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Spontaneous Formation of Stable Vesicles and Vesicle Gels in Polar Organic Solvents

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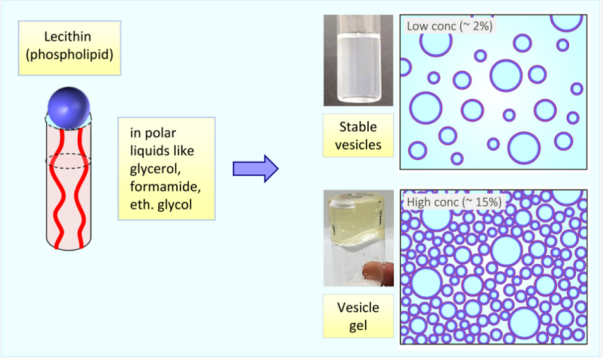
ABSTRACT: The self-assembly of lipids into nanoscale vesicles (liposomes) is routinely accomplished in water. However, reports of similar vesicles in polar organic solvents like glycerol, formamide, and ethylene glycol (EG) are scarce. Here, we demonstrate the formation of nanoscale vesicles in glycerol, formamide, and EG using the common phospholipid lecithin (derived from soy). The samples we study are simple binary mixtures of lecithin and the solvent, with no additional cosurfactants or salt. Lecithin dissolves readily in the solvents and spontaneously gives rise to viscous fluids at low lipid concentrations (~2−4%), with structures ~200 nm detected by dynamic light scattering. At higher concentrations (>10%), lecithin forms clear gels that are strongly birefringent at rest. Dynamic rheology confirms the elastic response of gels, with their elastic modulus being ~20 Pa at ~10% lipid. Images from cryo-scanning electron microscopy (cryo-SEM) indicate that concentrated samples are "vesicle gels," where multilamellar vesicles (MLVs, also called "onions"), with diameters between 50 and 600 nm, are close-packed across the sample volume. This structure can explain both the elastic rheology as well as the static birefringence of the samples. The discovery of vesicles and vesicle gels in polar solvents widens the scope of systems that can be created by self-assembly. Interestingly, it is much easier to form vesicles in polar solvents than in water, and the former are stable indefinitely, whereas the latter tend to aggregate or coalesce over time. The stability is attributed to refractive index-matching between lipid bilayers and the solvents, i.e., these vesicles are relatively "*invisible*" and thus experience only weak attractions. The ability to use lipids (which are "*green*" or eco-friendly molecules derived from renewable natural sources) to thicken and form gels in polar solvents could also prove useful in a variety of areas, including cosmetics, pharmaceuticals, and lubricants.



INTRODUCTION

Self-assembly, i.e., the spontaneous assembly of molecules into nanoscale assemblies, is of wide interest to scientists.[1−3] Molecules that self-assemble in water have both hydrophilic (water-loving) and hydrophobic (water-hating) parts. Examples of such amphiphilic molecules include surfactants and lipids, which have a hydrophilic head and one or two hydrophobic tails. Fundamental interest in self-assembly comes from the fact that it is ubiquitous in biology. For example, within a biological cell, lipids (i.e., two-tailed biological amphiphiles) assemble into membranes and vesicles.[4] Vesicles are the focus of this paper, and their structure is shown schematically in Figure 1A. They have a bilayer membrane (so-called because amphiphiles are arranged in a head-tail-tail-head fashion) that envelops an aqueous core. The bilayer thickness is ~5 nm, while the overall diameter of vesicles with a single bilayer (i.e., unilamellar vesicles) is ~100 nm.[1,2] The type of self-assembled structure formed by a given amphiphile is governed by its molecular geometry, as captured by the critical packing parameter CPP = a_{tail}/a_{head}.[1,2] That is, the CPP is the ratio of cross-sectional areas of the tail (a_{tail}) and the head (a_{head}). A CPP of 1 means that the molecule has a nearly equal head and tail areas, i.e., it is cylinder-shaped. This shape is readily achieved by lipids (Figure 1A) due to their having *two tails*, which explains why lipids form vesicles, which are also called liposomes.[5,6] Surfactants that have a head and a *single tail* tend to have a CPP much less than 1, and therefore tend to form micelles (spherical or cylindrical) instead of vesicles.[1,2]

In what solvents can self-assembly arise? It has been recognized for a long time that self-assembly can occur readily in solvents that are either *highly polar* (like water) or *highly nonpolar* (like oil).[7−9] Solvent polarity can be quantified by parameters such as the dielectric constant or the solubility parameter (which is a measure of the cohesive energy density).[1,2] A scale for such parameters is indicated schemati-

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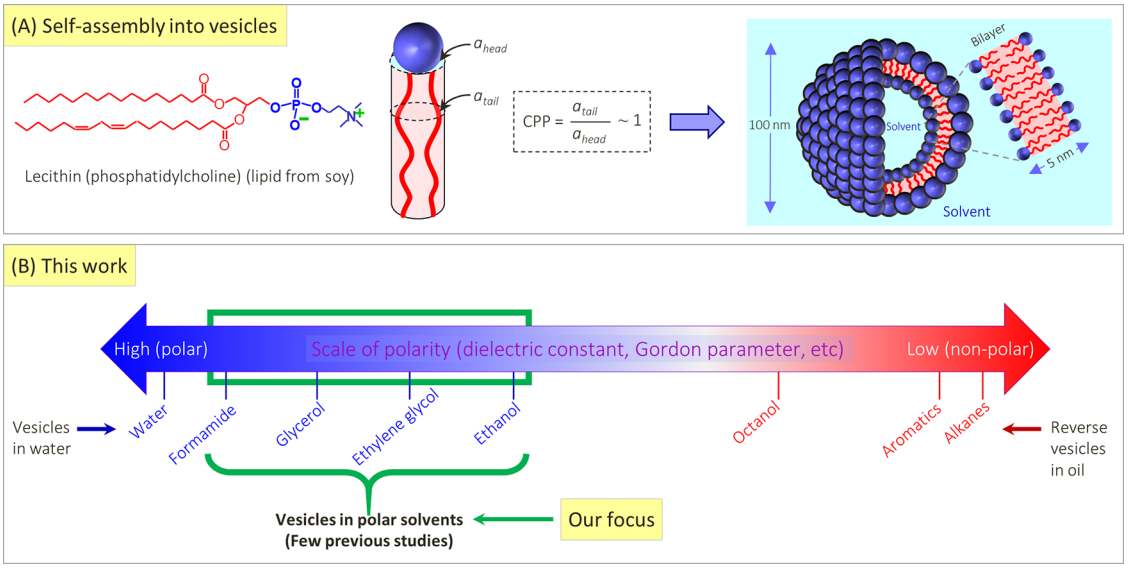

Figure 1. Overview of this work. (A) Amphiphiles (shown with their polar heads in blue and their nonpolar tails in red) can self-assemble into vesicles if they have a net cylindrical geometry, i.e., if their critical packing parameter (CPP) is ∼1. Phospholipids such as soy lecithin (structure shown) naturally have such a geometry due to their two tails. Vesicles in water are spheres (∼100 nm in diameter) with an aqueous core enveloped by a bilayer of lipids (∼5 nm thick). (B) A schematic scale of solvent polarity is shown. Vesicles have been formed in solvents at both extremes of this scale, i.e., in water (highly polar) as well as in oils (highly nonpolar). In solvents with lower polarity than water, there are few reports of vesicles thus far, and that is the focus of this work.

cally in Figure 1B, with water at the high end and oil at the low end of the scale. Self-assemblies such as micelles and vesicles are easily formed in water, while in oil, there are many examples of reverse micelles[10,11] and a few examples of reverse vesicles.[12] The term "reverse" refers to the inverted nature of these structures, with the hydrophobic tails of amphiphiles pointing outward (towards the nonpolar solvent) and the heads pointing inward. However, what about solvents that are not water or oil? Organic solvents that are close to water on the polarity scale include glycerol, formamide, and ethylene glycol (Figure 1B). In these polar solvents, there have been many reports of small, spherical micelles[7−9] and even some recent reports of long, cylindrical (wormlike) micelles.[13,14] Turning to *vesicles* in these solvents, which is our focus, there have been only a few reports,[15] which we discuss below.

Studies on self-assembly in polar solvents have concluded that the driving force is the "*solvophobic effect*," which is analogous to the hydrophobic effect in water.[16,17] Thus, amphiphiles will form "normal" micelles and vesicles in polar solvents, similar to those in water, and unlike the reverse structures in oil. The hydrophobic (nonpolar) parts of the amphiphiles will share a mutual dislike for the polar solvent, and therefore will arrange in a way that they are no longer in contact with the solvent.[16,17] Specifically, in the case of vesicles, the hydrophobic tails will be sequestered in the core of the bilayer membrane, while the heads will be in contact with the solvent on both sides of this membrane (Figure 1A). The same geometric principles (i.e., the CPP concept) are expected to hold for self-assembly in polar solvents as they do in water.[1,2] Hence, for vesicles to arise in such solvents, the amphiphile should have a CPP ∼ 1 (cylinder shape). As noted above, such a shape is easily realized by having two tails, rather than one, i.e., with lipids. (The one alternative is to combine single-tailed cationic and anionic surfactants in an equimolar ratio.[18]) Past studies on lipids in polar solvents have been conducted by Friberg,[19−21] McIntosh,[22,23] and Bergenstahl,[24,25] but the studies were confined to high lipid concentrations (above 20 wt %) where the lipids assembled into lamellar or other lyotoropic phases. McIntosh[23] mentions that multilamellar vesicles (MLVs) can be formed by lipids in solvents like glycerol, but no characterization of such vesicles is presented. Systematic studies on lipids at semidilute concentrations (<10 wt %) in pure polar solvents are scarce. The only study we have found was by Lattes,[15] which reported that lecithin could form MLVs around 500 nm diameter in formamide. Recently, there have been a couple of studies on lipid vesicles in ionic liquids.[26,27]

Here, we study the self-assembly of lecithin, a common phospholipid extracted from soybeans, in polar organic solvents such as glycerol, ethylene glycol, and formamide. Lecithin has a zwitterionic phosphocholine head, and one of its tails has *cis*-unsaturations (Figure 1A). Its self-assembly into vesicles has been extensively studied in water, typically at concentrations around 1−2 wt %. To form vesicles in water, lecithin is first dissolved in a polar solvent like ethanol or chloroform, and this solution is then introduced into water, followed by high shear (sonication or extrusion).[28,29] This multistep procedure is necessary because lecithin, like most lipids, is insoluble in water. On the other hand, in solvents like glycerol, we show that lecithin forms nanoscale vesicles *spontaneously* over a wide range of concentrations (∼2−15 wt %). The vesicles can be formed *in a single step*—simply by adding lecithin powder to the solvent and stirring under mild heat—which is in contrast to vesicles in water. At the higher end of this concentration range, the samples are gel-like and birefringent at rest. These properties are unusual and are not typically associated with vesicles in water. We will attribute these properties to the presence of close-packed MLVs in these samples. Our finding that vesicles can be readily formed in polar solvents has fundamental as well as practical implications, which we will discuss at the end of the paper.

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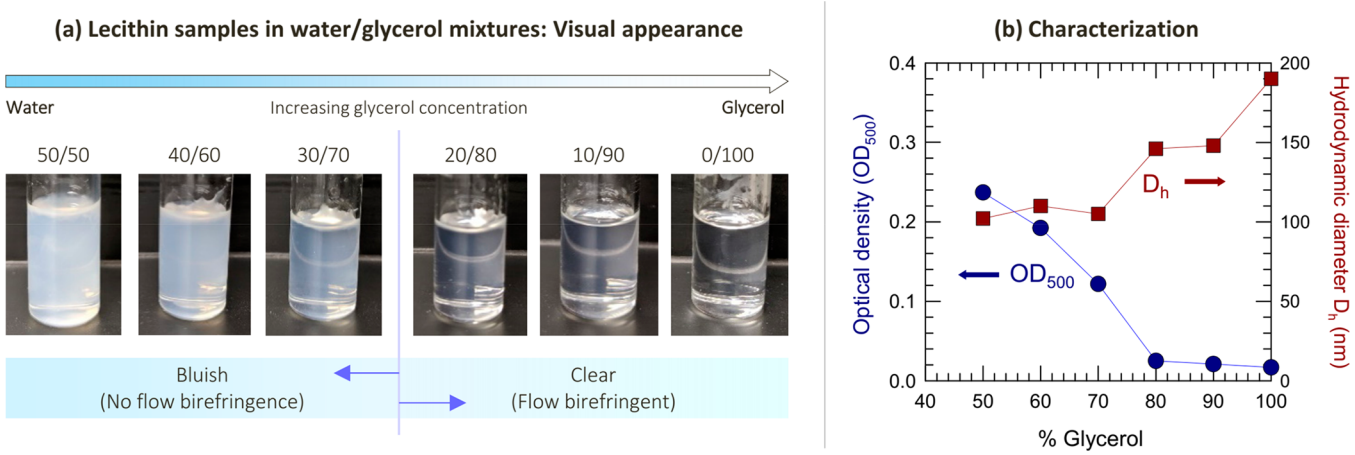

Figure 2. Lecithin self-assembly in water−glycerol mixtures. (a) Photos of samples containing 2% lecithin in water−glycerol mixtures from 50/50 to 0/100 are shown. Samples from 50/50 to 30/70 appear bluish and do not exhibit flow birefringence, whereas samples from 20/80 to 0/100 appear clear and exhibit flow birefringence. (b) Optical density at 500 nm (OD_{500}) from UV−vis spectroscopy and the hydrodynamic diameter D_h from DLS are plotted vs the glycerol content. OD_{500} (which is a measure of the turbidity) decreases with increasing glycerol content, consistent with the photos in (a). The D_h values are consistent with the presence of nanoscale vesicles in all of the samples.

EXPERIMENTAL SECTION

Materials. Lecithin (soy-phosphatidylcholine (95% purity)) was purchased from Avanti Polar Lipids. The solvents glycerol, ethylene glycol, and formamide were obtained from Sigma-Aldrich, while deuterated (d-8) glycerol ($C_3D_8O_3$) was from Cambridge Isotopes. Ultrapure deionized (DI) water from a Millipore filtration system was used to prepare aqueous samples.

Sample Preparation. Vesicles in water or water−solvent mixtures were prepared by the thin-film hydration method, as described in earlier studies.[30] Lecithin was dissolved in a 50−50 chloroform−methanol mixture, and the solvent was evaporated under nitrogen to yield a thin film of the lipid. The film was dried in a lyophilizer for 8−10 h and then contacted with the water−solvent mixture. Finally, the solution was sonicated using a Branson tip sonicator for 3−4 min. Alternatively, vesicles in water were prepared by the "solvent injection method,"[31] which also gave similar results for vesicle size. In this case, lecithin was mixed with ethanol in a 1:0.8 mass ratio. This solution was then added to water, followed by tip sonication for 3−4 min. Vesicles in pure solvents like glycerol were prepared by simply adding weighted amounts of lecithin and glycerol in a vial and heating to ∼60 °C on a hot plate under mild stirring for ∼24 h. After clear solutions were obtained, they were cooled and stored at room temperature. Samples were left at room temperature for at least a day before any measurements. All of the concentrations reported in this paper are on a weight % basis.

Rheology. Rheological experiments were conducted on an AR2000 stress-controlled rheometer (TA Instruments). A cone-and-plate geometry (2° stainless steel cone with 20 mm diameter) was used to perform steady-shear and oscillatory-shear experiments. The temperature was controlled by a Peltier assembly on the rheometer. Experiments were conducted at temperatures ranging from 25 to 65 °C. Dynamic frequency sweeps were conducted in the linear viscoelastic regime of each sample, which was first determined from strain sweeps.

Cryogenic Scanning Electron Microscopy (Cryo-SEM). A Hitachi S-4800 field emission scanning electron microscope with an operating voltage of 3 kV was used to obtain cryo-SEM images. The samples were placed into rivets mounted onto the cryo-SEM sample holder. The samples were then plunged into slushed liquid nitrogen for vitrification. This was followed by fracturing at −130 °C using a flat-edge cold knife and sublimation of the solvent at −95 °C for 15 min. The temperature was lowered back to −130 °C, and the sample was then sputtered with a gold−palladium composite at 10 mA for 132 s before imaging.

Small-Angle Neutron Scattering (SANS). SANS experiments were performed at the National Institute of Standards and Technology (NIST) in Gaithersburg, MD on the NG-B (30 m) beamline. Neutrons with a wavelength λ of 6 Å were selected. Samples were placed in 1 mm titanium cells with quartz windows. Scattering data were placed on an absolute scale of intensity using NIST calibration standards. The data shown are for the radially averaged intensity I as a function of the scattering vector $q = (4\pi/\lambda)\sin(\theta/2)$, where θ is the scattering angle. SANS fitting was done using the SasView software.

Small-Angle X-ray Scattering (SAXS). SAXS experiments were performed at 25 °C using a Xenocs Xeuss system with a Cu Kα X-ray source (λ = 1.54 Å). A Pilatus 300 K detector was used for collecting the scattered radiation, which was then converted to a plot of scattered intensity I vs scattering vector q using Igor Pro software.

Dynamic Light Scattering (DLS). Vesicle sizes were measured at 25 °C using a Photocor-FC instrument equipped with a 5 mW laser source at 633 nm, with the scattering angle being 90°. The autocorrelation function was measured using a logarithmic correlator and analyzed by the DynaLS software package to obtain the hydrodynamic size.

UV−Vis Spectroscopy. A Varian Cary 50 UV−vis spectrophotometer was used to determine the optical density (OD), i.e., the absorbance of vesicle solutions over a 1 cm path length, at a wavelength of 500 nm.

RESULTS AND DISCUSSION

Lecithin in Water−Solvent Mixtures. First, we study vesicle formation in water−solvent mixtures. Lipids like lecithin are known to form unilamellar vesicles in water. What happens if water is gradually replaced by a polar solvent like glycerol? To probe this, we prepared vesicles of 2% lecithin in water as well as in water/glycerol mixtures. The samples were first characterized by DLS, which yields the hydrodynamic diameter (D_h) of the vesicles. Lecithin vesicles in water had a D_h of about 160 nm, which is consistent with previous studies from our lab.[30] When water was replaced with water−glycerol mixtures, the vesicle diameter changed slightly.[31] In the case of a 50/50 water−glycerol sample, a D_h of about 100 nm was measured by DLS.

The photos of sample vials containing 2% lecithin in water−glycerol weight ratios from 50/50 to 0/100 are shown in Figure 2a. In this range of solvent compositions, some

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differences between the samples are apparent from the photos. It is well known that vesicle solutions appear turbid (bluish or bluish-white in color) due to the scattering of light by nanoscale vesicles.[30,31] An example is the 50/50 sample in Figure 2a (all samples at higher water−glycerol ratios looked just like this one). If vesicles are smaller or there are fewer of them, the sample will appear less turbid. Here, we find that the turbidity decreases as the glycerol content increases. Samples at 20/80, 10/90, and 0/100 water/glycerol are almost perfectly clear, i.e., they scatter light to a much lower extent (Figure 2a). These samples remain stable and clear weeks after preparation. Does this mean that there are fewer vesicles in these high-glycerol samples? We will offer a different explanation of this point later in the paper.

Figure 2b plots the optical density (OD), obtained by UV−vis spectroscopy, for each of the samples in Figure 2a. The OD is a measure of sample turbidity and the data confirm the observations from Figure 2a—that the samples become increasingly clear (i.e., OD drops to nearly zero) at higher glycerol content. In addition, the hydrodynamic diameter (D_h) from DLS for the samples is also plotted in Figure 2b. Note that, to obtain D_h, we have to use the correct viscosity of the solvent in the Stokes−Einstein equation (to relate the diffusivity measured by DLS to D_h).[1,2] The D_h from DLS is found to be around 100 nm for samples with 50−70% glycerol, and 150 nm or above for higher glycerol contents. Because similar diameters are obtained across the range of solvent compositions, we can conjecture that vesicles are present in all of the samples, including in pure glycerol. The presence of vesicles will be further confirmed later in the paper by data from scattering and microscopic techniques.

An unusual observation about the 20/80, 10/90, and 0/100 samples is that they exhibit flow birefringence. Birefringence implies that the sample has different refractive indices along perpendicular directions. To assess birefringence, samples are viewed under crossed polarizer plates. The above samples do not show birefringence at rest, but when vials are shaken, streaks of light become visible in them, and this is called flow birefringence. Vesicle samples are not expected to be flow-birefringent, and indeed this is not seen for samples from 100/0 to 30/70 water−glycerol. On the contrary, flow birefringence is associated with wormlike micelles (WLMs) because flow causes alignment of WLMs, and aligned chains will interact differently with light along directions parallel and perpendicular to their axis of alignment.[13,32] Here, we do not have WLMs, and yet we see this phenomenon. This peculiar aspect will be discussed below.

Lecithin in Polar Organic Solvents: Rheology. The previous results suggested that a sample of 2% lecithin in glycerol may contain vesicles. Next, we proceeded to study higher concentrations of lecithin in glycerol: from 2 to 15%. Typically, if such high lipid concentrations are added to water (through either the thin-film hydration or ethanol injection methods, followed by high shear via sonication or extrusion), the vesicles would convert from unilamellar to multilamellar.[5,6] Moreover, in the absence of charged lipids, the vesicles will tend to be unstable and aggregate, forming two phases over time. As an example, Figure S1 in the SI section shows that a sample of 12% lecithin in water is inhomogeneous right after preparation and separates into two distinct liquid phases within days. Conversely, in the case of glycerol, a solvent that is less polar than water, solid lecithin *can be dissolved completely even at 15% without the need for high shear, and the resulting samples are clear and homogeneous*. Thus, self-assembly occurs spontaneously in glycerol, making sample preparation much easier than in water.

We found, to our surprise, that most of these lecithin−glycerol samples were highly viscous or gel-like. This is shown by the steady-shear rheological data on the samples in Figure 3,

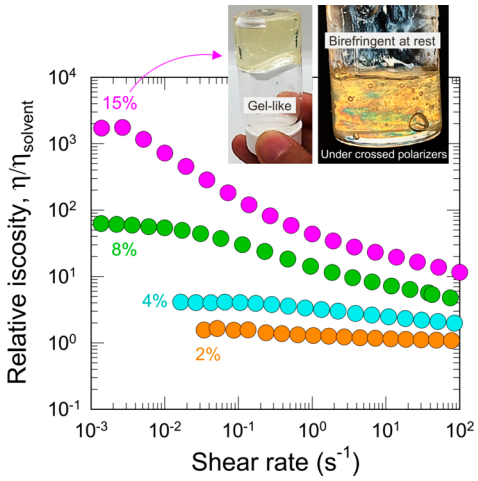

Figure 3. Steady-shear rheology of lecithin samples in glycerol. Plots of the relative viscosity $\eta/\eta_{solvent}$ as a function of shear rate are shown for 2, 4, 8, and 15% lecithin in glycerol at 25 °C. The inset shows photos of the 15% sample, which holds its weight in the inverted vial, indicating a gel-like response. This sample also exhibits birefringence at rest, which is apparent from the bright colors in the sample when viewed through crossed polarizers. See also Movie S1 in the SI.

where the relative viscosity ($\eta/\eta_{solvent}$) is plotted as a function of shear rate. For the 2% sample, there is hardly any change in viscosity relative to the solvent, i.e., $\eta/\eta_{solvent}$ is 1.6, and the sample shows Newtonian behavior (viscosity independent of shear rate). The 4 and 8% samples are moderately shear-thinning, with the viscosity being constant at low shear rates (this value is called the zero-shear viscosity η_0) and then decreasing at higher shear rates. The 15% sample is strongly shear-thinning, and its $\eta_0/\eta_{solvent}$ is 1880. Visual observation shows that this sample is gel-like and is able to hold its weight in an inverted vial (see inset to Figure 3). We had previously mentioned that the 2% lecithin sample (Figure 2a) was flow birefringent. This is also the case for the 4 and 8% samples. The 15% sample, however, is clear and *birefringent even at rest* (Figure 3 inset), with vivid colors seen in the sample when viewed under crossed polarizers. This can also be seen from Movie S1 in the SI. Static (at-rest) birefringence is generally indicative of a lyotropic liquid-crystalline phase.[1] In water, such phases are formed by surfactants of any kind only above 20 or 30 wt %.[1,3] So, what exactly is the nature of this birefringent 15% lecithin sample in glycerol: does it contain vesicles, wormlike micelles, or something else?

Dynamic rheological spectra for the samples in Figure 3 are shown in Figure 4 as plots of the elastic modulus G' and the viscous modulus G'' as functions of the frequency ω. The 4% sample (Figure 4a) shows a predominantly viscous response,[33,34] with G'' exceeding G' across the range of ω. Moreover, both moduli are strong functions of ω and $G'' \sim \omega^1$, as is typical of viscous systems (the deviation of G' from a $\sim \omega^2$ scaling does indicate some viscoelasticity in the sample).[33,34] The response of the 8% sample (Figure 4b) is

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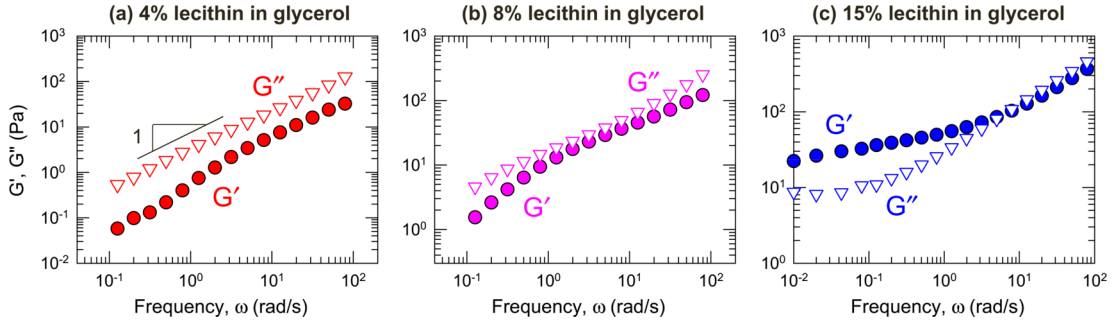

Figure 4. Dynamic rheology of lecithin samples in glycerol. Plots of the elastic modulus G' and the viscous modulus G'' vs frequency ω at 25 °C are shown for (a) 4%, (b) 8%, and (c) 15% lecithin in glycerol. The 15% sample exhibits a gel-like response.

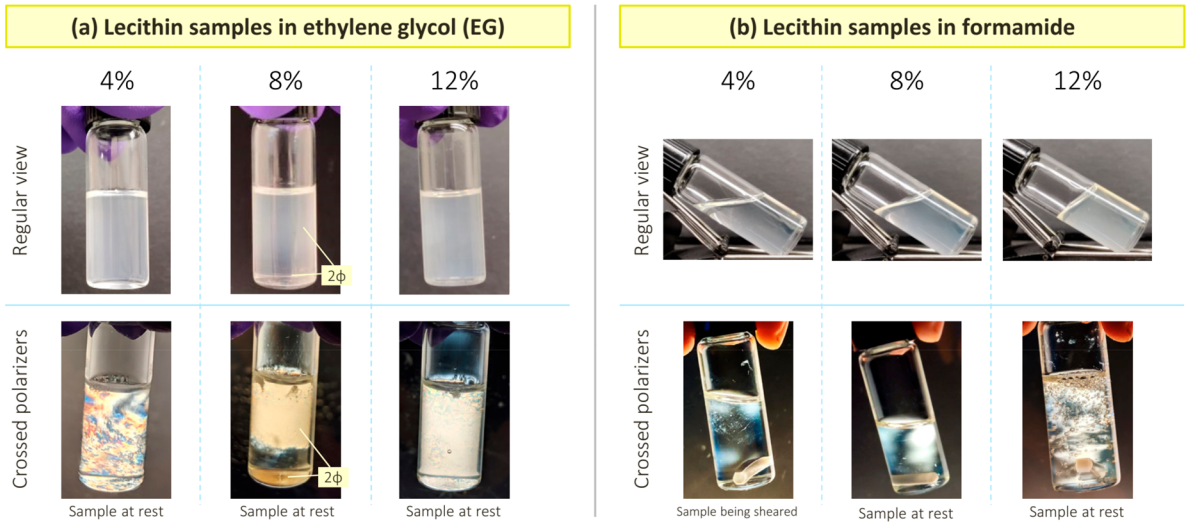

Figure 5. Lecithin samples in ethylene glycol (EG) and formamide. Photos of the samples viewed under ambient light and under crossed polarizers are shown. Samples are in (a) EG and (b) formamide and contain 4, 8, or 12% lecithin. All samples except one are birefringent at rest, as seen from the bright colors under crossed polarizers. The exception is the 4% sample in formamide, where birefringence is observed only when the sample is sheared (vial is shaken). Note that the 8% sample in EG is separated into two phases (2ϕ), with the top phase being birefringent. Also, note that the 8 and 12% samples in formamide samples are highly viscous or gel-like.

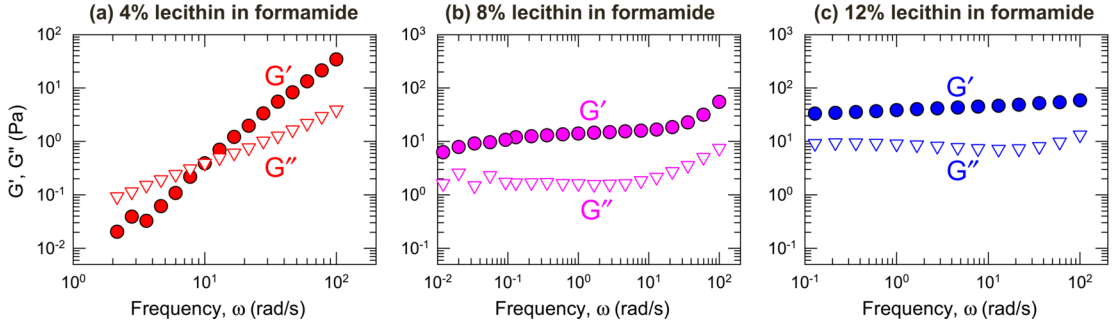

Figure 6. Dynamic rheology of lecithin samples in formamide. Plots of the elastic modulus G' and the viscous modulus G'' vs frequency ω at 25 °C are shown for (a) 4%, (b) 8%, and (c) 12% lecithin in formamide. The 4% sample shows a viscoelastic response, whereas the 8 and 12% samples show a gel-like response.

viscoelastic, with G'' and G' closer in magnitude to each other. On the other hand, the 15% sample (Figure 4c) shows the rheology of a weak gel, consistent with the photo in Figure 3, where the sample holds its weight in the inverted vial. In this case, at low ω, $G' > G''$, implying elastic behavior, and G' tends toward a constant (plateau) value of around 20 Pa. The plateau in G' (i.e., the fact that G' is frequency-independent) at low ω indicates that the nanostructure of the sample is persistent and does not relax over long timescales.[33,34]

Solutions of lecithin were also prepared in two other polar solvents: ethylene glycol (EG) and formamide. These two solvents are high on any polarity scale, comparable to water and glycerol (see Table S1 in the SI and further discussion below). The results in EG and formamide were broadly similar to those in glycerol, with some differences. In both cases, structures of ~200 nm diameter were detected by DLS at concentrations around 2% lecithin, similar to the data reported for glycerol in Figure 2b. At higher lecithin concentrations in

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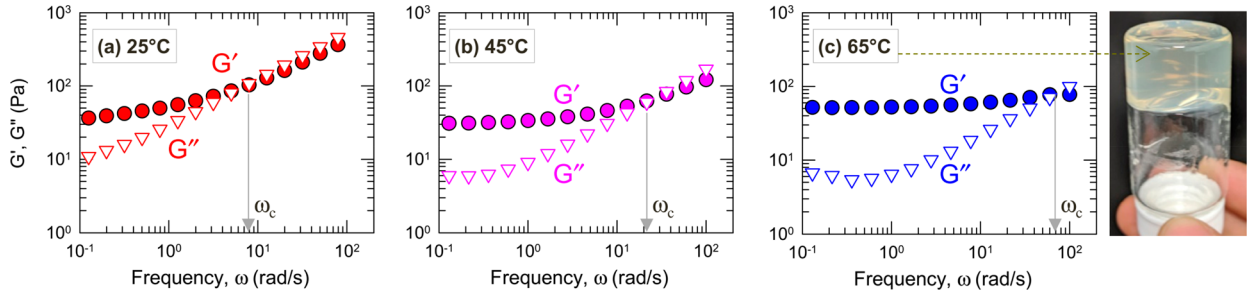

Figure 7. Dynamic rheology of the 15% lecithin sample in glycerol over a range of temperatures. Plots of the elastic modulus G' and the viscous modulus G'' vs frequency ω are shown for (a) 25 °C, (b) 45 °C, and (c) 65 °C. The sample remains gel-like at all temperatures. On the right, a photo of the sample at 65 °C is provided.

EG, the 4, 8, and 12% lecithin solutions are weakly turbid and viscous, but not gel-like (Figure 5a). Interestingly, even at 4%, the sample is highly birefringent at rest (bottom panel in Figure 5a). At 8% lecithin, a coexistence of two liquid phases is observed, with most of the lipids being confined to the upper phase, which is birefringent, whereas the lower phase is a thin EG solution (the interface between the phases is barely visible under ambient light). However, the 12% lecithin sample in EG is back to being a single phase and is also highly birefringent. Thus, there is an interesting pattern of phase behavior for lecithin in EG (1-phase to 2-phase to 1-phase, which is termed a re-entrant phase transition[1,3]).

In formamide, the 4, 8, and 12% lecithin solutions are more viscous than in EG (Figure 5b). The 8 and 12% samples are birefringent at rest, while the 4% sample is flow-birefringent. The dynamic rheology of the formamide samples is shown in Figure 6. The 4% sample shows a viscoelastic response, with $G'' > G'$ at low ω (indicating viscous behavior), while $G' > G''$ at high ω (indicating elastic behavior). In contrast, both the 8 and 12% samples exhibit gel-like rheology, with $G' > G''$ at all ω and G' showing a plateau at low ω. The plateau value of G' is ∼35 Pa for the 12% sample. We emphasize that the gel-like rheology for 15% lecithin in glycerol and 12% lecithin in formamide is unexpected and unusual. We had wondered whether these samples contained WLMs, but if that had been the case, the rheology would have been viscoelastic (with G' and G'' intersecting at a specific ω), not gel-like.[13,14,32] WLMs would also not show birefringence at rest.[13,32]

The rheology of the gel-like samples was also investigated as a function of temperature (T), and this was done for the sample of 15% lecithin in glycerol. Plots of G' and G'' vs ω at 25, 45, and 65 °C are shown in Figure 7. From the data, it is clear that the sample remains gel-like over the entire range of T, and the plateau value of G' remains nearly constant with T. This is also consistent with visual observations, and a photo of the gel at 65 °C is shown as an inset in Figure 7c. The only change with T is in the crossover frequency ω_c at which the moduli intersect. For $\omega > \omega_c$, the viscous solvent (glycerol) influences the rheology. From the data, ω_c shifts to higher ω with increasing T, reflecting the reduction in viscosity of glycerol with T. Still, the fact that the rheology stays about the same with increasing T is another indication that this sample does *not* contain WLMs. If the sample had WLMs, its viscosity would have significantly reduced upon heating (i.e., it would convert from a gel to a sol) because WLMs become exponentially shorter with increasing T.[13,14]

Lecithin in Polar Organic Solvents: Nanostructure. To make sense of the data shown up to this point, we need to elucidate the nanostructures present in the samples using microscopic or scattering techniques. With regard to microscopy, the technique of cryo-transmission electron microscopy (cryo-TEM) is the standard technique for visualizing the structure in aqueous solutions. However, cryo-TEM has not been used for solvents like glycerol because the high viscosity of the solvent complicates sample preparation (i.e., it is difficult to form a thin film of the sample). Therefore, we resorted to a related, but different technique, which is cryo-SEM. In this technique also, the sample is frozen rapidly so as to vitrify the solvent, which is then sublimed away. Structures in the sample are thereby preserved. Cryo-SEM has been used to image vesicles in several recent studies.[35,36]

Representative images from cryo-SEM are shown in Figure 8 for samples of 2 and 8% lecithin in glycerol. Both samples contain spherical structures with diameters ranging from 50 to 600 nm. The spheres are well-separated in the 2% sample

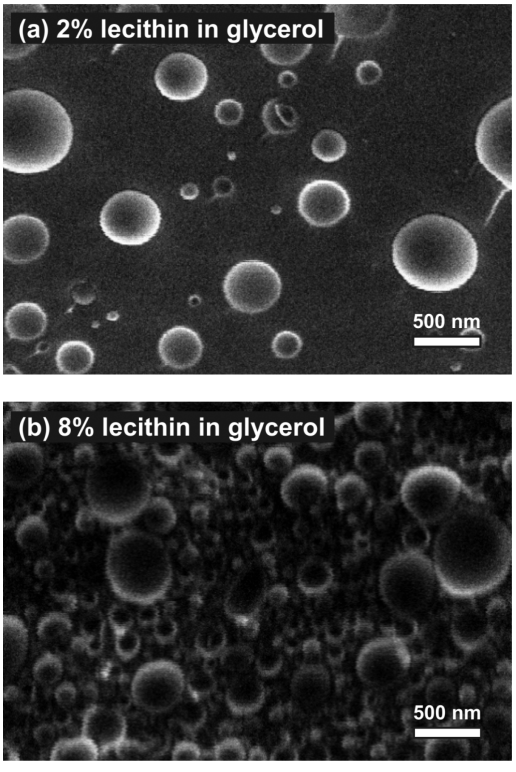

Figure 8. Cryo-SEM images of lecithin samples in glycerol. Samples contain (a) 2% and (b) 8% lecithin. Both show the presence of multilamellar vesicles, ranging from 50 to 600 nm in diameter.

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(Figure 8a). The smallest structures in this image appear to be in the background, and it is not clear if their actual sizes are larger. At the higher lipid concentration of 8% (Figure 8b), there are many more spheres in the field of view, and the spheres appear to be close-packed so as to fill up the volume.

A logical inference from the images in Figure 8 is that the structures seen are vesicles. They are too large to be spherical micelles (which are generally around 5 nm in diameter),[1,3] and there is no evidence for alternative possibilities such as cylindrical chains (WLMs) or lamellar sheets. The sizes of vesicles in these images are broadly consistent with the average D_h measured by DLS, which was 150 nm for the 2% lecithin sample (Figure 2). The sizes seen here are also comparable with the sizes of liposomes formed by lecithin in water, which generally fall in the 100−200 nm range.[30,31] Note that a 50 nm vesicle in the images is likely to be unilamellar, i.e., to have an outer shell of about 5 nm thickness corresponding to a single bilayer.[1,2] On the other hand, a 600 nm vesicle is likely to be multilamellar, i.e., it will be an onion-like structure with multiple concentric bilayers in its shell.[37,38] From these cryo-SEM images, it is not possible to distinguish between unilamellar vesicles (ULVs) and multilamellar vesicles (MLVs). However, it is more likely that they are MLVs because it would explain other observations, as discussed below. This interpretation is also consistent with the work of Lattes[15] who used electron microscopy to conclude the presence of lecithin MLVs in formamide.

We also used scattering techniques (SANS and SAXS) to probe the nanostructure in some lecithin samples. First, SANS data are shown in Figure S2 in the Supporting Information (SI) section for 0.5% lecithin in water−glycerol mixtures ranging from 90/10 to 60/40. The data plotted are for the scattering intensity I vs scattering vector q on a log−log scale. Deuterated water (D_2O) as well as deuterated glycerol was used to ensure sufficient contrast between the scattering objects and the background liquid. All of these plots show a characteristic slope of −2 at low and intermediate q, which is a signature of vesicles.[30,39] More specifically, the above slope implies that $I \sim q^{-2}$, which is the scattering pattern expected from bilayers (flat sheets) that envelop vesicle cores. It is difficult to extract the diameters of vesicles from the above SANS data because those values are expected to be in excess of 100 nm, which is a size range outside the window probed by SANS.[39] Nevertheless, an important conclusion from Figure S2 is that the data reflect the presence of vesicles in all cases, regardless of the glycerol content. This finding is consistent with the results of other recent studies.[31]

While the above data are for water−glycerol mixtures, we also ran experiments on lecithin in pure glycerol. Data from SANS for a sample of 3.2% lecithin in deuterated glycerol are shown in Figure 9a. The plot is similar to those for the water−glycerol mixtures, except that the slope for I vs q on the log−log plot is not a constant −2 at low and intermediate q; rather there appear to be two different slopes: a slope of −2.5 at low q and a slope around −1.5 or −1.7 at medium q. The different slopes suggest that the scattering over this q-range is influenced by interactions between the vesicles (structure-factor effects). At higher q, an inflection point is seen in the SANS data at q^* = 0.082 Å^{-1}. From Bragg's law,[39] this corresponds to a length scale of $2\pi/q^*$ = 7.6 nm. This length scale may represent the spacing between adjacent bilayers in MLVs. SAXS data are shown in Figure 9b, again as an I vs q plot. This data was acquired on a concentrated (15%) lecithin sample in glycerol,

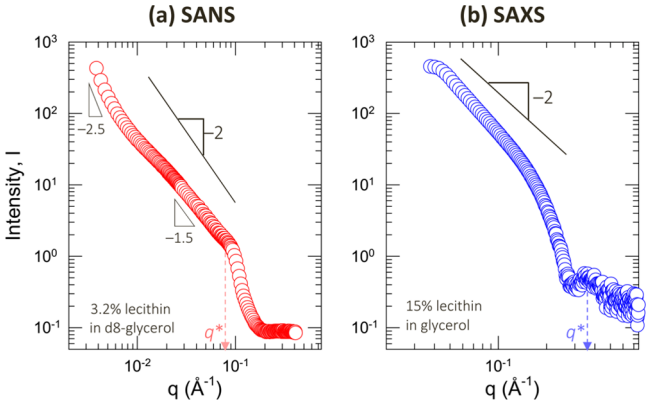

Figure 9. SANS and SAXS spectra for lecithin samples in glycerol. Each plot shows the scattered intensity I vs wave vector q, which is on an absolute scale in the case of SANS and on an arbitrary scale for SAXS. (a) SANS data for 3.2% lecithin in deuterated glycerol. (b) SAXS data for 15% lecithin in glycerol. A slope of −2, which reflects the presence of vesicles, is indicated on each plot.

which is gel-like and birefringent at rest (as per Figures 3 and 4). A slope in the vicinity of −2 is seen at intermediate q, and there is a weak peak at q^* = 0.35 Å^{-1}, corresponding to a length scale of 1.8 nm, which may again reflect the spacing between bilayers in MLVs. However, there are no multiple peaks to indicate that this sample is a well-ordered (liquid-crystalline) phase.[37,39]

Discussion: Lecithin Forms Close-Packed MLVs in Polar Solvents. Based on all of the results, what can we conclude regarding the nature of lecithin solutions in polar liquids? Considering that lecithin is a lipid and therefore has the right geometry (CPP ∼ 1, see Figure 1A) to form vesicles in water,[1,2] it is reasonable to expect that it could also form vesicles in liquids that are polar enough to facilitate self-assembly. The cryo-SEM images (Figure 8) seem to conclusively prove that vesicles are indeed present in these samples. The scattering data (Figure 9) too are more consistent with vesicles than any other structures. But if vesicles are present, a few of our results remain to be explained, including:

(1) the gel-like rheology at high lipid concentrations;
(2) the birefringence at rest or under flow; and
(3) the clarity of the samples (weak intensity of light scattering).

We believe the nanostructure proposed in Figure 10 can explain all of the above results. In both low and high lecithin concentrations, we suggest that multilamellar vesicles (MLVs), which are also termed "onions" in the literature, are present.[37,38,40−44] At low lecithin content, the MLVs will be spaced widely apart and hence will not interact with each other (Figure 10A). The sample will then be akin to a suspension of spherical particles occupying a volume fraction ϕ_{MLV} of at most 40%, and in such cases, the viscosity will be low and the rheology will be Newtonian[37,38] (such as for the 2% lecithin sample in Figure 3). As the lecithin concentration is raised, ϕ_{MLV} will increase to more than 60%, and the vesicles will become close-packed, i.e., they will fill up the entire volume (Figure 10B). At this point, the sample will start to show gel-like rheology.[37,38] That is, when a stress is applied to the sample, the stress will be transmitted throughout the volume because each MLV will be in contact with its neighbors. The

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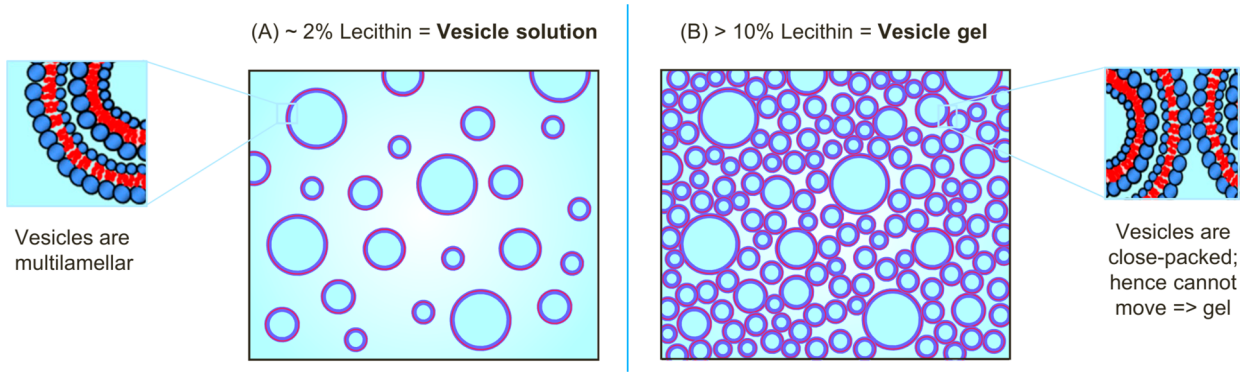

Figure 10. Schematics of the nanostructure in lecithin−polar solvent mixtures. Lecithin forms multilamellar vesicles (MLVs or "onions") at all concentrations. (a) At low lecithin concentrations (~2%), the MLVs are well separated and the viscosity of the solution is low. (b) At high lecithin content (>10%), the MLVs are close-packed, thereby giving rise to a "vesicle gel" that shows elastic rheology.

close-packed MLVs will thereby act like a connected or correlated structure, similar to a network of chains, and the structure will not relax over long timescales.[38,41] This explains why G' shows a plateau at low ω for samples like the 15% lecithin in glycerol (Figure 4c).

Support for the presence of close-packed MLVs comes from the cryo-SEM image in Figure 8b, which is for 8% lecithin in glycerol. Our rheological data indicated that this sample was viscoelastic and shear-thinning, but not a gel (Figures 3 and 4). This suggests that the ϕ_{MLV} in this sample may be in the range of 40−50% (i.e., below close-packing). If we extrapolate to a 15% lecithin sample, vesicles would be even more densely packed and the ϕ_{MLV} could easily increase above 60% (i.e., above close-packing), which would explain the gel-like rheology of this sample. The above estimates are consistent with back-of-the-envelope calculations. For example, if we assume an average vesicle diameter of 300 nm and a bilayer thickness of 4 nm, then the volume fraction of vesicles[1,2] in a 8% lecithin sample would be 102% if the vesicles were all unilamellar and 51% if the vesicles all had two concentric bilayers. Note that the average number of layers in the MLVs is not easily determined, either from cryo-SEM or SANS, but if this parameter was available, the vesicle volume fraction could be calculated more accurately.

The presence of close-packed MLVs can thus explain the gel-like rheology at high lipid concentration, which was point (1) above. In this regard, previous studies on MLVs (onions) in water have also observed gel-like rheology at high MLV volume fractions.[40−44] Such systems with MLVs have been prepared in some cases with lipids,[44] but as mentioned earlier, these MLVs tend to be unstable. However, stable MLVs have been formed in multicomponent mixtures of a single-tailed surfactant, a cosurfactant (like an alcohol), and/or salt.[40−43] The term "*vesicle gel*" has been used to describe these systems.[37,38] The elastic modulus of these gels has been shown to increase with ϕ_{MLV} and with decreasing MLV size.[37,38] Moreover, many of these vesicle gels are reported to be birefringent at rest when ϕ_{MLV} is high and flow-birefringent at lower ϕ_{MLV}, which is exactly what we observe.[40,43] Birefringence presumably arises when light interacts with the multiple concentric lamellae in MLVs, which if aligned will resemble a cut section of a lamellar phase. There will hence be anisotropy in refractive indices along mutually perpendicular directions. Flow birefringence, which arises when the vial is titled or shaken, may arise because the lamellae in the MLVs get more aligned when stress is applied. Thus, point (2) above is also consistent with the presence of MLVs.

Finally, we come to point (3), which is the clarity of lecithin vesicles in polar solvents. That is, while vesicles in water give rise to a highly turbid sample, vesicles in glycerol are optically clear (Figure 2). Why the difference? In the context of Figure 2, where lecithin was maintained at 2%, we wondered if the lower turbidity in glycerol was because the vesicle volume fraction was lower compared to water (i.e., that there were fewer objects to scatter light in the former). However, even at 15% lecithin in glycerol, the sample (see the photo in Figure 3) is much less turbid than a sample of 2% lecithin in water. Therefore, the lack of turbidity is *not* related to the vesicle volume fraction. Instead, we suggest that the reason lies in the refractive indices (n) of the lipid versus those of the solvents. The refractive index n for lecithin has been estimated to be around 1.43,[45] which is similar to the n of long alkanes, since the lipid has two long alkyl tails (n for hexadecane is also 1.43). In the case of the solvents, n for water is 1.33 and n for glycerol is 1.47 (see Table S1 in the SI). Thus, the n for the lipid is much closer to that for glycerol, which means that the optical contrast between the vesicle bilayer and the solvent will be low. The intensity of scattered light will be proportional to this optical contrast.[39,45] This could explain why vesicle solutions and gels in glycerol do not scatter light strongly, i.e., why they are much less turbid compared to vesicles in water. The same arguments extend to vesicles in formamide (n = 1.45) as well as EG (n = 1.46). Stated differently, lecithin vesicles in these polar solvents are more or less "invisible"—similar to the lecithin vesicles in aqueous solutions containing sucrose that were prepared by Ardhammar et al.[45]

An important corollary of the above point pertains to the *stability* of vesicles. It is well known that suspended particles in a solvent will experience attractive van der Waals (vdW) interactions.[1,2] Thus, when vesicles approach each other by diffusion (Brownian motion), vdW forces will induce vesicles to aggregate or coalesce. To keep vesicles stable, attractive vdW forces must be counteracted by repulsive forces, which are typically either via steric repulsions (due to undulations of vesicle bilayers) or electrostatic repulsions (in the case of charged bilayers).[1,2] Even so, lipid vesicles in water are rather unstable and must usually be stored at low temperatures (<5 °C) to ensure their stability. The strength of vdW attractions is dictated by the Hamaker constant A_{121} (where 1 refers to vesicles and 2 the solvent), which is given by[1,2]

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$$A_{121} = \frac{3}{4}k_B T\left(\frac{\varepsilon_1 - \varepsilon_2}{\varepsilon_1 + \varepsilon_2}\right)^2 + \frac{3h\nu_e}{16\sqrt{2}}\frac{(n_1^2 - n_2^2)^2}{(n_1^2 + n_2^2)^{3/2}} \quad (1)$$

Here, ε_1 is the dielectric constant, n_1 the refractive index of the vesicles, and ε_2 and n_2 are the corresponding parameters of the solvent. k_B is Boltzmann's constant, T is the absolute temperature, h is Planck's constant, and ν_e is the main absorption frequency of the material. Of the two terms in eq 1, the second term is generally the larger contribution to A_{121}.[1,2] Accordingly, eq 1 implies that by matching the refractive indices n_1 and n_2, the Hamaker constant A_{121}, and thereby the vdW attractions between vesicles, can be greatly reduced. This concept is termed "index-matching" in the literature.[1,2] Extending this to our case, since n_1 (lecithin vesicles) and n_2 (glycerol) are very close, the vesicles will experience weaker vdW attractions in glycerol. Therefore, the vesicles will be more stable in glycerol compared to water. In addition to the weakened vdW attractions, another factor contributing to stability in glycerol is expected to be the high viscosity of the solvent (~900 times higher than that of water),[13] which will slow down the approach of vesicles toward each other by Brownian motion.

The above prediction is confirmed by our experiments. The vesicle samples we have prepared in glycerol have *remained stable for over 6 months*. That is, we see no evidence of phase separation, increases in turbidity, changes in vesicle size for dilute lecithin samples, or changes in rheology for concentrated lecithin samples. These observations are in stark contrast to vesicles in water, prepared from lecithin or other lipids. (If lecithin is the sole lipid because it is zwitterionic, its vesicles would lack a surface charge, which would make them harder to stabilize in water.) As noted above, aqueous vesicles have to be stored in a fridge to maintain their stability, and even with that, they have to be used for biomedical applications within at most a week after preparation. Thus, a practical implication from this paper is that *it could be advantageous to prepare vesicles in glycerol (or other polar solvents) and then transfer these into water at the time of use*. We have noted that preparing lecithin vesicles in glycerol is very easy because it is spontaneous—one could simply dissolve solid lecithin in glycerol without using other solvents or high shear. These vesicles can then be stored at room temperature, rather than a fridge, and will still remain stable for months. If a sample of 15% lecithin in glycerol is prepared, this can be used like a "stock solution" and diluted with water or aqueous buffer at the time of use. This procedure could greatly simplify vesicle preparation and in turn enhance biomedical applications. Note also that glycerol is known to be a biocompatible solvent and a cryoprotectant for cells.

Another practical aspect emerging from our study is the use of lipids as rheology modifiers (i.e., thickeners) for nonpolar liquids. Many aqueous formulations, such as fabric softeners, contain MLVs, and indeed Kuhnle et al.[46] have pointed out that MLVs can act as rheology modifiers in water due to their ability to close-pack at the nanoscale. Our study suggests that the same possibility can be extended to polar liquids like glycerol and EG. Glycerol is used as a hydrating agent in cosmetic formulations,[31] while both glycerol and EG are used in antifreeze fluids and lubricants.[47,48] In many such formulations, polymers or other materials are used as rheology modifiers, but based on our study, we could achieve similar effects using lipids. Because lipids are derived from natural sources, they would fall under the "green," i.e., eco-friendly, category of chemicals, and could therefore present a viable alternative to existing thickeners for use in lubricants.

Finally, we discuss the fundamental implications of our study regarding self-assembly. As noted in the Introduction section, until recently, only small spherical micelles had been self-assembled in polar liquids. However, we have recently demonstrated the self-assembly of WLMs in glycerol and other polar liquids,[13,14] and here we have shown the self-assembly of vesicles in the same (our work substantiates and builds on earlier work by Lattes[15]). *Thus, it is now clear that all of the three major classes of self-assemblies—spherical micelles, cylindrical (wormlike) micelles, and vesicles—can be formed in polar liquids*. The liquids do need to have sufficient polarity and cohesive energy density (CED) to promote self-assembly. Table S1 in the SI shows the dielectric constant, which is a key measure of solvent polarity, and the Gordon parameter G, which is a measure of the CED, for several common solvents. G is defined by[2,9]

$$G = \frac{\gamma}{(V_s^{mol})^{1/3}} \quad (2)$$

where γ is the surface tension of the solvent and V_s^{mol} is its molar volume. Water has the highest value of G and is thus the most favorable solvent for self-assembly. Glycerol, formamide, and EG all have high G, which explains why we were able to form lecithin vesicles in these solvents. Note also that, from our study, the viscosity (or the elastic modulus) of vesicle samples at a given lecithin concentration follows the order: formamide > glycerol > EG, which is the same trend as for their Gordon parameters. Conversely, we have been unable to find any evidence of lecithin self-assembly in ethanol or methanol, which have lower G values. In the latter solvents, lecithin dissolves readily but forms thin, clear liquids that show no signs of large aggregates. Inducing self-assembly in those solvents may require a different strategy, as has been suggested by Huang et al.[18]

CONCLUSIONS

This study has demonstrated that vesicles can be self-assembled in a variety of polar organic solvents including glycerol, formamide, and EG. The amphiphilic molecule used to create these vesicles is the simple phospholipid, lecithin, or soy-phosphatidylcholine. Lecithin is insoluble in water but dissolves readily in polar solvents at concentrations up to 15%, with the resulting fluids being colorless and relatively clear (nonturbid). At low lecithin concentrations (~2%), the fluids are viscous and flow-birefringent, and structures around 200 nm are found in them by DLS. At higher concentrations (>10%), the fluids are gel-like and strongly birefringent at rest. Dynamic rheology of the latter reveals an elastic, gel-like response, with $G' > G''$ and G' being frequency-independent at low frequencies. Images from cryo-scanning electron microscopy (cryo-SEM) indicate that concentrated samples are "vesicle gels," where MLVs (onions), with sizes between 50 and 600 nm, are close-packed across the entire sample volume. This structure explains both the rheology and the birefringence. To our knowledge, this is the first report of both vesicles as well as "vesicle gels" in pure polar solvents. Our study significantly expands the possibilities for self-assembly in polar solvents, and our new formulations may open new avenues for applications in pharmaceutics, cosmetics, and as lubricants or antifreeze agents.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.langmuir.1c00628.

Visual appearance of concentrated lecithin samples in water (Figure S1), SANS data for lecithin vesicles in water−glycerol mixtures (Figure S2), selected properties of various polar solvents (Table S1) (PDF)

Birefringence of a 15% lecithin sample in glycerol (Movie S1) (MP4)

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Notes

The authors declare no competing financial interest.

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Supporting Information for:

Spontaneous Formation of Stable Vesicles and Vesicle-Gels in Polar Organic Solvents

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Contents

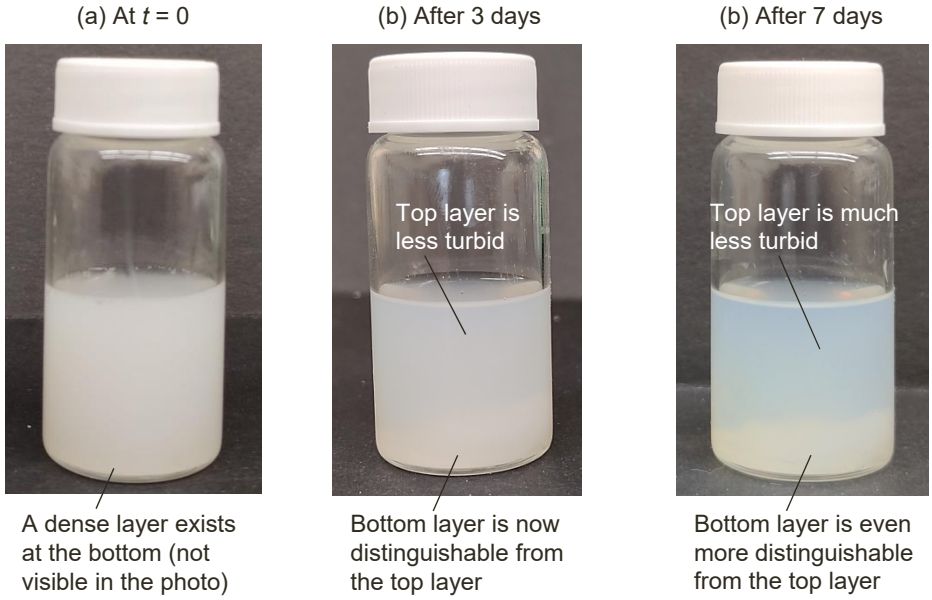

Figure S1. Visual appearance of concentrated lecithin samples in water. Photos are shown of a sample of 12% lecithin in water, prepared by the thin-film hydration method, followed by sonication. The sample is observed on the lab bench under ambient conditions. (a) Right after preparation, the sample is inhomogeneous with a dense layer at the bottom, but this is not perceptible in the photo. (b) After 3 days, a clear demarcation of the two layers (phases) can be seen, with the top being less turbid. (c) After 7 days, the contrast between the phases is more apparent, with the top layer being much less turbid than the bottom. Both phases are of low-viscosity, i.e., the sample readily flows if the vial is tilted.

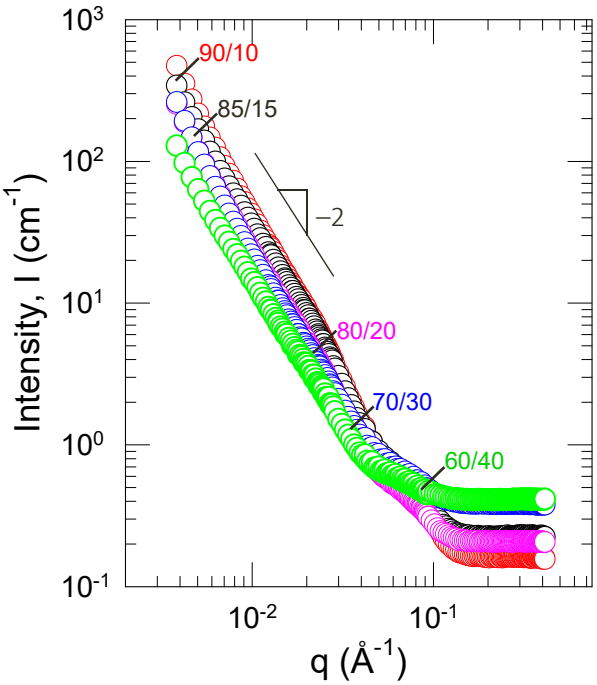

Figure S2. SANS data for lecithin vesicles in water-glycerol mixtures. The scattering intensity *I* versus wave vector *q* is plotted for 0.5% lecithin vesicles in water(W)-glycerol (Gly) mixtures ranging from 90/10 W/Gly to 60/40 W/Gly. Deuterated water (D_2O) and glycerol ($C_3D_8O_3$) were used for contrast. A slope of –2, which reflects the presence of vesicles, is indicated on the plot.

Solvent	**Dielectric Constant** ε (at 20°C)	**Gordon Parameter** G ($Jmol^{1/3}m^{-3}$)	**Refractive index** n (-)
Water	80	2.74	1.330
Formamide	109	1.73	1.445
Glycerol	47	1.52	1.473
Ethylene glycol	37	1.36	1.463
Methanol	33	0.66	1.330
Ethanol	25	0.58	1.360

Table S1. Selected properties of various polar solvents. The dielectric constant ε and the Gordon parameter G are both measures of solvent polarity. The refractive index n is important in dictating the colloidal interactions of vesicles in the solvents.