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LETTER TO THE EDITOR

Lipid organization and the morphology of solid-like domains in phase-separating binary lipid membranes

V D Gordon[1], P A Beales[1], Z Zhao[1], C Blake[1], F C MacKintosh[2], P D Olmsted[3], M E Cates[1], S U Egelhaaf[1,4] and W C K Poon[1]

[1] SUPA, School of Physics and Collaborative Optical Spectroscopy, Micromanipulation and Imaging Centre (COSMIC), The University of Edinburgh, James Clerk Maxwell Building, Kings Buildings, Mayfield Road, Edinburgh EH9 3JZ, UK
[2] Vrije Universiteit, De Boelelaan 1081, 1081 HV Amsterdam, The Netherlands
[3] Department of Physics and Astronomy, University of Leeds, Leeds LS2 9JT, UK
[4] Condensed Matter Physics Laboratory, Heinrich-Heine-University, D-40225 Düsseldorf, Germany

E-mail: vernita@post.harvard.edu and w.poon@ed.ac.uk

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Abstract

In multi-component lipid membranes, phase separation can lead to the formation of domains. The morphology of fluid-like domains has been rationalized in terms of membrane elasticity and line tension. We show that the morphology of solid-like domains is governed by different physics, and instead reflects the molecular ordering of the lipids. An understanding of this link opens new possibilities for the rational design of patterned membranes.

Membranes are widely studied in soft-matter and biological physics [1–7] and exploited as tunable, responsive structures in materials science [8]. In particular, membranes made of two or more lipid species have recently attracted considerable attention [9, 1–3]. In some of these mixed membranes, lateral phase separation produces two coexisting fluid-like phases [1, 2]. For such fluid–fluid phase separation, line tension between phases favours circular domains [1, 2], while more complex patterns may emerge from the interplay between line tension and other membrane properties [1] such as tension and bending stiffness [7]. In contrast, the shapes of solid-like domains [9, 3] have not been successfully rationalized within this framework of elastic properties [5, 10, 6]. Here we demonstrate that qualitatively different physics dominates the problem: lipid packing on the molecular level determines mesoscopic domain shape [3]. This link is reminiscent of a well known effect in hard condensed matter, the way unit cell symmetries determine the macroscopic shape of three-dimensional crystals [11]. Our new physical insight has important implications for the development and understanding of membrane-based materials [3, 5, 10, 6, 8, 9]. Such systems are widely used both as biotechnological containers or delivery vectors [12–14] and as models for a variety of biological structures [1, 15, 16] in which demixing produces heterogeneities with vital functions [15–19].

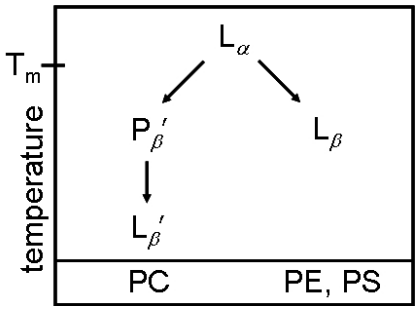

Figure 1. Schematic diagram of the phase behaviour of unitary bilayer systems made of the lipids studied here, as described in the text.

At high temperatures, unitary bilayer membranes made from the lipid species we study here are in a 2D fluid phase (L_α), in which lipids have conformationally disordered hydrocarbon tails. Below their melting temperature T_m, such bilayers can form various solid-like phases with ordered tails [20] (figure 1). Bilayers of the phosphocholine (PC) lipids discussed in this work form a phase with 10 nm sawtooth ripples, P'_β, in which the lipids have near-hexagonal ordering, which can extend over large areas [4, 21]. Upon further cooling, these bilayers form a flat phase with tilted tails, L'_β, in which lipids have near-hexagonal ordering over large areas [22]. Bilayers made of the phosphoethanolamine (PE) and of the phosphoserine (PS) lipids studied here instead form a flat untilted phase, L_β [23–26], for which to our knowledge no long-range order has been reported.

Here we investigate solid-like domains in unsupported lipid membranes which are made of two different lipid species (designated using the usual nomenclature[5]) (Avanti Polar Lipids). These membranes are in the form of giant unilamellar vesicles (GUVs), 10–80 μm in diameter, which are prepared using standard electroformation techniques [27, 2, 9]. Our GUVs are formed at temperatures above the higher T_m of the two lipids, creating well mixed fluid membranes. When the temperature is lowered (typically at 0.2–0.4 °C min^{-1}), the lipids demix laterally to form solid domains in a fluid matrix [9, 3]. These domains are imaged with a confocal microscope (BIORAD) using trace amounts of amphiphilic fluorescent dyes (0.1–0.5 mol%, Molecular Probes) that partition differently into fluid and solid-like phases; one dye (type 1) has a fluorophore in the hydrophobic region (BODIPY) and two others (type 2) have fluorophores in the hydrophilic region (Rh-DPPE and Di-I-C18)[6].

In each lipid mixture, we observe one of three characteristic domain morphologies: stripes, hexagons, and circles. We now discuss in turn the occurrence of these three domain shapes in relation to the lipid organization in the solid phases.

Our first observation is that stripe-like domains arise in all systems that consist of PC lipids with short and intermediate tails (figures 2(a)–(e)). (Data for DLPC:DPPC have already been reported [9, 3].) These stripes often bend sharply in the plane of the membrane; in the DLPC:DPPC mixture, the bending angles cluster around 60° and 120° (figure 2(f)), with the distribution broadened as growing domains push and distort each other [28]. Furthermore,

[5] We use conventional four-letter abbreviations of the form DxPy, where here 'x' indicates the length of each lipid's two identical saturated acyl tails and 'y' specifies the type of polar headgroup. We study lipids with the polar headgroups phosphocholine (PC), phosphoserine (PS), and phosphoethanolamine (PE). The length of tails, measured in number of carbon atoms, is given by $L = 12$, $M = 14$, $P = 16$, and $S = 18$.

[6] Dyes are lissamine rhodamine B 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (Rh-DPPE), 2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-pentanoyl)-hexadecanoyl-*sn*-glycero-3-phosphocholine (BODIPY), and 1, 1′-dioctadecyl-3, 3, 3′,3′-tetramethylindocarbocyanine perchlorate (DiI-C-18).

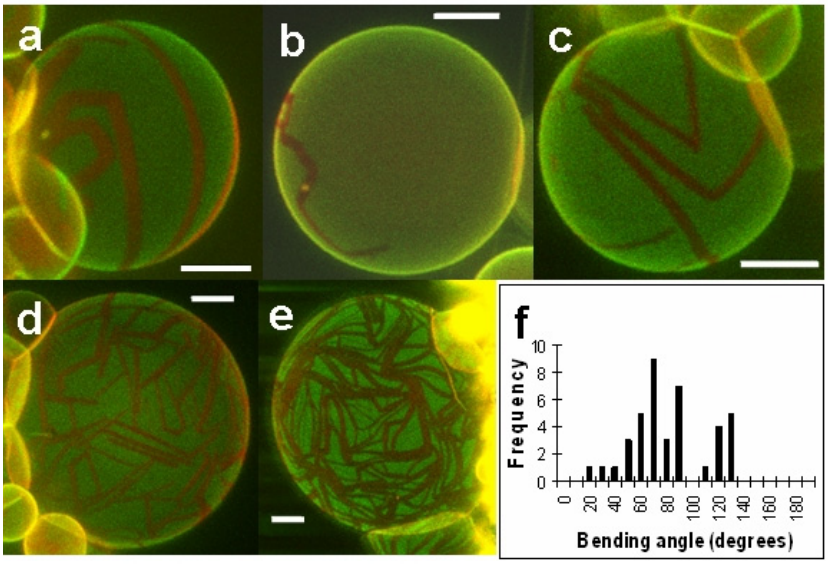

Figure 2. Stripe-like domains in vesicles made of binary PC mixtures. (a) DLPC:DPPC (1:1), 29.5 °C; (b) DLPC:DSPC (1:1), 37.7 °C; (c) DMPC:DSPC (1:1), 42.5 °C; (d) DMPC:DPPC (1:1), 36.4 °C; (e) DPPC:DSPC (1:1), 43.9 °C. BODIPY, shown in green, is excluded from solid-like domains, while Rh-DPPE, shown in red, preferentially partitions into solid-like stripes. Scale bars = 5 μm. (f) Bending angles of stripe-like domains in DLPC:DPPC (1:3) membranes.

stripes contain *more* of the type 2 dyes than the surrounding fluid. This is *a priori* surprising since we should expect that dyes, as generic impurities, will be expelled from an ordered phase (as is our type 1 dye).

Membranes made of any one of these stripe-forming PC species exhibit the P'_β phase below T_m [22], as do binary PC mixtures [29, 30]. Thus the solid-like stripes observed in our mixtures are made of P'_β. This is consistent with their observed enrichment by type 2 dyes: these dyes disrupt P'_β less than they disrupt the locally flat L_α phase as the crest of each ripple [20] provides free volume to accommodate their large hydrophilic fluorophore. We argue that the micron-sized stripe domains are thus a mesoscale manifestation of the molecular-scale anisotropy that directs the nanoscale ripples in P'_β; the resulting anisotropy of line tension, or perhaps of bending moduli, could explain the formation of stripes [11]. An additional link between lipid organization and domain properties is seen in the 'quantized' bending angles (figure 2(f)), which presumably arise from the hexagonal ordering of lipids in P'_β. Such bending angles were previously observed for elongated, rippled domains in supported multibilayers [30] and elongated, rippled grains of single-component small vesicles [4].

Our second observation is that, in contrast to the binary-PC mixtures, domains in DPPC:DPPS membranes exclude *all* our dyes and are polygonal and often hexagonal (figure 3), with angles of vertices that are sharply peaked at 120° (data not shown). These domains clearly reflect hexagonal ordering over a length scale of microns. The structure of the solid-like phase for DPPC:DPPS mixtures has not been determined [31], but the presence of L'_β-forming DPPC makes it plausible that L'_β should coexist with L_α in this mixture (since P'_β occurs in only a small region of the phase diagram [31]). This can explain our observations of polygonal domains so long as our hypothesis, that domain shapes reflect molecular organizations of lipid phases, holds. It further implies that lipid ordering determines the growth rates of domain edges [11, 32].

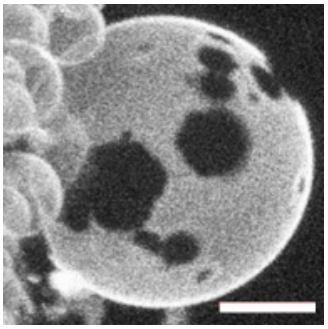

Figure 3. Polygonal, often hexagonal domains in DPPC:DPPS (3:1) vesicles, $T = 39.9\,^{\circ}\mathrm{C}$. These domains exclude the dye Rh-DPPE and therefore appear dark. Scale bar = 10 μm.

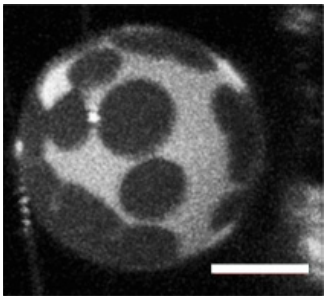

Figure 4. Circular domains in DPPC:DPPE (3:1) vesicles, $T = 53.0\,^{\circ}\mathrm{C}$. These domains exclude the dye Rh-DPPE and therefore appear dark. Scale bar = 10 μm.

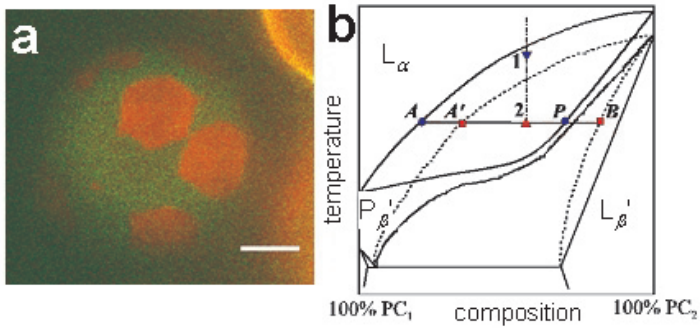

Figure 5. (a) Fast cooling (0.8 °C min^{-1}) produces hexagonal domains in a DLPC:DPPC (1:1) vesicle ($T = 25.0\,^{\circ}\mathrm{C}$). These domains contain the dye DiI-C-18. Scale bar = 4 μm. (b) Schematic phase diagram for a binary PC mixture, constructed after an experimental phase diagram [29]. Equilibrium phase boundaries are given by solid curves, while dotted curves outline the metastable $L_\alpha + L'_\beta$ coexistence region. A membrane quenched to point '1' can only separate into $L_\alpha + P'_\beta$ coexistence. A membrane quenched to point '2' may partially lower its free energy first by separating into coexisting $L_\alpha + L'_\beta$ regions with compositions given by points A' and B, before finally reaching equilibrium by separating into coexisting $L_\alpha + P'_\beta$ with composition given by points A and P.

Our third observation is that *circular* dye-excluding domains are found both in DPPC:DPPE (figure 4) and in DLPE:DPPE (not shown) membranes. These domains sometimes aggregate to form more complex shapes, similar to previous observations of

domains in such vesicles [9]. In the DPPC:DPPE system, DPPC-rich L_α coexists with solid-like, untilted, DPPE-rich L_β [33], in which the lipids have significant rotational freedom [24] and ordering over large areas has not been reported. For a solid-like phase with an isotropic or multicrystalline ordering on the domain scale, we expect the domains to be shaped by line tension, similar to fluid–fluid systems. The L_β phase and fluid phase both lack in-plane order over micron length scales; consistent with our hypothesis, a circular domain shape is seen for both.

To summarize these observations, in all three classes of system, we find that *solid-like domain morphologies reflect the molecular organizations of lipids in the corresponding phases*. We next show that kinetic effects provide further strong evidence that lipid phase organization indeed controls the shape of solid-like domains.

Recall that our DLPC:DPPC system consistently forms dye-containing stripes upon slow cooling (about $0.2\,^\circ\mathrm{C\ min^{-1}}$) (figure 2(a)). However, faster quenches (about $0.8\,^\circ\mathrm{C\ min^{-1}}$) frequently result in dye-containing hexagonal domains instead of stripes (figure 5(a)). A sufficiently fast quench should take the system deep into the $L_\alpha + P'_\beta$ coexistence region before domains can nucleate (figure 5(b), point 2). This part of the equilibrium $L_\alpha + P'_\beta$ coexistence region overlaps with the *metastable* $L_\alpha + L'_\beta$ coexistence region (dotted lines, figure 5(b)) [34]. This $L_\alpha + L'_\beta$ phase coexistence can thus be established as a metastable intermediate between the high-temperature L_α phase and low-temperature $L_\alpha + P'_\beta$ coexistence. This is an instance of Ostwald's rule of stages, whereby a phase-separating system may take a path through a succession of intermediate metastable phases that decreases the free energy gradually [35]. Therefore, the homogeneous mixture may reduce its free energy by first nucleating hexagonal, dye-excluding domains of L'_β. These metastable L'_β domains may either template further growth of the P'_β phase, giving rise to the dye-containing hexagons, or transform into P'_β (point B) without mesoscopic morphological change because the intra-domain rearrangements needed are slower than the $L'_\beta \rightarrow P'_\beta$ conversion [32, 36].

Thus the observed morphologies can again be explained in terms of the molecular organization of lipids in the solid phase. Such a relationship is unexpected for lipid mixtures, but is well known in solid state physics, where unit cell symmetry controls crystal morphology. Interestingly, the Wulff construction, which predicts *equilibrium* crystal shapes from unit-cell symmetry [11], often does not hold: instead, growth kinetics determine the crystal shape and the fastest-growing faces disappear in the final crystal [11]. For lipid domains, we have above shown an instance of kinetic control, whereby the metastable L'_β phase determines the morphology of mesoscale P'_β domains. Other kinetic effects are also likely to exist.

In conclusion, our work demonstrates that the symmetries of molecular lipid packing determine the properties of mesoscopic solid-like domains in lipid membranes, with the resulting domain morphology subject to a fine degree of kinetic control. This understanding opens pathways to tailored membranes and/or the rational design of membrane patterns, based on studies of lipid phase behaviour, for which a substantial body of data already exists.

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