension was automatically calculated by the instrument software (IT Instruments; Lyon, France) using the standard pendant drop analysis method.⁶ Both the aqueous and oil phases were equilibrated at the temperature of the experiment for an hour before injecting the water droplet. During the measurement, a temperature bath maintained the reservoir containing the sample vial to within $0.1\text{ }^{\circ}\text{C}$ to prevent temperature gradients and any resulting convection currents.

Microscopy. Bright field microscopy observations of the dodecane suspensions were carried out with an inverted microscope (Leica) a few hours after injection of the water drop into the dodecane solution. Both phase-contrast images and images formed with crossed polarizers were acquired to determine the nature of the spontaneously formed particles. The composition of the onionlike structures formed during the spontaneous emulsification was determined using CARS microscopy.²⁴ A detailed description of this technique can be found elsewhere.²⁵ Briefly, CARS is a third-order nonlinear optical process in which a pump excitation field at frequency ω_p and a Stokes excitation field at frequency ω_s are mixed in a sample to generate an anti-Stokes signal field at frequency $\omega_{\text{as}} = 2\omega_p - \omega_s$. CARS microscopy provides a vibrational contrast based on the enhancement of the signal when $\omega_p - \omega_s$ is tuned to a Raman active molecular vibration. Because CARS is a multiphoton process, the signal is generated only at the center of the focus, which permits three-dimensional sectioning of a sample. In our experiment, CARS images of the onionlike features were acquired by raster scanning two near-infrared picosecond laser beams.²⁶ The distributions of lipid, oil (fully deuterated dodecane), and water in the onions

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were mapped by tuning $\omega_p - \omega_s$ to the CH stretching vibration at 2845 cm^{-1} , the CD stretching vibration at 2125 cm^{-1} , and the OH stretching vibration at 3445 cm^{-1} , respectively.

Light Scattering Experiments. To study the kinetics of the spontaneous emulsification and the size of the particles formed, a pendant drop was formed by the same method as described for the surface tension measurements but in a 3.5 mL cylindrical glass vial placed in a light scattering instrument, an ALV DLS/SLS-5000 spectrometer/goniometer (ALV-Laser GmbH, Langen, Germany). Dynamic light scattering²⁷ (DLS) was used to determine the size of the small emulsion droplets formed spontaneously in dodecane. The temperature was held constant by a thermal bath. The light scattered at 90° was collected, and the temporal autocorrelation function of the intensity fluctuation was determined. A cumulants analysis²⁸ was performed on the initial decay to obtain the average size. For data taken during the first hour, this analysis provides a reasonable estimate, but after 2 h, the distribution became very polydisperse and a CONTIN fit^{29,30} was used to determine the particle size distribution.

Results and Discussion

The time evolution of the surface tension $\gamma(t)$ of a dodecane–water interface during adsorption of POPC, a nonionic phospholipid, was measured for three different lipid concentrations at 25°C . Figure 1 shows the results plotted as a function of the square root of time, as suggested by eq 1. Triangles, circles, and squares correspond to samples with lipid concentrations of 0.01, 0.05, and 0.10 mg/mL, respectively. The critical micellar concentration (cmc) of this lipid in dodecane is about 0.05 mg/mL. The dynamic surface tension decreases the fastest for the highest concentration, which is consistent with eq 1. For times shorter than 30 min, the three curves decrease almost linearly with the square root of time; this time dependence is consistent with a diffusion-controlled adsorption mechanism. However, at longer times the slopes of these curves change dramatically, indicating a slower decrease in surface tension with time, and an equilibrium value is not reached even after 6 h. This change in slope is reproducible, ruling out spurious effects such as those caused by dust in the sample. For concentrations of 0.01 and 0.05 mg/mL, which are smaller than or equal to the cmc, the diffusion coefficients obtained by fitting eq 1 to the short-time part of the data are 4.5×10^{-12} and $4.8 \times 10^{-12}\text{ m}^2/\text{s}$, respectively. For the 0.1 mg/mL POPC sample, there are sudden jumps in the surface tension at early times; using the very early time data, the diffusion coefficient obtained is $12 \times 10^{-12}\text{ m}^2/\text{s}$. This larger value presumably reflects the adsorption of micelles, which increases the adsorption rate. However, all of these values of the diffusion coefficient are about 2 orders of magnitude smaller than the value of $D = 5 \times 10^{-10}\text{ m}^2/\text{s}$ reported for the adsorption of lipid at air–water interfaces,³¹ suggesting that the evolution of the surface tension does not result from the simple diffusion of lipids to the interface.

The second indication of unusual behavior was first observed when the temperature of a typical sample was lowered to 20°C ; scattering from a dodecane suspension containing 0.1 mg/mL POPC cooled to 20°C increased, the suspension becoming turbid after 1 h. Phase contrast microscopy of the dodecane suspension collected after a few hours revealed the presence of two types of structures: submicron droplets, which were determined to be

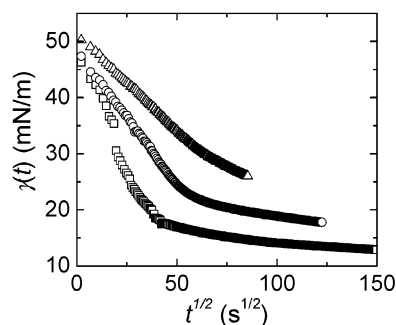


Figure 1. Time evolution of the surface tension of a dodecane–water interface for three POPC concentrations: (triangles) 0.01 mg/mL, (circles) 0.05 mg/mL, and (squares) 0.1 mg/mL. At short times, the surface tension decreases linearly with the square root of time. At longer times, the surface tension decreases slowly without reaching an equilibrium value within the 6 h duration of the experiment.

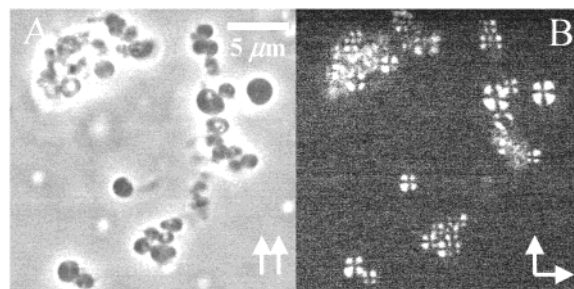


Figure 2. Optical microscopy image of the dodecane suspension after a few hours. Image A shows a phase microscopy image of multimellar onions that appear in the dodecane suspension a few hours after injection of a water drop. Image B shows the same objects between crossed polarizers. The defects observed confirm that these droplets are ordered multimellar concentric shells.

emulsion droplets, and larger droplets. Both of these structures were formed by spontaneous emulsification from the surface of the water drop. A typical image of the larger droplets is shown in Figure 2A. When imaged between crossed polarizers, as shown in Figure 2B, these droplets are clearly birefringent and have defects characteristic of molecules ordered normal to the interface, consistent with previous observations of multimellar onionlike structures.^{17,18} Though the turbidity was more apparent at 20°C , closer inspection reveals that over time the scattering intensity increases for all samples.

To monitor the appearance of the spontaneously formed submicron particles, we repeated the pendant drop experiment in a goniometer and used dynamic light scattering to measure their size. For these experiments, POPC was dispersed in dodecane at a concentration of 0.05 mg/mL, and the temperature was set to $(30 \pm 0.05)^\circ\text{C}$. Since these experiments were thermostated, convection currents were minimized and external perturbations of the pendant drop were eliminated. We measured the time evolution of the scattering intensity at 90° every 10 min for 6 h. These results are plotted in Figure 3A. In addition, we used DLS to measure the time evolution of the mean radius for these droplets; the results are plotted in Figure 3B. In Figure 3B, the circles and triangles represent measurements performed for two distinct pendant drops under identical experimental conditions. Both measurements agree at short times when the spontaneously formed drops are not too polydisperse. The data can be divided into three regimes. In the first 40 min, the scattering intensity increases while the mean radius of the particles remains nearly constant, indicating that the number of

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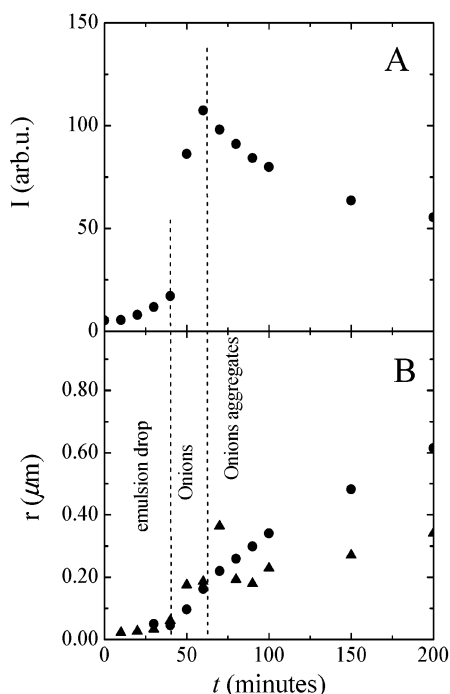


Figure 3. Light scattering measurements at 30 °C of the dodecane suspension right below the pendant water drop formed in dodecane containing 0.05 mg/mL POPC. (A) The time evolution of the static intensity at a scattering angle of 90°. (B) The corresponding evolution of the mean radius of the particles present in the dodecane suspension obtained by DLS. Triangles and circles correspond to results obtained with two different lipid batches. Before the droplets become larger than 2 μm , both measurements are consistent, confirming that the time evolution of spontaneous droplet formation is set by the initial concentration and the ambient temperature.

particles present in suspension increases and their size remains constant. During the next 20 min, both the scattering intensity and the mean radius of the particles increase. Imaging with optical microscopy reveals the presence of spontaneously formed onionlike droplets. At even longer times, we observe a decrease of the scattering intensity and significant disparities between the mean droplet size measured for both samples. This suggests that large droplets ($> 2 \mu\text{m}$) are sedimenting to the bottom of the vial thereby decreasing the intensity. At these longer times, light scattering is no longer appropriate to study the particles formed, and optical microscopy provides a more accurate assessment of the particles present in suspension.

The reproducible behavior of this spontaneous emulsification allows us to investigate its origin and the underlying mechanisms. One possible mechanism of spontaneous emulsification is diffusion of water into the dodecane. Water is known to be partially soluble in anhydrous dodecane; diffusion of water from the pendant drop into the dodecane could decrease the lipid diffusion coefficient and could result in the nucleation of water droplets in dodecane or cause swelling of any existing micelles. To examine whether the diffusion of water contributes to spontaneous emulsification, we prepared two samples: one with dodecane saturated with water and the other with dodecane containing 1% silicone oil, which prevents water from diffusing into the anhydrous dodecane. POPC was dispersed in each phase at 0.25 mg/mL, about 5 times its cmc, and a water drop was added to each sample. After a few hours, a whitish film covered each drop. We found that this film is made of onionlike particles still tethered to the lyotropic film. Gently stirring



Figure 4. Contour of a pendant water drop after injection into a dodecane suspension containing 0.25 mg/mL POPC. This image shows the presence of droplets at the dodecane–water interface. The fact that the droplets grow into the dodecane confirms that the spontaneous formation of droplets happens at the interface.

the oil surrounding the aqueous drops causes these particles to separate from the film and become suspended in the oil phase. Optical microscopy showed that each sample contained both emulsion droplets and birefringent onionlike structures of comparable size. Additional DLS measurements confirmed that the spontaneous emulsification of similar structures occurs in both saturated dodecane and dry dodecane within the same time scale. These results provide strong evidence that the diffusion of water does not affect the spontaneous emulsification of water in dodecane. Thus the spontaneous emulsification is not due to the nucleation or swelling of micelles in dodecane and is thus not a bulk mechanism.

Other possible mechanisms entail a perturbation of the interface, either by convection currents or fluctuations due to low surface tension. Convection currents driven by temperature gradients in the oil phase do not play a role in our system as all of the pendant drop experiments were performed in a temperature-controlled environment. The temperature gradient across the sample cell was smaller than 0.05 K/cm, and the resulting temperature difference across a 1 mm drop was thus too small to drive the Marangoni effect⁶ necessary for surface instability. Thus, the spontaneous emulsification is not due to the presence of convection currents.

To investigate the impact of fluctuations, we imaged the surface directly with a microscope. Using a micropipet, we injected a water droplet into a microscope chamber filled with dodecane containing POPC at a concentration of 0.25 mg/mL and imaged the contour of the droplet by optical microscopy. We did not observe any shape fluctuations indicative of very low surface tension. Instead, we saw very distinct and well-defined droplets form on the oil side of the oil–water interface, as shown in Figure 4. The edge of the large droplet is composed of a concentric stack of POPC bilayers with an onionlike structure, as evidenced by the bright band just inside the droplet, while an oil–lipid–water isotropic phase is located within the droplet core. As they become hydrated, these multilayers swell by diffusion of water through the film; the water must spread laterally to form droplets. These droplets eventually detach from the interface and remain in

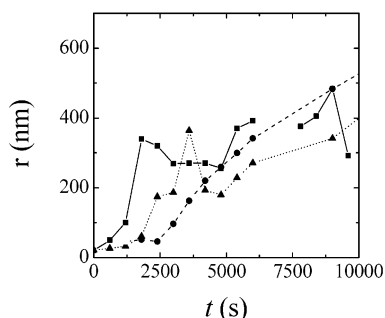


Figure 5. The time evolution of the radius of the spontaneously formed droplets in water-saturated dodecane at 30 °C for three different concentrations of POPC: (squares) 0.75 mg/mL, (triangles) 0.25 mg/mL, and (circles) 0.05 mg/mL. The mean radius was obtained by fitting the short-time part of the autocorrelation function to a single exponential.

dodecane. This strongly suggests that spontaneous emulsification results from the presence of lyotropic liquid-crystalline phases at the dodecane–water interface. To investigate the lyotropic semicrystalline films involved in the spontaneous emulsification process, we studied the particles produced at 35 °C for different lipid concentrations and compositions. We varied the initial concentration of POPC in saturated dodecane and repeated the light scattering measurements of the pendant drop. We observed that the higher the concentration, the faster the growth of the droplets produced. Typical results for the time dependence of the radius of spontaneously formed emulsion droplets are shown in Figure 5 for three initial POPC concentrations. The kinetics of the spontaneous emulsification is controlled by the initial lipid concentration, which determines the number of lipid molecules reaching the interface per unit time to form the lyotropic phase, and hence the growth rate of the film; as the film gets thicker, the onion particles formed get larger.

To elucidate the influence of lipid packing properties on the spontaneous emulsification process, we measured the time evolution of the droplet size for two lipids that have the same acyl chains but different polar headgroups. We used POPS, which has a negatively charged phosphatidyl serine headgroup and a negative³² spontaneous curvature and is best suited for stabilization of inverted emulsions, and POPC, which has a nonionic phosphatidyl choline headgroup, is characterized by almost zero curvature, and is best suited for the formation of bilayers. We used DLS to monitor the size of the emulsion that forms spontaneously after injection of a water drop into dodecane containing lipids at 0.25 mg/mL at 35 °C. At this temperature, the size of POPC and POPS lipid aggregates initially present in dodecane is about 20 nm. Figure 6 shows the evolution of the mean size of the particles formed after injection of the water drop; the triangles correspond to the results obtained for POPC, and the squares correspond to the results obtained for POPS. We observe that before 1000 s, the size of the POPS aggregates remains unchanged, whereas the POPC aggregates grow continuously in time to much larger sizes, suggesting that the lipid polar head changes the time evolution of the particles produced.

Additional differences between POPC and POPS samples were observed when we examined the content of the water drop. The pendant drop was removed from dodecane after 2–3 h and studied by phase contrast microscopy. For a sample prepared at 25 °C with a

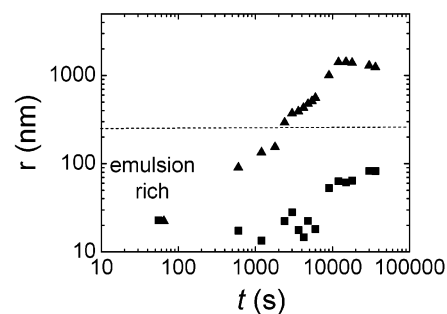


Figure 6. Comparison of the time evolution of the mean radius of spontaneously formed droplets in water-saturated dodecane containing POPC (triangles) and POPS (squares), both at a concentration of 0.05 mg/mL. The radius was obtained by fitting the short-time part of the autocorrelation function measured at 90°. The mean size of the droplets formed in the POPC suspension increased faster than the mean size of the droplets formed in the POPS suspension when the temperature was set at $T = 35$ °C.

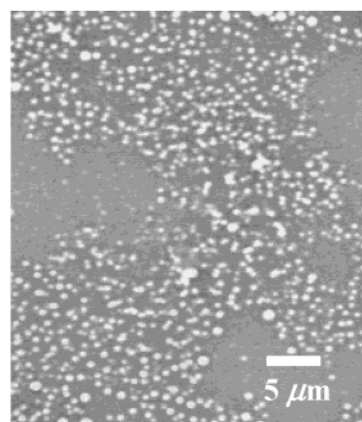


Figure 7. Phase contrast optical image of spontaneously formed oil emulsion droplets in the water pendant drop 6 h after the water drop was injected into a dodecane suspension at 30 °C containing POPC at a concentration of 0.75 mg/mL.

dodecane phase containing 0.1 mg/mL POPC, we observed a significant number of vesicles in the water drop and this number increased with time. It appears that the POPC diffuses into the water phase where it self-assembles into bilayers. In addition to vesicles, micron-sized oil droplets were also observed in the pendant water drop, as shown in Figure 7, for samples prepared between 27 and 30 °C only. The coexistence of water droplets spontaneously formed in oil and oil droplets spontaneously formed in water observed in this temperature range resembles the behavior of microemulsions near the phase inversion temperature (PIT).³³ A PIT occurs in a microemulsion when the balance between hydrophilic and hydrophobic forces switches with temperature. This can cause the packing properties of the surfactant molecules to change so that a direct microemulsion can become an inverted microemulsion. Here a similar phenomenon may be occurring as the hydration of the lipid headgroups changes with temperature. This behavior was observed only for POPC. Very few vesicles were observed in the water phase of the POPS samples, and no direct emulsions were observed; POPS is known to have a negative spontaneous radius of curvature³² and is therefore more likely to stabilize an inverted emulsion and remain in dodecane. The abundance of vesicles observed in the water phase of

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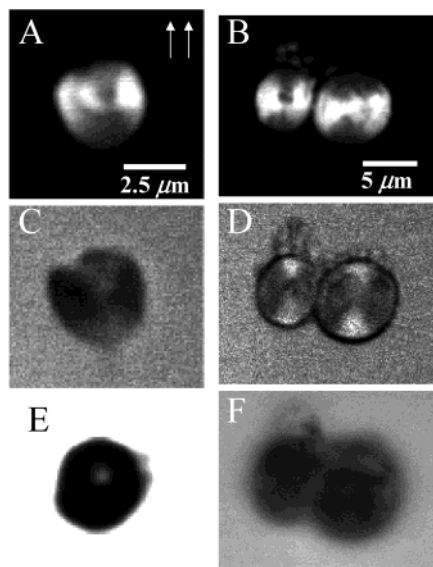


Figure 8. (A,C,E) CARS images of a POPC onion and (B,D,F) CARS images of two POPS onions prepared at 27 °C. The arrows indicate the polarization of the pump and Stokes beams for all the images. Images A and B map the CARS signal intensity with $\omega_p - \omega_s$ tuned to 2845 cm^{-1} . Images C and D map the CARS signal intensity with $\omega_p - \omega_s$ tuned to 3445 cm^{-1} . Images E and F map the CARS signal intensity with $\omega_p - \omega_s$ tuned to 2125 cm^{-1} . The laser beams were focused on the equatorial plane of the onion. The pump frequency (ω_p) was fixed at 14 212 cm^{-1} . The average powers of the pump and the Stokes beam were 100 and 50 mW, respectively, at a repetition rate of 80 MHz. Each image of 512×512 pixels was acquired at a dwell time of 10 μs per pixel and was an average of five scans.

the POPC samples at temperatures greater than 25 °C is consistent with the fact that POPC molecules have almost no spontaneous curvature, making it possible for them to stabilize either direct or inverted emulsions or to self-assemble into bilayers.

More information about the microscopic composition of the interfacial lyotropic film can be obtained by examining the multilamellar onions that are produced from this film. We have observed that for a given phospholipid, the initial lipid concentration and the ambient temperature set the morphology of the onions. In general, onions formed with POPS are smaller than 2 μm in diameter with a very small isotropic core that does not change significantly as we change the temperature. By contrast, onion structures observed for POPC are highly dependent on temperature and concentration. The diameter of the onion structure increases with the initial concentration of POPC in dodecane and with the temperature. To investigate the differences in microscopic composition, we used CARS microscopy.^{24–26} We investigated the chemical composition for POPC and POPS onions prepared at 27 °C and an initial lipid concentration of 0.25 mg/mL. Images A and B of Figure 8 show the distribution of the CARS signal from the lipid molecules, in particular the CH_2 symmetric stretching vibration mode, for onions formed in POPC and POPS, respectively. The images exhibit a high contrast, which is bipolar and perpendicular to the excitation field polarization. This confirms that both POPC and POPS lipids are organized in ordered concentric bilayers. The existence of water inside the onions is confirmed by the CARS images with $\omega_p - \omega_s$ tuned to the OH symmetric stretching band at 3445 cm^{-1} , shown in Figure 8C,D. The water signal for the POPC onions, shown

in Figure 8C, exhibits less contrast than that for the POPS onions, shown in Figure 8D, indicating the POPC onions contain less water than those of POPS prepared under the same conditions. The CARS contrast for water is also bipolar and is perpendicular to that for the lipids, indicating that most of the interbilayer water is oriented with its dipole perpendicular to that of the CH_2 group.³⁴ Images E and F of Figure 8 show the dodecane signal, based on the C–D stretching vibration, for these structures. Dodecane is also present within the structure though its distribution varies with the lipid polar headgroup. It is tightly confined in the core of the POPC onion structure (Figure 8E), whereas oil inclusions are found throughout the POPS onions (Figure 8F). The CARS measurements show that the polar head of the lipid does affect the chemical composition of these spontaneously formed onions. Further study of the POPC onions shows that the water content of the onions increases with temperature,³⁴ which reinforces our hypothesis that a change in hydration of the POPC bilayer may be occurring with temperature.

Conclusions

We have shown that when a water drop is placed in contact with a dodecane solution containing POPC or POPS, emulsification occurs spontaneously at the interface, creating either a water-in-oil emulsion or an oil-in-water emulsion. The spontaneous emulsification of the interface explains the apparently slow dynamics of lipid adsorption at the oil/water interface. It also accounts for the difficulty experienced in controlling the size of inverted emulsions produced by shear or mixing. Invariably a bidisperse distribution of sizes²⁷ is obtained: a population of large droplets corresponding to emulsion droplets formed due to the shear applied and a population of smaller droplets corresponding to the droplets formed by spontaneous emulsification.

In addition to POPC and POPS, we have observed spontaneous emulsification with other phospholipids such as 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (SOPC), and the phenomenon is certainly more general than these four lipids. We have identified the mechanism as being due to formation and swelling of the liquid-crystalline lipid structures. Lipids can self-assemble over several layers at an oil–water interface forming a thick film, which seems to be responsible for the formation of the multilamellar onions observed in the oil phase. The study of these inverse onions with CARS microscopy shows that variations in composition are related to changes in hydration of the lipid bilayers due to the lipid polar head due to temperature changes. These results confirm that the microscopic organization of the particles formed is dictated by the hydrophilic lyophilic balance of the lipid used at the experimental temperature and by the lipid initial concentration in dodecane.

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Abbreviations

AOT	Aerosol (OT); sodium bis(2-ethylhexyl) sulfosuccinate	PIT	phase inversion temperature
C ₁₂ E ₆	dodecylhexaglycol polyoxyethylene 6 lauryl ether	POPC	1-palmitoyl-2-oleoyl- <i>sn</i> -glycero-3-phosphocholine
CARS	coherent anti-Stokes Raman scattering	POPS	1-palmitoyl-2-oleoyl- <i>sn</i> -glycero-3-phospho-L-serine
DLS	dynamic light scattering	LA034532F	