Phase behaviour of lipid-cholesterol membranes

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Abstract

The proposed existence of cholesterol-rich lipid rafts in eucaryotic plasma membranes has led to a large number of studies on the influence of cholesterol on the structure and phase behaviour of model membranes. In this article we first give a brief overview of the phase behaviour of membranes made up of binary and ternary lipid-cholesterol mixtures determined from these experiments, and then present the results of our recent x-ray diffraction and fluorescence microscopy studies on these systems. Our observations suggest a possible resolution of the reported discrepancy in the phase behaviour of these systems obtained using different experimental techniques.

1 Introduction

Cholesterol is a major component of plasma membranes of eucaryotic cells [1]. The distribution of cholesterol in these membranes is believed to be inhomogeneous, and microdomains called lipid rafts, which are rich in cholesterol and in lipids with long saturated hydrocarbon chains, such as sphingolipids, have been proposed to exist in them [2, 3, 4, 5]. These lipid rafts have been implicated in many cellular processes, such as membrane protein sorting and the construction of signaling complexes.

The proposed existence of lipid rafts in biomembranes has led to a large number of studies on the influence of cholesterol on the structure and phase behaviour of lipid bilayers, using a variety of experimental techniques. In this article we give a brief overview of earlier investigations on the phase behaviour of binary and ternary lipid-cholesterol mixtures, and also present the

results of our x-ray diffraction and fluorescence microscopy studies on these systems. In some binary lipid-cholesterol mixtures we observe a modulated phase at lower water content, which melts, in excess water, into a lamellar phase with lower bilayer rigidity compared to the higher-temperature lamellar phase. These observations indicate the presence of concentration fluctuations in the system, which can account for the discrepancy in the reported phase behaviour of these systems, obtained using different experimental techniques. In ternary lipid-cholesterol mixtures we have observed the coexistence of two fluid phases in bulk samples, consistent with earlier observations on single bilayers.

2 Binary Mixtures

The phase behaviour of binary lipid-cholesterol mixtures has been extensively studied using a variety of experimental techniques [6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19]. Most of these studies have been on lipids, such as phosphatidylcholine (PC) and sphingomyelin (SM), since they along with cholesterol are the major components of plasma membranes of many mammalian cells. Further, lipids with saturated hydrocarbon chains have been widely studied as their transition temperatures usually fall in an experimentally convenient temperature range.

Differential scanning calorimetry studies show that the main- and pretransitions of PC-cholesterol mixtures broaden and the transition temperatures and the corresponding enthalpies decrease progressively with cholesterol concentration (x_c) [10, 18]. The DSC endotherms are found to consist of a sharp component, which disappears at $x_c \sim 20$ mol%, and a broad component which gets broader with increasing x_c, and is observed even up to $x_c \sim 50$ mol%. Freeze fracture electron microscopy studies on PC-cholesterol mixtures show that the ripple phase, occurring between the main- and pretransitions, exists up to $x_c \sim 20$ mol%, with the ripple wavelength increasing with cholesterol concentration and diverging near $x_c \sim 20$ mol% [8].

Partial phase diagrams of binary dipalmitoylphosphatidylcholine (DPPC)-cholesterol and SM-cholesterol mixtures have been determined using nuclear magnetic resonance (NMR) and electron spin resonance (ESR) techniques [10, 13]. These studies show that the main-transition temperature (T_m) of the lipid is not significantly affected by cholesterol up to $x_c \sim 20$ mol%, beyond which it drops abruptly. Below the main transition a pure gel phase is

found for $x_c < \sim 5$ mol%, beyond which it coexists with a fluid phase. The hydrocarbon chains of the lipid molecules are more ordered in this phase, compared to those in the fluid phase of the pure lipid. Hence this phase is often referred to as the liquid-ordered (l_o) phase, in contrast to the liquid-disordered (l_d) phase of the pure lipid above T_m. The gel-l_o coexistence region disappears at $x_c \sim$ 20-25 mol%. The two boundaries of this coexistence region are practically independent of temperature. The most intriguing feature of these phase diagrams is the l_d-l_o coexistence region found at temperatures above the main-transition. Just above T_m the coexistence region extends over $\sim 5 < x_c < \sim 20$ mol %. Its width decreases with increasing temperature and it disappears at a few tens of degrees above T_m.

The structure and phase behaviour of these mixtures have also been probed using x-ray diffraction [7, 15, 19, 20, 21] and neutron scattering [9]. A partial phase diagram of dimyristoylphosphatidylcholine (DMPC)-cholesterol mixtures has been determined by Mortensen et al. from neutron scattering data [9]. In agreement with the spectroscopy results, the main-transition temperature is found to decrease slightly with cholesterol content up to $x_c \sim 20$ mol%, beyond which only a fluid phase is observed. The modulation wavelength in the ripple phase was also found to increase with x_c and diverge near 20 mol%, consistent with the freeze fracture electron microscopy results. However, no fluid-fluid phase separation was observed above the main-transition, contrary to the spectroscopy results.

X-ray diffraction studies on PC-cholesterol mixtures also show only a single phase above T_m [7, 15, 19, 21, 22, 23]. At these temperatures the lamellar periodicity is found to increase slightly with cholesterol content. Electron density distribution in the bilayers, calculated from the diffraction data, show enhanced electron density in the hydrocarbon region due to the presence of cholesterol molecules [7]. These studies also show that the incorporation of cholesterol in the bilayer increases the ordering of the hydrocarbon chains, confirming the results of spectroscopy studies. Similar results have also been obtained for SM-cholesterol bilayers [24].

Systematic deviations of some membrane properties at specific sterol concentrations have been seen in a number of experiments. In order to explain this behaviour a superlattice model of sterol organization in bilayers has been proposed [25]. The formation of lipid-cholesterol complexes at specific stoichiometric ratios has also been suggested by some studies on monolayers made up of PC-cholesterol mixtures [16, 17].

We have recently carried out a systematic study of the influence of choles-

terol on the phase behaviour of DPPC, DMPC, dioleoylphosphatidylcholine (DOPC) and dilauroylphosphatidylethanolamine (DLPE) bilayers, using x-ray diffraction [19, 20, 21, 26]. The different phases were identified from their characteristic diffraction patterns. DPPC-cholesterol and DMPC-cholesterol mixtures were also probed at different levels of hydration. The phase diagram of the DPPC-cholesterol system in excess water is shown in Fig. 1. In agreement with earlier studies: (1) the main transition temperature is found to decrease slightly with cholesterol content up to about 20 mol%, beyond which it drops sharply, (2) the ripple ($P_{\beta'}$) phase is seen up to $\sim$ 20 mol%, and (3) the gel ($L_{\beta'}$) phase is not observed even at 5 mol% cholesterol. We were not able to observe a gel-fluid coexistence region, probably due to the coarse steps along the composition axis ($\sim$ 2.5 mol%). The most interesting region of the phase diagram is the one we have denoted as L'_α. This is a fluid phase with a larger lamellar periodicity, compared to the high temperature L_α phase. It also has a higher degree of chain ordering compared to the latter, as indicated by the condensed wide angle reflections. The abrupt increase in the lamellar periodicity by about 0.5 nm across the L_α - L'_α transition, suggests that the latter is a distinct phase [26]. The need to distinguish this region of the phase diagram is further highlighted by the phase behaviour at a slightly lower hydration of 98 % relative humidity [19]. At this hydration this region is occupied by a modulated phase (P_β), which is distinct from the higher temperature $P_{\beta'}$ phase. The P_β phase persists even at lower hydrations, whereas the $P_{\beta'}$ phase is seen only very close to full hydration (Fig. 2). Electron density maps of this phase suggest spatially periodic modulations of the cholesterol concentration in the two monolayers making up each bilayer, which are 180° out of phase [21]. At much lower levels of hydration the P_β disappears and the phase behaviour resembles that of the DLPE-cholesterol system at high hydrations, with a direct L_β - L_α transition on increasing x_c [21].

The non-uniform distribution of cholesterol in DPPC bilayers, inferred from the electron density map of the P_β phase, can couple to the local curvature of the bilayer and produce the periodic undulation of the bilayer seen in this phase. One possible reason for such a distribution is that a definite amount of cholesterol, which is intercalated between the chains in the bilayer, is need to remove the chain tilt, which arises from the mismatch between the effective headgroup area and the cross-sectional area of the chains. Taking the headgroup area to be 0.48 nm^2, tilt = 30° [27], and the cross-sectional area of cholesterol to be 0.38 nm^2 [25], this cholesterol concentration turns

out to be about 16 mol%. Thus, according this model, about 16 mol % of cholesterol is required to completely remove the chain tilt. It is interesting that the P_β - L_α boundary is found at a value of x_c not too far from this estimate. At lower cholesterol content, this model would suggest either a phase separation between cholesterol-rich and cholesterol-poor bilayers, or a non-uniform cholesterol distribution. The system seems to prefer a periodic distribution like the one proposed in Fig. 3, which does not perturb the lamellar stacking significantly. Coupling of such a modulation in the cholesterol concentration to the local curvature can lead to the formation of the P_β phase with undulating bilayers. Interbilayer interactions are needed to hold this structure together, and it melts in excess water, where the bilayer separation is larger. However, the resulting lamellar phase has a larger d spacing compared to the higher-temperature L_α phase, indicating a higher bilayer flexibility. In view of the above discussion, it is possible that the cholesterol concentration in these bilayers exhibit significant thermal fluctuations. The coupling of these concentration fluctuations to the local bilayer curvature can result in pronounced lowering of the bilayer rigidity. It is conceivable that similar concentration fluctuations are present even above the main transition at these cholesterol concentrations. Such a scenario would explain the observation of phase-coexistence above T_m only by techniques using local probes.

The dependence of the phase behaviour of the PC-cholesterol system on the relative humidity can be rationalized in terms of the decrease in the chain tilt with decreasing degree of hydration. Lower amounts of cholesterol, therefore, is required to remove the tilt of the bilayers, thus shifting the P_β - L_α boundary to lower values of x_c. At very low hydrations the tilt of the PC molecules vanishes [7] and the phase behaviour of the PC-cholesterol system resembles that of the PE-cholesterol system, with a direct L_β - L_α transition on increasing x_c [21, 26]. Thus the proposed model provides a framework to understand the various experimental observations on these binary mixtures.

The results of fluorescence microscopy studies on giant unilamellar vesicles (GUVs) made up of PC-cholesterol mixtures are consistent with the phase behaviour inferred from the scattering experiments. The GUVs do not give any indication of a two-phase region above the main transition. As expected, they show solid-like gel domains at lower temperatures at low cholesterol concentrations. However, this behaviour is found only in GUVs of diameter less than ~ 20 μm (Fig. 4a). Larger GUVs, on the other hand, exhibit significant thermal shape fluctuations, but do not show gel domains

(Fig. 4b) [20]. The decrease in the bilayer rigidity implied by this observation is consistent with the larger d spacing observed in the L'_α phase, since in these uncharged lipids the interbilayer repulsion can only be increased through the Helfrich repulsion, arising from thermal undulations of the bilayers. However, the size-dependence of the behaviour of the GUVs is very surprising and is currently under investigation.

3 Ternary Mixtures

Ternary mixtures of cholesterol with two lipids with very different main transition temperatures have been widely studied in order to understand the formation of lipid rafts in plasma membranes. One of the lipids used in these so-called raft mixtures is usually DPPC or SM, both of which have long saturated hydrocarbon chains. The other component is often a lipid with unsaturated chains, such as DOPC. The only criterion for choosing the two lipids is that their mixtures exhibit a region of gel-fluid coexistence. The addition of cholesterol then converts the gel-fluid coexistence region into a fluid-fluid coexistence region by melting the gel phase.

Phase diagrams of such raft mixtures have been determined from fluorescence microscopy studies on GUVs [28, 29, 30, 31] and fluorescence anisotropy measurements on multilamellar vesicles (MLVs) [32]. Preferential partitioning of the dyes reveals fluid-fluid coexistence in the form of micrometer-sized fluid domains on the GUVs below a demixing temperature. In some cases budding of these domains has been seen, driven by the line tension between the two phases [33]. One of the coexisting phases is found to be rich in the high-T_m lipid, the other phase being rich in the low-T_m lipid. Studies on the partitioning of cholesterol into these two phases suggest that the former is only slightly richer in cholesterol [30]. These two phases are often referred to as l_o and l_d in the literature. In our view this terminology is somewhat misleading as it has also been used to describe the phase separation suggested by spectroscopy experiments in binary lipid-cholesterol mixtures above the main-transition. As discussed in the previous section, it is unlikely that a true phase separation is taking place in the binary system, and these experiments most probably indicate the existence of concentration fluctuations in the bilayer. On the other hand, in ternary mixtures an unambiguous fluid-fluid coexistence is observed, but at temperatures below the T_m of the lipid with saturated chains. Further, the phases are more aptly described as rich in the

high T_m lipid and the low T_m lipid, respectively, instead of as cholesterol-rich and cholesterol-poor.

The differences in the phase behaviour of binary lipid-cholesterol mixtures found using different experimental techniques are also reflected in phase diagrams of ternary mixtures reported in the literature. For example, phase diagrams derived from fluorescence microscopy studies show a closed fluid-fluid coexistence region, below a demixing temperature. On the other hand, those inferred from fluorescence anisotropy measurements show the two-phase region extending to the high-T_m lipid-cholesterol axis of the ternary phase diagram, since according to this technique even this binary system exhibits such a coexistence.

Somewhat surprisingly, the fluid-fluid coexistence was not observed in an earlier x-ray diffraction experiment on a ternary raft mixture even at partial hydration [34]. Therefore, it was suggested that the two types of domains, which differ in their bilayer thickness, might order in such a way as to produce a single lamellar periodicity. However, we have recently obtained evidence for a two-phase region from x-ray diffraction studies on aligned bilayers made up of ternary lipid-cholesterol mixtures (Fig. 5) [26]. Electron density profiles of the bilayers in the two phases suggest that one of them is rich in DPPC, whereas the other is rich in DOPC (Figs. 6 and 7). Furthermore, our results are in broad agreement with those of fluorescence microscopy studies on GUVs made from such mixtures.

Although ternary lipid-cholesterol mixtures have been rather widely used to model cholesterol containing plasma membranes, some striking differences between the behaviours of the two systems should not be overlooked. As mentioned above, the domains seen on GUVs made from ternary raft mixtures are typically micrometer-sized (Fig. 8). On the other hand, domains in plasma membranes are at best $\sim$ 100 nm in size. It has also been suggested that the lipid organization in cell membranes is unlikely to be the result of equilibrium phase separation [35]. Active cellular processes might maintain small-scale dynamic inhomogeneities in the membrane, which may cluster together to form functional rafts as and when required by specific cellular functions. Thus it is fairly clear at present that the fluid-fluid phase separation seen in model ternary systems is not identical to the lipid rafts in plasma membranes. This, however, does not make studies on model systems irrelevant, since a proper understanding of their behaviour is a prerequisite for deciphering the much more complex behaviour of plasma membranes.

4 Conclusions

We have observed a modulated phase of binary PC-cholesterol bilayers over wide ranges of cholesterol and water contents. In excess water it melts into a lamellar phase with larger periodicity compared to the higher-temperature L_α phase. These results indicate the presence of concentration fluctuations in the bilayers that can account for the reported differences in the phase behaviour of these systems obtained using different experimental techniques. In ternary lipid-cholesterol mixtures we have observed fluid-fluid coexistence in a bulk sample, for the first time, which is consistent with the results of earlier fluorescence microscopy experiments on this system.

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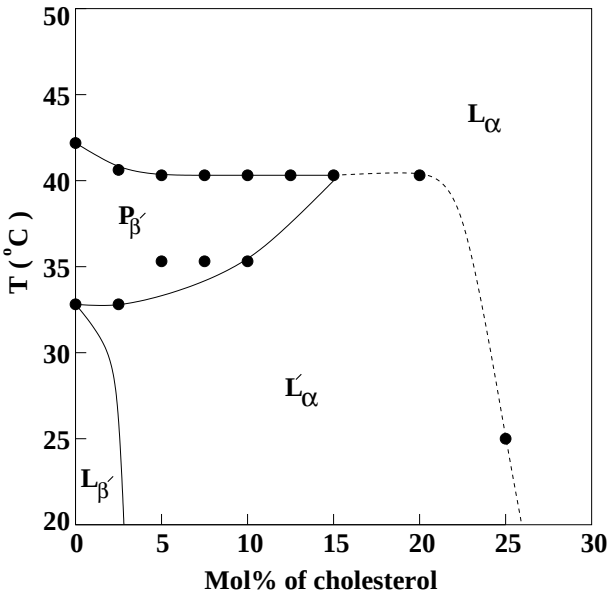


Figure 1: Phase diagram of DPPC-cholesterol bilayers in excess water. L_α: fluid phase; $P_{\beta'}$: ripple phase; $L_{\beta'}$: gel phase; L'_α: fluid phase with a larger periodicity. The dotted line is the phase boundary suggested by an abrupt change in the lamellar periodicity.

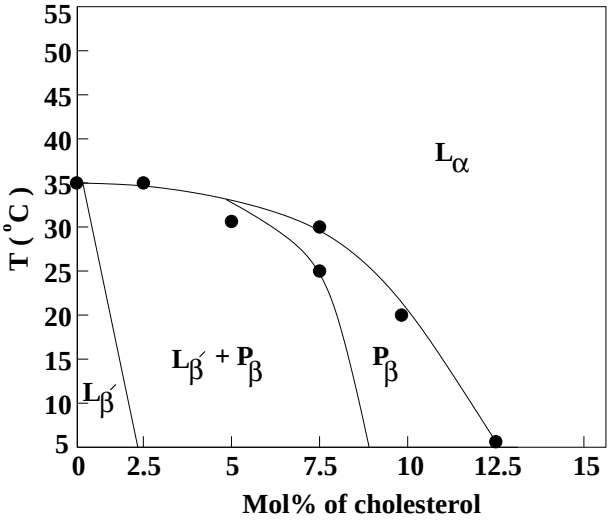


Figure 2: Phase diagram of DMPC-cholesterol bilayers at 65 % relative humidity. Note the absence of the ripple $P_{\beta'}$ phase at this hydration level.

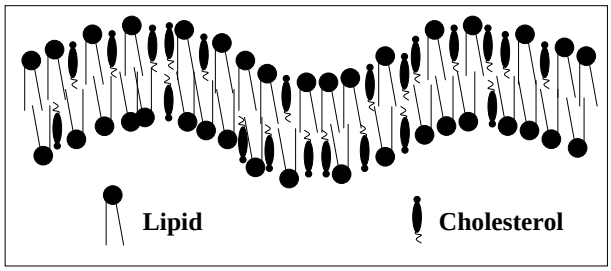
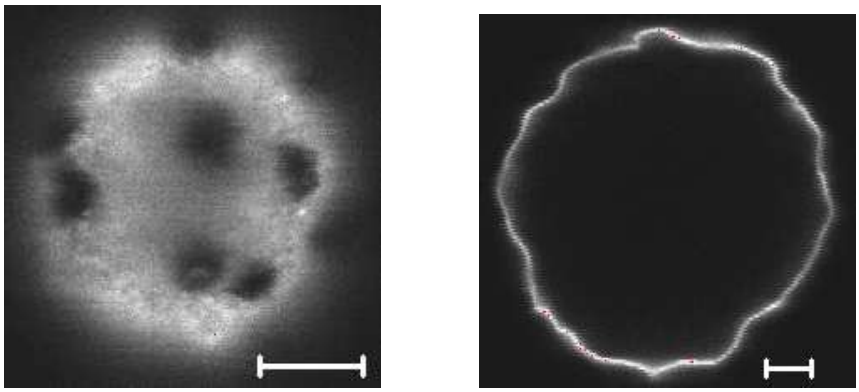


Figure 3: Proposed model for the organization of cholesterol in the P_β phase of lipid-cholesterol membranes. The spatial modulations in the cholesterol concentration in the two monolayers making up a bilayer are 180 ° out of phase. The wavelength of the modulation, obtained from the diffraction data, is $\sim$ 6 nm, whereas its amplitude, estimated from the electron density maps, is $\sim$ 0.2 nm.



(a) (b)

Figure 4: Fluorescence micrographs of GUVs made from DPPC containing 1.5 mol% cholesterol at 23°C. The scale bars correspond to 5 μm. The smaller GUV in (a) exhibits gel-L_α coexistence, but no thermal shape fluctuations, whereas the bigger one in (b) exhibits thermal shape fluctuations, but no phase separation.

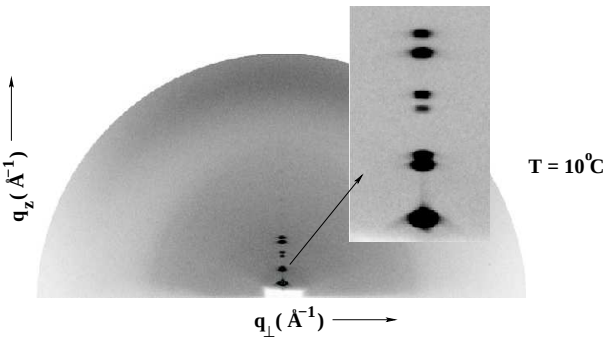


Figure 5: Diffraction pattern of the equimolar mixture of DPPC, DOPC and cholesterol at 10°C, showing the coexistence of two lamellar structures with periodicities of 5.64 and 6.13 nm. The absence of sharp wide angle peaks indicates that both these phases are fluid. A single phase is seen at higher temperatures.

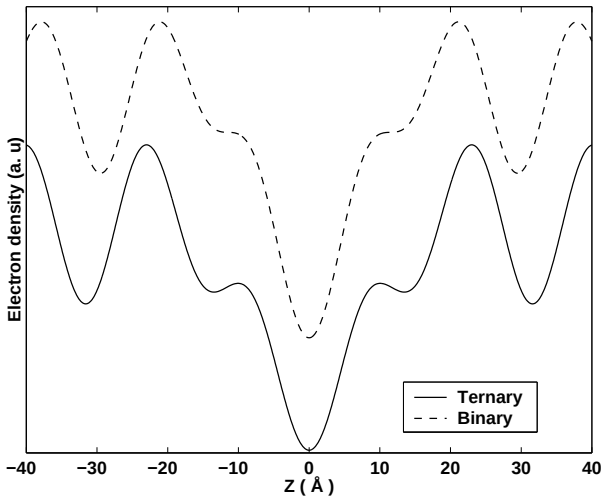


Figure 6: Transbilayer electron density profile of the coexisting fluid phase with larger spacing, observed in DPPC-DOPC mixtures at 20 mol % cholesterol (solid line). This phase can be identified as DPPC-rich from the similarity of the profile to that of DPPC-cholesterol bilayers (dotted line).

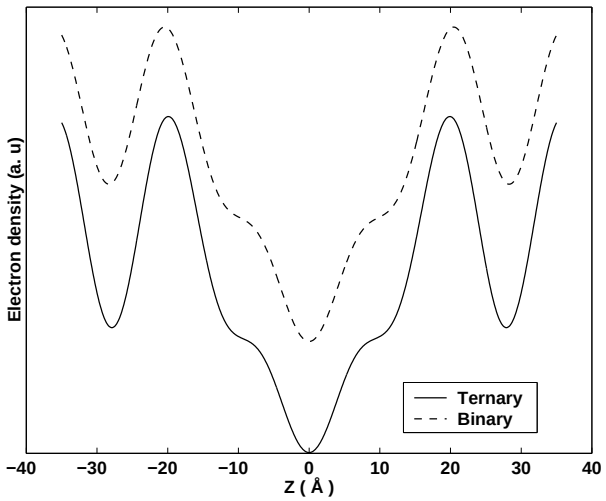
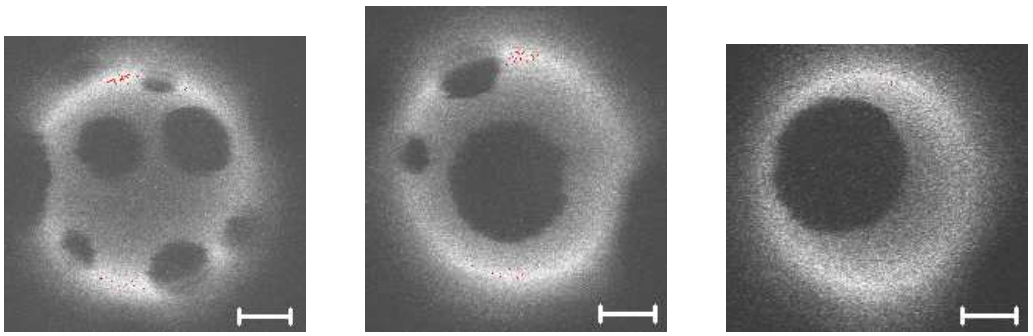


Figure 7: Transbilayer electron density profile of the coexisting fluid phase with smaller spacing, observed in DPPC-DOPC mixtures at 20 mol % cholesterol (solid line). This phase can be identified as DOPC-rich from the similarity of the profile to that of DOPC-cholesterol bilayers (dotted line).



(a) (b) (c)

Figure 8: Fluorescence micrographs of a GUV made from 1:1:1 DPPC-DOPC-cholesterol mixture showing the coalescence of domains. The time lag between between the first and last figures is $\sim$ 60 s. The scale bars correspond to 5 μm.