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INVITED ARTICLE

The effects of fluorescent probes on model membrane organization: photo-induced lipid sorting and soft structure formation

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Liquid crystalline phase behaviour in model cell membranes prepared in-vitro has generated an enormous amount of interest in recent years and the phase diagrams of various bio-mimetic mixtures explored. Most recently photo-induced oxidation in fluorescently labelled membranes has been shown to influence these phase diagrams and can be used to generate interesting membrane-based geometries based on differences in membrane composition. In this article the different phases observed in model membranes will be discussed. Methods used to study these systems and recent developments in photo-stimulated phase changes are described along with areas for future investigation.

Keywords: lipid bilayers; rafts; fluorescence; membranes

1. Introduction to lipid phase behaviour in model membranes

The cell membrane consists of a lipid bilayer in which a complex mixture of different lipids, sterols and membrane proteins are organized, resulting in a highly selective membrane for transport of materials in and out of the living cell. This membrane is commonly known to exist in the so-called liquid crystalline phase as the lipid molecules exhibit fluid-like properties in the plane of the two-dimensional (2D) membrane with short-range packing organization. In aqueous solution single lipids behave as lyotropic liquid crystals, forming a variety of different phases, such as micellar solutions, lamellar and hexagonal phases as a function of concentration (*1*). This phase behaviour results from their amphiphillic nature, as most lipids consist of a hydrophilic head group and hydrophobic tails. As an example, Figure 1 shows the molecular structure of some different lipids commonly used in mixtures to model the cell membrane.

Figure 2 shows three of the different lyotropic phases which can occur as we vary lipid concentration in a polar solvent such as water. The exact temperature at which a phase will occur depends on the molecular details of the lipids in the solution. Lipids can also behave as thermotropics and phase changes occur as a result of changes in the packing of the lipid tails in the bilayers as the temperature is increased. Above the so-called 'melting temperature' (T_m), in any of the lyotropic phases (such as shown in Figure 2) the lipid chains are in a liquid-like state, with no positional order. At fixed concentration where the lipid will exhibit stacking of sheet-like bilayers (the lamellar phase – Figure 2(b)) we can define several thermotropic sub-phases (the lamellar lyotropic phase is chosen here as a basis as it most closely represents the cell membrane).

At the lowest temperatures lipids in a bilayer crystallize in the sub-gel phases (*1*). In this case the molecules are arranged in a crystalline-like lattice and are restricted in both rotational and translational motion. On increasing the temperature we may observe a transition to the gel phase ($L_{\beta'}$). The gel phase has long-range order in the membrane plane, but some rotational motion of the individual molecules can occur. Lipid chains are hexagonally packed and the hydrophobic tails may be tilted in the bilayer. The individual molecules are not free to diffuse in the plane of the bilayers. At higher temperatures the more unusual ripple phase ($P_{\beta'}$) can occur. The phase is similar to the gel phase in viscosity but the in-plane head groups have an orthorhombic packing configuration. This leads to an out of plane 'ripple' in the membrane sheet (*2,3*). At the highest temperatures the membrane will form the liquid crystalline phase (L_α) and this phase is most relevant to the membranes of the living cell. The melting temperature (T_m) is defined as the transition point from the gel phase (or ripple phase if present) to the liquid crystalline phase, where the membrane lipids are fluid-like. The two most commonly observed thermotropic subphases of the lipid lamellar phase (gel and liquid crystalline) are shown in Figure 3.

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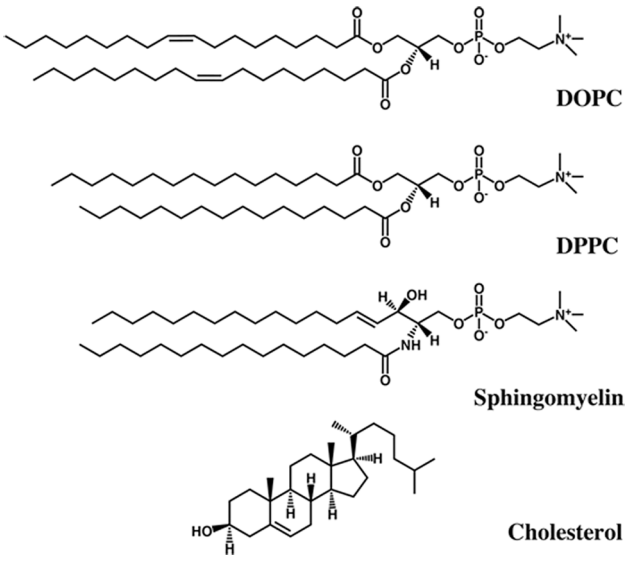

Figure 1. Molecular structures for some lipids commonly used in model membrane studies. DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) is an unsaturated phospholipid and DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphocholine) and sphingomyelin ((2S,3R,4E)-2-acylaminooctadec-4-ene-3-hydroxy-1-phosphocholine) are saturated lipids. The molecular structure for cholesterol is also shown.

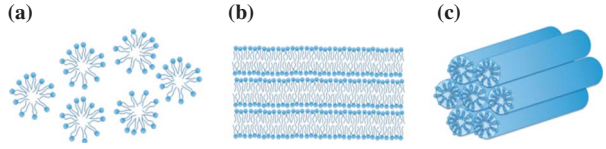

Figure 2. Cartoon depicting the organisation of lipids in the (a) micellar, (b) lamellar and (c) hexagonal (H_α) phases for a lipid/water system. Water molecules are not shown. Individual lipids are depicted as composed of a hydrophilic head-group with two hydrophobic 'tails'.

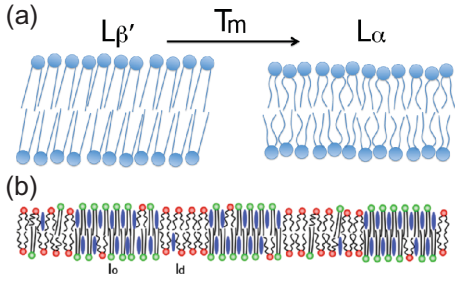

Figure 3. (Colour online). (a) Cartoon showing the melting transition (T_m) from the 'gel' phase ($L_{\beta'}$) to the 'liquid crystalline' phase (L_α) and (b) domains of the l_o and l_d phase in a three component bilayer. Green lipids – high T_m, red lipids – low T_m, blue lipids – cholesterol.

2. Lipid rafts and membrane phase separation

The original and widely accepted model for the cell membrane until fairly recently was the fluid mosaic model, in which lipids and embedded proteins were described as randomly distributed (*4*). It is, however, now known that this is not the case; biological membranes exhibit a much more complicated in-plane organization and that phase behaviour of the constituent lipids plays a role in membrane structure. In recent years a new hypothesis for the structure of the cell membrane has come about in which the membrane's phase behaviour is described as influencing the organization of membrane proteins. In particular, membrane proteins may laterally cluster into submicrometre domains (*5–7*). This is known as the 'raft hypothesis' and these lipid domains, often referred to as 'lipid rafts', are postulated to take the form of lateral patches in the membrane, of differing composition to the surrounding areas (*8*). This is an attractive idea as it provides a simplified model for how protein organization may be controlled by the cell.

It was described by Simons and Ikonen (*8*) in 1997 that in mixtures of lipids used as a mimic for the cell membrane (typically consisting of a saturated lipid–gel phase at room temperature and an unsaturated lipid–LC phase at room temperature) the incorporation of cholesterol resulted in in-plane bilayer domain formation (*9,10*). These domains are observed in model systems to be micrometre-scaled and therefore the phenomena can be thought of as a phase separation. This phase separation was initially observed as more ordered domains in model membranes by fluorescence microscopy on giant unilamellar vesicles (first observed by Dietrich *et al.* (*9*)). Two different phases can be observed in these vesicles, defined as the l_o (liquid ordered) and l_d (liquid disordered) phases, although it should be clarified here that the l_d phase is equivalent to the L_α, or liquid crystalline phase. These sub-phases (l_o and l_d) are considered liquid crystalline as they both exhibit short-range in-plane ordering and they arise as a result of selective partitioning of cholesterol to the more ordered chains of the high-T_m lipid. Current thinking is that the lipid rafts which may occur in the membrane are composed of lipids in the more ordered (liquid ordered – l_o) phase, where the alkyl chains are more highly ordered than in the L_α phase, but the molecules are still free to diffuse laterally (*11,12*). This phase is more fluid-like than the gel phase and clearly distinguishable experimentally by X-ray diffraction or microscopy.

Both cholesterol and sphingolipids have been hypothesized to be essential for lipid raft formation in the cellular membrane (*13–16*) and it is in systems where these molecules are present that much of the work on model membranes has been carried out. Current interest in biological membrane rafts relates to their potential functional role in cell biology. Rafts were first proposed to mediate sorting in the trans-Golgi network (*8*) and recent results suggest that rafts

may be important for sorting in the endocytic pathway, serving as docking sites for certain pathogens and toxins (*17*). Additional theories discuss how sorting of proteins via rafts may help to concentrate and localize proteins in the membrane, facilitating intermolecular interactions (*8,18,19*). The ordered lipid environment of the raft may also influence their function, possibly by altering protein conformation.

Cholesterol is an essential component of living membranes and a large number of studies have been carried out, implicating this molecule in a variety of cellular functions, including cell permeability (*20,21*) membrane fusion (*22*) and receptor function. Cholesterol is an amphiphilic, fairly rigid molecule and is able to localize in the membrane, influencing phase behaviour. The amount of cholesterol which can be incorporated into a bilayer varies greatly between cell types and certain biological membranes can actually contain up to 50% cholesterol (*23*). In model systems the upper limit above which no further cholesterol can be dissolved in the membrane is around this value (*24,25*). In the cell the distribution of cholesterol is not homogenous in the membrane and there is now strong evidence that cholesterol-rich lipid rafts do form in cell membranes (*26*). This phenomenon has been documented by several authors in recent years (*27–29*), although direct and unambiguous imaging of rafts in a living cell is still to be achieved. More indirect studies of protein sorting in the proximity of potential domains, however, have been carried out by several groups (*30*).

Problems with imaging this phenomenon stem from the dynamic nature and small size of the proposed domains. It is currently presumed that membrane domains in the cell are nano-scale in size, dynamically arising and dispersing as a function of local lipid composition. In model systems a great deal of work has been carried out and phase diagrams for various lipid mixtures designed to represent a simplified model of the cell membrane with cholesterol have been investigated in detail (*31–35*). Techniques commonly used to study these model membranes include fluorescence microscopy, nuclear magnetic resonance (NMR), atomic force microscopy (AFM) (*35–37*) and x-ray diffraction (XRD).

Although it is well accepted that the lipid rafts postulated in the living cell are mainly composed of sphingolipids and cholesterol, lateral phase separation is a feature not restricted to membranes containing sphingolipids. Model membrane studies on ternary lipid mixtures composed of cholesterol and two phospholipids with a high T_m and a low T_m respectively show that coexistence of the l_o phase and l_d phase can occur in the absence of sphingolipids (*38*). In these mixtures, l_o phase domains are enriched in the high-T_m lipid and cholesterol separates from surrounding l_d phase regions (enriched in the low-T_m lipid). Recent work using time-of-flight secondary ion mass spectrometry (TOF-SIMS) to look at ternary lipid mixtures and their phase behaviour revealed that the alkyl chain saturation, rather than the head group difference, is the dominant factor in determining phase separation (*39,40*). It was found that sphingolipid is not essential to raft formation, but that any saturated phospholipids with similar alkyl chains such as 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) should be capable of fulfilling the same role.

3. Photo-induced effects in fluorescently labelled systems

Since the discovery of this phase separation effect in ideal lipid bilayers and its potential relevance to cell structure, an enormous amount of effort has been dedicated to the elucidation of this ternary phase behaviour and the potential applicability to cell function.

A common method used to look at phase behaviour in bilayers is to carry out fluorescence microscope imaging on giant unilamellar vesicles (GUVs). Giant vesicles are used as a model for the cell as they consist of a spherical single bilayer of large diameter ($\sim$10–50 μm) in aqueous solution. Such vesicles represent a simplified model of the cell membrane, or an 'empty cell'. Their composition can be varied from a single lipid to a complex mixture including proteins and other molecules of interest. The different phases are visualized by selective labelling of the mixture with fluorescently conjugated lipids or other fluorescent dye molecules. For example, a common technique is to add into the lipid mixture a small amount ($<$0.5 mol%) of Rh-DPPE (1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt)). This lipid preferentially partitions to the l_d phase and has been widely used to visualize phase separation in these system. Other labels, such as perylene and NBD-DPPE (1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (ammonium salt)) are able to partition into the l_o phase, although contrast between the two phases can still typically be seen (Figure 4(b)). Figure 4 shows a GUV exhibiting phase separation labelled by this method.

Although the use of fluorescence microscopy on GUVs to determine the presence or absence of phase-separation or domains has become fairly ubiquitous as a biophysical technique in the field and is widely used, recently some doubts have been cast on the usefulness of this method to accurately determine thermodynamic phase boundaries. In 2006, Ayuyan and Cohen (*41*) examined the effects of fluorescence

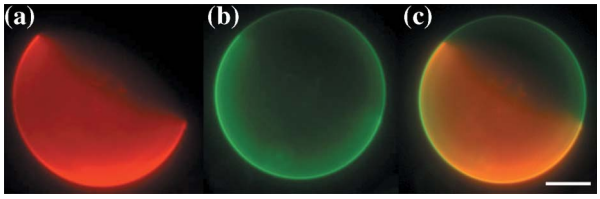

Figure 4. A single giant unilamellar vesicle (GUV) labelled as described in the text. (a) Rh-DPPE, (b) NBD - DPPE and (c) a superposition of the two. (Scale bar = 10 μm.)

illumination as typically applied in imaging on vesicle morphology and obtained the surprising result that apparently uniform vesicles can be induced to phase separate on sustained illumination of several seconds as a result of lipid oxidization. This proposed mechanism introduces unknown parameters to the experiment in the form of various amounts of oxidation products. In order to avoid these obvious problems in using GUVs to elucidate the intrinsic phase behaviour of lipid mixtures, experimental modifications have been proposed. Anti-oxidative ingredients may be included, such as N-propyl gallate (NPG) to retard the light induced oxidation, although the efficacy of this strategy has not been quantified. Alternatively a lower intensity light can be used for imaging, although minimal effects cannot be discounted and may have a large effect on phase behaviour. Small compositional changes near the miscibility critical point (*42*,*43*), as described recently by the Keller group, will likely have a large effect on whether or not domains are observed and their size.

Additional problems with the GUV method come from the fact that we do not have a good model to determine intrinsic domain size in a given mixture. It is not clear if the equilibrium domain size should be below the resolution of light microscopy (~200 nm) in some, or even any, relevant mixtures. To solve this problem a solution x-ray scattering technique was proposed by the author (*44*). Phase diagrams produced by this technique are in approximate agreement with fluorescence GUV results, except XRD reveals the region of phase coexistence to be larger. This suggests that not only do mixtures exist on the phase diagram in which domain size is below the optical resolution but also that intrinsic domain size is a function of membrane composition.

4. A potential mechanism for light-induced phase separation

Ternary lipid mixtures for these studies are prepared consisting of different lipids and cholesterol and typical mixtures exhibiting phase separation include an unsaturated lipid, a saturated lipid and cholesterol. It is of course possible to investigate an enormous number of different lipid combinations; however, a small sub-set of these have been studied in detail and the ternary phase diagram completed. In the images shown in this paper we focus on a mixture of DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine)(T_m: –20°C), (egg sphingomyelin (eSM)) (2S,3R,4E)-2- acylaminooctadec-4-ene-3-hydroxy-1-phosphocholine (T_m: 40°C), and cholesterol as a well studied example. In order to visualize the different in-plane phases in this system, the labelled lipids, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (ammonium salt) (NBD-DPPE), and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) (Rh-DPPE) can be added in small amounts to the mixture (0.5–0.01 mol%). Lipid materials may be purchased from Avanti Polar Lipids (Alabaster, Al) and used without further purification. The lipids are dissolved in chloroform (high-performance liquid chromatography grade) and stored under –20°C with a total lipid concentration of 0.5 mM. Mixtures can then be prepared in chloroform with varying molar ratios of the different components. The chloroform is removed by drying overnight under vacuum, and then rehydration with water produces aqueous lipid phases. When preparing lipid mixtures, it is important that all residual chloroform must be removed from the system.

On observation of GUVs prepared from such a lipid mixture, micrometre-scale domains are observed on the surface of the vesicle at certain lipid ratios. The size of the domains observed may vary, depending on lipid composition and preparation technique, but of course only micrometre-sized domains can be observed by fluorescence microscopy. The mechanism by which photo-induced phase separation can be initiated originates in the labelled lipid molecules and although not fully understood it has been discussed recently in the literature as mentioned earlier in this paper. There have been just a couple of theories for the mechanisms involved in this photo-induced domain formation/coalescence as this is a relatively newly observed phenomenon. Ayuyan and Cohen (*41*) report that peroxidation of the fluorescently labelled lipid leads to gradual oxidation of the unsaturated lipids in GUVs. Excitation of the fluorophore (e.g., Rh-DPPE in our case) in the presence of oxygen may result in peroxidation at the unsaturated bond and the production of free radicals in the solution. These radicals are highly reactive and are able to oxidize the double bonds of unsaturated lipids in the mixture, for example in the DOPC. Another group (*45*) reports similar findings based on their observations of lipid monolayers. This group also confirms that oxidation of cholesterol is not a factor and therefore deduce that is it the oxidation products of the other lipids creating

the observed changes. Unfortunately, lipid oxidation is a complex process, which can be initiated by various peroxidation-induced reactive oxygen species and will induce a variety of oxidation products. The chemistry of these processes is well understood (*46*,*47*) but it is extremely hard to determine, in any given mixture, what the new composition after oxidation will be. The products may be numerous and are virtually impossible to extract and quantify. It is likely, following lipid oxidation by this mechanism, that widespread changes in the lipid tail conformations result, producing a change in the intrinsic phase behaviour of the mixtures. These conformational changes, such as *cis* to *trans* conversions (*48*) or cleavage of the alkyl chains at the site of an initial double bond, will lead to packing rearrangements in the domains. The next step (linking these phenomena to macroscopic phase separation) has not been explored extensively and represents an area for future investigation. Some recent theoretical work (*49*) has successfully described the growth and stabilization of domains in such lipid systems as being dependent on line tension. Oxidization products may increase the line tension, thus allowing the growth of large domains (*45*). In addition, it is certain that the presence of oxidation products will increase the disorder in the DOPC dominated l_d domains, possibly making it a less favourable environment for the more ordered sphingomyelin/cholesterol packing and promoting phase separation (*50*).

Elucidating the intrinsic size of domains in ternary lipid systems and the parameters which control this is an open question. Many of the phase diagram studies carried out in the literature use fluorescently labelled GUVs to determine the phase boundaries and regions of phase coexistence. This technique, however, does not take into account the very real possibility that domains may be much smaller than can be optically resolved, or that photo-oxidation is artificially producing large domains and inducing phase separation. Other potential techniques include NMR and XRD. NMR can be used to deduce the presence of different domains and map the phase diagram (*51*); however, a detailed determination of domain size is not possible using this technique. Recent work by the author (*44*) and others (*52*) has shown that x-ray diffraction from solution samples can be used also to verify the phase diagram. Technically x-ray scattering could provide information on domain size, although this has not yet been achieved.

5. Photo-effects in different geometries

A new direction which has recently been explored by the author involves the harnessing of photo-induced phase separation in lipid membranes for the creation of new structures. The photo-induced phase separation phenomena in ternary lipid mixtures described above is not only interesting from a fundamental science point of view but may also have some novel applications. In recent years work has been carried out in the area of 'soft micro-fluidics', in which networks of vesicles and tubules can be created entirely from lipid assemblies in solution (*53*,*54*). Such systems may find applications as chemical micro-reactors or models for cellular processes. Figure 5 shows some examples of these structures. Up to now the creation of membrane based 'shapes' has been a hands-on procedure in which tubules are manually 'pulled' from vesicles and connected to each other. Shape controlled in this way is restricted to certain low-energy configurations, e.g. spheres, cylinders etc. By introducing domains of a second, stiffer phase into the membranes which comprise these shapes it is now possible to add a much richer library of morphologies (*55*). Some examples of the effects domains can have on equilibrium vesicle shape can be seen in the recent paper by Baumgart *et al.* (*56*). Selective control of membrane stiffness by lipid sorting could provide a mechanism for better control of membrane morphologies.

Lipid tubules can be formed and stabilized in many lipid mixtures, provided the energy costs are not too great. These can last for more than 24 hours if prepared using the following method. Lipid tubules can be formed with different diameters and may be unilamellar or multilamellar. They are essentially very long cylindrical vesicles and can be formed easily in the lab for microscopy. One simple method for generating tubules is by flow-assisted rehydration (*57*). To

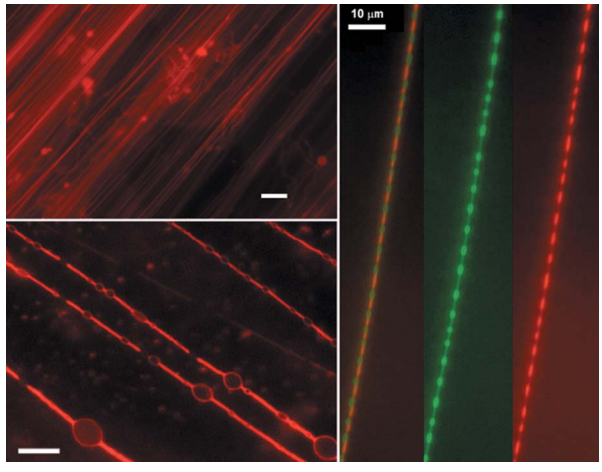

Figure 5. (a) Lipid tubules formed by flow-assisted hydration and imaged with fluorescence microscopy, (b) photo-induced phase separation in lipid tubules and (c) two-phase lipid bilayer discs. Red indicates the l_d phase and green the l_o domains. (Scale bars =10 μm.)

prepare lipid tubules in solution on a glass microscope slide, a lipid solution with a total lipid concentration of ~0.5 mM is carefully dip-coated on a clean glass slide surface as small isolated droplets. After 2 hours under vacuum, the dry lipid film is re-hydrated with a drop of nano-pure water or 100 mM sucrose solution at a temperature above T_m, and then covered with a piece of cover glass. As the glass cover is placed, flow of solution towards edges will generate a large number of tubules. Following hydration, the formation of lipid tubules can be directly observed with a fluorescence microscope provided a fluorescent probe is incorporated. Fluorescent probes such as Rh-DPPE or NBD-DOPE (probes for the L_d phase) may be added to the lipid mixture before the drying stage at ~0.2 mol% to visualize lipid tubules on formation. Fluorescence microscopy images of lipid tubules labelled with Rh-DPPE can be seen in Figure 5. The tubules produced using this method are typically oriented parallel to each other and often remain anchored to the lipid film, providing a nice array of tubules for observation. Free tubules in solution are highly mobile and difficult to image or target for illumination. It is therefore particularly desirable when carrying out this work that the tubules be somehow anchored to reduce excessive thermal motion. GUVs are slightly easier to immobilize as they can be prepared with sucrose solution inside. This allows them to sink in water reducing their mobility.

An alternative method for tubule formation is to 'pull' a single tubule from a GUV manually, as performed by the Orwar group (*53*,*54*) and by careful manipulation of GUVs they show that complex networks of vesicles and tubules can be created. This technique could provide us with a well anchored tubule for imaging. Curvature can also induce lipid sorting – another complication. This has been observed looking at narrow lipid tubules with high curvature pulled from low curvature GUVs (*58*). In further evidence, curvature induced sorting on supported bilayers can also be induced by using a patterned surface (*59*).

Figure 5 shows examples of new shapes with can be formed by the new 'two phase' membranes. On the formation of domains in the lipid tubule two clear domain organizations can be observed, a 'band' structure (Figure 5(b)), where domains form an alternating pattern along the tubule, and the 'disc instability' (Figure 5(c)) (*55*). Lipid bilayer discs are formed along the length of the tubule, their flat surfaces consisting of islands of the l_o phase. There is a great deal of work still to be carried out in understanding the mechanisms which lead to either the 'band' or the 'disc' structures. Currently it seems that differentiation between the two is driven by the spatial distribution of the initial growing domains on illumination. Isolated domains may grow and wrap around the tubule creating a 'band' whereas two growing domains in close proximity could interact, forming a disc structure. Further detailed microscopy will allow us to understand this process more quantitatively.

6. Conclusion

In this paper, we have described the phase behaviour of lipid membranes and some of the interesting macroscopic membrane structures which can be formed as a result of in-plane domain formation in the lipid bilayer in ternary lipid mixtures. Two main areas of interest can be identified for future study. Firstly, a thorough investigation of the mechanisms behind this photo-induced phase separation phenomenon needs to be undertaken. What is the chemistry involved and can the process be controlled and used to generate new materials and mixtures? Secondly, the question of how this effect can be utilized in the formation of new macroscopic structures needs to be addressed. The lower curvature of the l_o phase over the l_d phase (compositionally different sub-phases of the classic L_α liquid crystalline phase) can lead to the formation of non-spherical geometries such as a bilayer disc structure and banded tubules. As changes in morphology can be photo-induced it should now be possible to create custom structures based on inducing localized changes in curvature for 'soft' membrane-based nanofluidic devices using this technique. Most promisingly such changes in structure can be induced remotely without mechanical interference to the delicate system.

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