A Phase of Liposomes with Entangled Tubular Vesicles

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An equilibrium phase belonging to the family of bilayer liposomes in ternary mixtures of dimyristoylphosphatidylcholine (DMPC), water, and geraniol (a biological alcohol derived from oil-soluble vitamins that acts as a cosurfactant) has been identified. Electron and optical microscopy reveal the phase, labeled \( L_{tv} \), to be composed of highly entangled tubular vesicles. In situ x-ray diffraction confirms that the tubule walls are multilamellar with the lipids in the chain-melted state. Macroscopic observations show that the \( L_{tv} \) phase coexists with the well-known \( L_{v} \) phase of spherical vesicles and a bulk \( L_{v} \) phase. However, the defining characteristic of the \( L_{tv} \) phase is the Weissenberg rod climbing effect under shear, which results from its polymer-like entangled microstructure.

Phospholipid molecules form closed bilayer shells known as liposomes or vesicles when dispersed in water because of the amphiphilic nature of the molecules (1). Since their discovery by Bangham et al. (2), uni- and multilamellar vesicles have received much attention because of their similarities to living cells and their potential for encapsulating and segregating water-soluble materials from a bulk solution (1). Vesicles are used extensively as models of adhesion, de-adhesion, and fusion of interacting cells (1, 3) and in fundamental studies of colloidal interactions and stability (4, 5). From a technological viewpoint, vesicles are increasingly used in the cosmetics industry as controlled chemical release agents (such as in formulations of lotions, gels, and creams) (6); they continue to be explored for their utility in the food and agricultural industries and are likely to dramatically impact the medical field as drug and gene carriers (7).

We report on the discovery of another vesicle phase in room-temperature mixtures of DMPC, water, and geraniol. Geraniol is a branched long-chain biological alcohol, derived from oil-soluble vitamins, that acts as a cosurfactant (Fig. 1). In the DMPC-rich corner of the phase diagram, we observe a lyotropic liquid-crystal multilamellar \( L_{m} \) phase, and in the water-rich corner, an extremely dilute phase of spherical unilamellar vesicles that is similar to the equilibrium vesicle phases \( (L_{v}) \) recently observed in similar systems (8–10). A phase consisting of entangled multilamellar tubular vesicles, which we have labeled the \( L_{tv} \) phase, emerges in the region between the \( L_{m} \) and \( L_{v} \) phases. The \( L_{tv} \) phase responds dramatically to applied flow fields, which strongly indicates that its microstructure is similar to that of a semidi-
lute, entangled polymer solution. Typical di-lute uni- and multimellar vesicles cannot
become entangled because of their closed, spherical topology and hence have rather
simple, solvent-dominated behavior under shear (10).

Myel-in-like cylindrical vesicles similar to the L_{rv} phase have been observed in the
early stages of hydration of lecithin samples (11). However, the formation of these het-
erogeneous samples depended on the method of lipid hydration and were primarily
made up of large spherical multimellar vesicles; the nonspherical structures gradually
evolved into spherical structures on equil-
ibration. Here we report on a distinct situation associated with the equilibrium phase
behavior of a ternary system. Upon simply
mixing DMPC, geraniol, and water at the
appropriate ratios and storing the solution at 25°C without agitation, tubular vesicles
form spontaneously. Over a wide variety of
concentrations, long-term phase coexist-
ence is observed, either between the L_{rv}
and L_{α} phases or the L_{α} and L_{rv} phases
(Figs. 1 and 2).

A key ingredient leading to the formation of this new phase is the presence of an
alcohol or "cosurfactant." Cosurfactants,
such as alcohols, act in a fashion similar to
that of surfactants in that they partition preferentially into the bilayer, although they
typically do not undergo extensive self-as-
semble. Alcohol cosurfactants are known to

qualitatively alter phase behavior when present as a majority component in the bilayer,
typically two to four cosurfactant mole-
cules per surfactant (12–15). The stiffness
or bending rigidity (k_{b} = 50 k_{b}T, where k_{b}
is Boltzmann's constant and T is temperature)
of DMPC-water multilayers in the L_{α} phase
(16, 17) may be strongly reduced by the
addition of the cosurfactant pentanol (18),
which decreases the bilayer thickness and
makes the membranes more flexible. As a
result, an enhanced undulation repulsion
(15, 18–21) between mixed lipid-cosurfac-
tant bilayers enables dilation of the stacked
membrane phase to interlayer spacings much
larger than the membrane thickness (which
is typical of rigid membranes (4, 22)).

The ternary phase diagram of DMPC-
geraniol-water system at 25°C (Fig. 1) has
four single-phase domains along with regions
(shaded) where two or more of these phases
cocexist. Single-phase domains occur in each
of the DMPC-water, and geraniol-rich cor-
ers of the phase diagram; they are, respec-
tively, the well-known L_{α}, lamellar phase,
an extremely dilute vesicle phase (L_{q}), and a
reverse-micellar phase. In binary DMPC-wa-
ter mixtures, at concentrations less than
about 40 weight % water, an L_{α} phase is
formed (22); however, in ternary DMPC-
geraniol-water mixtures at ~14 weight %
geraniol, the L_{α} phase can be diluted to ~46
weight % water (resulting in an interlayer spacing d = 63 Å). At about the same ratio

of DMPC to geraniol, dilution of the lamel-
lar phase to greater than 46 weight % water
leads to phase separation, giving rise to a
bluish and transparent L_{rv} phase on the sur-
face that coexists with a lamellar L_{α} phase
that settles to the bottom of the solution. In
Fig. 1, the shaded area below the L_{α} phase
is a two-phase region where the L_{rv} phase
coexists with the lamellar phase.

When samples are prepared at approxi-
mately 75 weight % water, 15 weight %
DMPC, and 10 weight % geraniol, the pre-
dominant phase forms (>90% by volume)
is the bluish L_{α} phase. The L_{rv} phase
appears to be an equilibrium phase; sealed
solutions of this phase have remained sta-
ble for more than 2 years. The new phase
is stable at 25°C, appearing isotropic be-
tween cross-polarizers, and is temperature-


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**Fig. 1.** Ternary phase diagram of DMPC-geraniol-water at 25°C [DMPC and geraniol were purchased from Avanti Polar Lipids (Alabaster, Alabama) and Sigma (St. Louis, Missouri), respectively]. The two-phase regions are shown as shaded areas and the one-phase lamellar (L_{α}) and reverse-micellar phases are outlined by solid lines. Probable single-phase regions of the tubular L_{q} and the unilamellar spherical L_{α} phase vesicles are indicated by black dots because they were found only in restricted regimes of concentration. The structure at the top right is DMPC, a double-tailed bilayer-forming lipid, and the structure at the bottom right is geraniol, a branched biological alcohol.

**Fig. 2.** (A) Photograph of a test tube showing the interface of a two-phase sample with a tubular vesicle phase L_{α} (top) and a dilute L_{q} spherical vesicle phase (bottom). (B) When a rotating rod (not shown) is inserted into the upper phase, the observed response is characteristic of a densely entangled semidilute polymer liquid: The interface moves inward and upward as described in the text. (C) Viscosity of the L_{α} phase (80 weight % water, 14 weight % DMPC, and 6 weight % geraniol) in centipoise (1 P = 1 dynes cm^{-2} s^{-1}) plotted versus the shear rate at 25°C. The viscous phase shows slight thickening over the four decades of measured shear rate. The instrument used was a low-shear rotational couette rheometer (Contraves LS-30, AG Zurich, Switzerland).
sensitive, turning cloudy upon cooling.

Particularly interesting is the flow behavior at the interface of the L_{ov} and L_{o} phases. We made a mixture of 76 weight % water, 18 weight % DMPC, and 6 weight % geraniol with a well-defined interface (Fig. 2A). Cryogenic transmission electron microscopy (cryo-TEM) has shown that the bottom phase consists of dilute spherical vesicles (L_{o}) (Fig. 1) and that the upper phase is the L_{ov} phase. The top phase shows rather dramatic flow effects (Fig. 2B); a rotating rod inserted into the top phase leads to an immediate response in which the interface moves inward and climbs toward the rod at the center. This behavior, although uncommon in typical vesicle dispersions, is reminiscent of the so-called Weissenberg effect (23) observed in semidilute polymer solutions. We note, however, that the L_{ov} phase exhibits an "inverse" Weissenberg effect; the typical Weissenberg effect would lead to the interface moving inward and dipping at the center. This inverted behavior may arise from a larger second normal stress difference of the L_{ov} phase under flow than is usually found in semidilute polymeric solutions (24). The Weissenberg effect is a consequence of flow-induced normal stresses induced by an entangled network. Hence, this flow response of the L_{ov} phase is strongly suggestive of a highly entangled microstructure. The Weissenberg effect is the most distinctive and unusual signature of this phase. More detailed measurements of the L_{ov} phase (Fig. 2C) show that it is quite viscous, between 22 and 24 times the viscosity of water, and shows a slight thickening tendency over four decades of shear rate.

Analyses with cryo-TEM, optical microscopy, and x-ray scattering show, in fact, that the L_{ov} phase consists of bilayer sheets in the form of tubular vesicles, unlike the "living polymer" (25) systems consisting of self-assembled cylindrical micelles. This difference may account for the observation of the inverse Weissenberg effect under flow (Fig. 2B). We prepared the cryo-TEM samples by spreading the sample into a thin (<100 nm) film on a holey-carbon grid (Pella, Redding, California); the grid was then frozen by plunging it into liquid ethane cooled by liquid nitrogen. Plunging resulted in vitrification of the solvent, and the solution microstructure was preserved. We avoided evaporation losses and temperature effects by preparing the films in a temperature- and humidity-controlled environmental chamber (26). The films were examined at −170°C in a JEM 200FX (Peabody, Massachusetts) scanning transmission electron microscope with a GATAN (Pleasanton, California) cryotransfer system.

A cryo-TEM image of a relatively thin area of one such sample of L_{ov} phase (Fig. 3A) reveals extended cylindrical structures spanning the field of view (>10 μm long). The image shows concentrically wrapped bilayers that bulge at regular intervals. A majority of these vesicles have spindle-shaped structures with the cylinder diameter varying along their length from greater than 100 nm to almost the width of a single membrane tether. The individual bilayers are well separated from each other, indicating a more repulsive interaction [most likely enhanced undulation repulsion (15, 18–21)] between the bilayers than found in the lamellar phase, in which the bilayer spacing is about the same as the bilayer thickness (22). A thicker area of the cryo-TEM specimen (Fig. 3B) shows that these tubular vesicles can be highly entangled and take on a variety of spherical and cylindrical shapes. This level of entanglement is consistent with the Weissenberg effect observed in the L_{ov} phase under shear.

On larger length scales, we observed tubular and elongated multimellar vesicles with optical microscopy using both differential interference contrast (DIC) (Fig. 4A) and phase contrast (Fig. 4B). Here, however, the tubules were observed on a much larger scale both in diameter (~1 μm) and length (~100 μm), although the general tubular morphologies observed seem independent of the length scale. The microscopy images show that the predominant morphology for the L_{ov} phase is a dense cross-linked network of multimellar tubular vesicles. The images reveal an abundance of conformations of tubular vesicles, most of them appearing entangled, but occasionally flow-induced alignment was observed. On rare occasions, we observed breakage of the long vesicles followed by a slow retraction of the ends. Microscopy also reveals that these cylindrical vesicles coexist with some extremely large spherical vesicles that are often highly convoluted and multimellar. In the case of accidental flow, the vesicle structures elongated and with-
stood tension, as seen by the absence of out-of-plane fluctuations.

The similarities of the cryo-TEM and optical micrographs is striking even though the dimensions differ by almost two orders of magnitude. In thin specimens prepared for electron microscopy, vesicles larger than a few micrometers in diameter were excluded from films, and hence, only smaller vesicles were imaged. Vesicles of such size were not observed in optical microscopy images because they were beyond the resolution limit. However, the combined electron and optical micrographs show that the tubule diameters span a range from tens of nanometers to many micrometers, with their length approaching macroscopic dimensions.

In situ structural studies were carried out with x-ray scattering on samples of the L_{TV} and L_{HP} phases both at our in-house Materials Research Laboratory x-ray facility and, at much smaller vector sizes, on beam line VI-2 at the Stanford Synchrotron Radiation Laboratory (SSRL). The solutions were contained in sealed quartz capillaries with diameters of 1.5 and 2.0 mm, which yielded randomly oriented lamellar domains for samples in the L_{HP} phase. Scattering profiles from longitudinal scans for the L_{TV} phase (triangles) and the L_{HP} phase (squares) are shown in Fig. 5. The multiple harmonics observed in the L_{HP} phase are typical of the scattering from a low water content L_{HP} sample; here, at 20 weight % water, they show an interlayer spacing d = 44 Å. In the monolayer limit of water dilution for the L_{TV} phase (46 weight % water with 14 weight % geraniol), d = 63 Å. From the sharpness and position of the peak at small wave vectors in the L_{TV} phase (Fig. 5) combined with the microscopy images, we infer that the tubules consist of multilayers

REFERENCES AND NOTES

6. See, for example, chap. 19 of (1), which contains a list of commercial cosmetics products.
7. See, for example, chap. 11 of (1).
12. See, for example, A. Ben-Shaul, W. Gebert, D. Roux, Eds., Micelles, Membranes, Micromulsions, and Monolayers (Springer-Verlag, New York, 1994).
27. Light-scattering experiments on the shape fluctuations of monodisperse vesicles prepared by extrusion from L_{HP} phase bilayers indicate that the bending modulus k_{s} is reduced to about 2k_{B}T (M. Speo-
29. Preliminary results in our laboratory show the existence of this phase in DMPC-water mixtures with other surfactants, such as pentanol, hexanol, and heptanol.
30. We thank P. Pincus, J. Israelachvili, E. Keller, and D. Roux for useful comments. In particular, M. Cates is acknowledged for discussions on the Weisssenborn flow effects. C.R.S., H.E.W., and S.H.I. gratefully acknowledge partial support by the National Science Foundation (NSF) under grant DMR-91-01399 and the Petroleum Research Fund (27337-AC2). J.A.Z., S.C., and E.N. acknowledge partial support by the National Institutes of Health under grant GM-1354 and NSF under grant CTS-9101719. The Materials Research Laboratory at Santa Barbara is supported by NSF under grant DMR-91-23048. The synchrotron x-ray scattering experiments were carried out on beam line VI-2 at the SSRL, which is supported by the U.S. Department of Energy.
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