Cholesterol in Condensed and Fluid Phosphatidylcholine Monolayers Studied by Epifluorescence Microscopy

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ABSTRACT Epifluorescence microscopy was used to investigate the effect of cholesterol on monolayers of dipalmitoylphosphatidylcholine (DPPC) and 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC) at 21 ± 2°C using 1 mol% 1-palmitoyl-2-[12-[(7-nitro-2-1, 3-benzoxadizole-4-yl)amino]docanoyl]phosphatidylcholine (NBD-PC) as a fluorophore. Up to 30 mol% cholesterol in DPPC monolayers decreased the amounts of probe-excluded liquid-condensed (LC) phase at all surface pressures (π), but did not effect the monolayers of POPC, which remained in the liquid-expanded (LE) phase at all π. At low π (2–5 mN/m), 10 mol% or more cholesterol in DPPC induced a lateral phase separation into dark probe-excluded and light probe-rich regions. In POPC monolayers, phase separation was observed at low π when ≥40 mol% or more cholesterol was present. The lateral phase separation observed with increased cholesterol concentrations in these lipid monolayers may be a result of the segregation of cholesterol-rich domains in ordered fluid phases that preferentially exclude the fluorescent probe. With increasing π, monolayers could be transformed from a heterogeneous dark and light appearance into a homogeneous fluorescent phase, in a manner that was dependent on π and cholesterol content. The packing density of the acyl chains may be a determinant in the interaction of cholesterol with phosphatidylcholine (PC), because the transformations in monolayer surface texture were observed in phospholipid (PL)/sterol mixtures having similar molecular areas. At high π (41 mN/m), elongated crystal-like structures were observed in monolayers containing 80–100 mol% cholesterol, and these structures grew in size when the monolayers were compressed after collapse. This observation could be associated with the segregation and crystallization of cholesterol after monolayer collapse.

INTRODUCTION

Cholesterol is an important constituent of biological membranes. Mammalian cells may utilize endogenous or exogenous sources of cholesterol for the maintenance of membrane structure and for cellular growth, and cholesterol may determine certain physical and functional properties of biological membranes, such as fluidity and permeability (see Yeagle, 1985, 1993, for reviews). Studies of model phospholipid membranes containing high concentrations of cholesterol have shown a lateral separation of lipid into domains that may be either rich or poor in cholesterol (Estep et al., 1978; Smutzer and Yeagle, 1985), but the distribution of high amounts of cholesterol in phospholipid membranes or in biological membranes is not yet well delineated.

Despite its essential role in the cell, overloading of some cells with cholesterol has been attributed to the onset of atherosclerosis (Yeagle, 1985). Particularly relevant to this phenomenon is the deposition of high levels of cholesterol in the lamellar bodies found in atherosclerotic lesions. Multilamellar lipid structures consisting of unesterified cholesterol and phospholipid in ratios of up to 2.6:1 have been isolated from the extracellular space of diseased human arterial walls; however, it is not known whether these lamellar bodies are formed in the extracellular space or within the cell (Chao et al., 1988). A metastable phase with cholesterol:phospholipid ratios of 2:1 has been described (Bar- ton, 1976), and more recent studies have found that cholesterol-phospholipid dispersions of this ratio or greater tend to aggregate and precipitate in water (Collins and Phillips, 1982). If this observation were extrapolated to lamellar bodies, it may explain the deposition of cholesterol crystals in the extracellular space of atherosclerotic lesions.

Monolayers of cholesterol and phospholipids have been studied in great detail in the last three decades as models for lipid distributions in biological membranes (Albrecht et al., 1981; Cadenhead, 1985; Chapman et al., 1969; Müller-Landau and Cadenhead, 1979; Phillips and Chapman, 1968). These monolayer studies have indicated that the mixing properties of cholesterol with saturated chain phosphatidylcholines are different from those of their unsaturated chain counterparts. Recently new techniques for visualizing such monolayers have allowed direct observation of the various phase segregation and transitions occurring in such cholesterol/phospholipid monolayers.

Fluorescence microscopy of phospholipid monolayers at an air-water interface has become a convenient tool for studying model biological membranes. One can visually observe lipid-lipid interactions and two-dimensional phase transitions occurring in such monolayers with different lipid packing states (Knobler, 1990; Möhwald, 1990; Nag and Keough, 1993; Stine, 1994; von Tscharner and McConnell, 1981; Weis, 1991; Yu and Hui, 1992). Studies of different phosphatidylcholine monolayers with low to intermediate
cholesterol contents have been used in this technique (Hirschfeld and Seul, 1990; Weis and McConnell, 1985), and the phase diagrams and miscibility of the lipids derived from surface pressure-area profiles have been confirmed by fluorescence microscopy (Hirschfeld and Seul, 1990). The cholesterol amounts used in such studies rarely exceeded the critical miscibility limit of the lipids in bilayers, and only recently have studies been performed with higher (≥30 mol%) cholesterol in phosphatidylcholine monolayers (Merkel and Sackmann, 1994; Mattjus and Slotte, 1994; Slotte and Mattjus, 1995; Slotte, 1995a). These studies indicated that cholesterol may induce a lateral separation into cholesterol-rich and cholesterol-poor regions in the phospholipid matrix at critical concentrations and surface pressures, depending on the chain length of the phosphatidylcholine (Mattjus et al., 1994; Slotte, 1995b). However, the studies were done with saturated phospholipids, and the distributions of cholesterol in saturated versus unsaturated phosphatidylcholine monolayers are not clear from these studies. Using epifluorescence microscopy, we have studied the distribution and association of cholesterol in dipalmitoylphosphatidylcholine (DPPC) and 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) monolayers, using the exclusion of a fluorescent probe from the different phases to indicate changes in domain structures. Using image analysis, we estimated the distribution of possible “cholesterol-rich” and “cholesterol-poor” phases in such monolayers at different packing densities and at different surface pressures (π).

MATERIALS AND METHODS

Materials

The lipids, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and cholesterol, were purchased from Sigma Chemicals (St. Louis, MO). Cholesterol was recrystallized from ethanol before use. 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and the fluorescent lipid probe 1-palmitoyl-2-[12-(7-nitro-2,1,3-benzoxadiazole-4-yl)amino] dodecanoyl]-sn-glycero-3-phosphocholine (NBD-PC) were purchased from Avanti Polar Lipids (Pelham, AL).

The lipids and probes were determined to be pure by thin-layer chromatography (TLC) and were used as received. The concentration of the phospholipid samples was determined by a modified version (Keough and Kariel, 1987) of the analysis for organic phosphorus of Bartlett (1959). The lipids and the probes were dissolved in chloroform:methanol (3:1 v/v), mixed in desired molar proportions.

Double-distilled water was used as the subphase on which the monolayer was spread, the second distillation being performed from dilute permanganate solution.

Epifluorescence microscopy of monolayers

Monolayers were spread on clean water subphases in an epifluorescence microscopic surface balance, the design and construction of which are discussed elsewhere (Nag et al., 1990). All experiments were performed at a temperature of 21 ± 2°C. Mixtures were formed from a chloroform:methanol (3:1, v/v) solution of DPPC or POPC containing the desired proportions of cholesterol and NBD-PC. The monolayers were formed by evenly spreading 25-nmol aliquots of lipid in chloroform:methanol solution on the surface, and 30 min was allowed for solvent evaporation and monolayer equilibration before compression. The monolayers were compressed in steps at an initial rate of 0.13 Å²/molecule/s. Monolayer features were video recorded for 1 min at selected surface pressures before the next compression step. A complete compression of the monolayer from maximum to minimum area of the trough (171.6 cm²) took 212 min. The monolayer features were analyzed using digital image analysis software JAVAVA 1.3 (Jandel Scientific, Corte Madera, CA). The percentage of the dark regions was estimated by division of the total amount of probe-excluded regions by the total area of a frame. This methodology has been used previously to observe lipid monolayers under quasiequilibrium conditions and has been discussed in detail elsewhere (Nag and Keough, 1993; Nag et al., 1991).

RESULTS

Fig. 1 shows the surface pressure (π)-average area per molecule (A) isotherms of monolayers DPPC (Fig. 1 A) and POPC (Fig. 1 B) containing 10–80 mol% cholesterol and 1 mol% NBD-PC. The isotherms indicated that DPPC underwent an liquid expanded (LE) to liquid condensed (LC) phase transition between π of 5 and 15 mN/m, as indicated by the plateau region of the isotherm, whereas POPC remained in the LE or fluid phase, and cholesterol remained in the solid condensed phase up to the points of monolayer collapse. At cholesterol concentrations above 10 mol%, the LE-LC plateau of DPPC was no longer detectable in the isotherms. Increasing amounts of cholesterol shifted both DPPC and POPC isotherms to the left by reducing the mean

![FIGURE 1](image)

area per molecule ($\AA^2$/molecule). The steep slopes of the isotherms of DPPC and POPC monolayers containing $\geq 50$ and $\geq 70$ mol% cholesterol, respectively, were indicative of an ordered cholesterol-like isotherm. The probe NBD-PC did not significantly alter the $\pi$-$A$ properties of the lipids, because the $\pi$/A isotherms of the DPPC/cholesterol/NBD-PC or POPC/cholesterol/NBD-PC mixtures were comparable to previously studied isotherms of similar phospholipid/cholesterol contents (Ghosh and Tinoco, 1972; Lund-Katz et al., 1988; Müller-Landau and Cadenhead, 1979).

Fig. 2 shows plots of area per molecule as a function of composition for these lipid monolayers as calculated from their respective isotherms. They indicated that at a low $\pi$ of 5 mN/m, cholesterol did not mix ideally with DPPC (Fig. 2 A) or POPC (Fig. 2 B). There was a negative deviation from the ideal mixing line for the lipids, consistent with the conventional interpretation that cholesterol condensed DPPC or POPC monolayer isotherms at low $\pi$. At higher surface pressures of 15 and 30 mN/m, DPPC seemed to mix ideally with cholesterol (or the components were completely segregated), whereas negative deviations from ideal mixing persisted for POPC/cholesterol at these pressures. Such mixing of cholesterol with DPPC or POPC in monolayers was very similar to the patterns observed previously by others (Chapman et al., 1969; Lund-Katz et al., 1988; Smaby et al., 1994).

![FIGURE 2](image)

**FIGURE 2** Mean molecular area as a function of mole percent for the monolayers of DPPC/cholesterol (top) and POPC/cholesterol (bottom). The surface pressures are 5 mN/m (O), 15 mN/m (●), and 30 mN/m (▼) for each mixture. The solid lines represent the mean molecular areas for ideal mixing of cholesterol and phosphatidylcholine.

Fig. 3 shows typical images seen in monolayers of DPPC containing 0–80 mol% cholesterol (Fig. 3 A–F) and of pure cholesterol (Fig. 3 G), each containing 1 mol% of the fluorescent probe NBD-PC. DPPC monolayers showed LE plus LC domain structures between $\pi$ of 6 and 15 mN/m, the latter being typically kidney-bean shaped. The LC domains changed to spiral or circular shapes with the addition of 2–6 mol% cholesterol (data not shown), which have been observed previously by Weis and McConnell (1985). DPPC monolayers containing 10 and 20 mol% cholesterol showed remnants of such a spiral condensed phase at higher pressures ($\pi \approx 10–20$ mN/m). With 30 mol% or more cholesterol in DPPC monolayers, the LC phase was not observed at any surface pressure up to the point of monolayer collapse. The images obtained indicate that at comparative $\pi$, the area occupied by the LC phase decreased as the molar concentration of cholesterol increased, implying that lower concentrations (0–30 mol%) of cholesterol fluidized or increased the expanded phase in DPPC monolayers.

Epifluorescent microscopy has previously indicated the coexistence of two fluid phases in monolayers of phospholipid mixtures with critical cholesterol concentrations (Benvenug and McConnell, 1993; Hirshfeld and Seul, 1990; Rice and McConnell, 1989; Slotte, 1995a,b; Subramaniam and McConnell, 1987). At low pressures (1–5 mN/m), pure DPPC monolayers showed a homogeneous fluorescent phase (Fig. 3 A); however, lateral phase separation was observed at similar low pressures in DPPC/cholesterol mixtures containing 10, 20, and 30 mol% cholesterol (Fig. 3, B–D). At these concentrations of cholesterol, the appearance of the film at low pressures was heterogeneous in that fields showed two types of lateral probe distributions. Typical images showed circular “drop-like” probe-excluded domains in a “sea” of light fluorescence. If the pressure was increased in the monolayers, the heterogeneous appearance “melted” into a homogeneous fluorescent phase. As surface pressure was increased further in monolayers of DPPC containing 10 and 20 mol% cholesterol, probe-excluded regions again appeared in the fields (see the third and fourth frames in rows B and C of Fig. 2). These regions were likely in the liquid-condensed phase. The elongated narrow or lattice-like forms of the dark domains suggested that the line tension between them and the fluorescent phase was lower than between the liquid-condensed and liquid-expanded regions of pure DPPC. The appearance may represent relatively pure condensed DPPC-rich domains being excluded from somewhat more fluid DPPC/cholesterol domains. At low surface pressures in monolayers with 10, 20, and 30 mol% cholesterol, distortion of the phase boundaries occurred continually and domain coalescence was observed in the circular probe-excluded regions formed. These properties of the probe-excluded domains suggested that they constituted a fluid phase as opposed to a typical condensed phase. Subramaniam and McConnell (1987) have also reported similar immiscible fluid-phase domains in monolayers of dimyristoylphosphatidylcholine (DMPC) containing 30 mol% cholesterol at 25°C. At higher pressures (>20
FIGURE 3  Typical images of monolayers of DPPC:NBD-PC 99:1 (A: $\pi = 4.8, 7.3, 10.1$, and 43.4 mN/m); DPPC:chol:NBD-PC 89:10:1 (B: $\pi = 2.6, 4.3, 12.4$, and 44.5 mN/m); 79:20:1 (C: $\pi = 2.5, 3.5, 19.1$, and 37.5 mN/m); 69:30:1 (D: $\pi = 3.6, 5.6, 19.2$, and 33.9 mN/m); 49:50:1 (E: $\pi = 2.1, 12.0$, 29.3, and 41.8 mN/m); 19:80:1 (F: $\pi = 0.9, 15.1, 42.0$, and 46.0 mN/m); and chol:NBD-PC 99:1 (G: $\pi = 2.5, 19.6, 39.6$, and 41.9 mN/m). The light areas indicate the phases containing the fluorescent probe NBD-PC. The number in the lower right-hand corner of each image is the surface pressure in mN/m. The scale bar is 25 $\mu$m.
mN/m) the fluorescence of the DPPC monolayer with 30 mol% cholesterol was reduced, yet a faint homogeneously distributed fluorescence emission was still observed. The occurrence of the probe throughout the monolayer was indicative of the presence of a fluid phase; however, lack of monolayer flow suggested it had a viscous, somewhat condensed nature.

With 50 mol% cholesterol in DPPC at low \( \pi \), the texture of the monolayer changed (Fig. 3E). The monolayers displayed a complex arrangement. On long-range sections the appearance of the monolayer was “inverted” from that observed at lower cholesterol concentrations, with the occurrence of circular fluorescent domains in a dark background, whereas within the fluorescent domains there were dark circular regions in patterns reminiscent of those seen for whole fields with monolayers containing \( \leq 30 \) mol% cholesterol. At higher \( \pi \), the fluorescent domains were considerably reduced in area, and the film displayed a lattice-like appearance. With 80 mol% cholesterol, the monolayer exhibited a probe-excluded phase with crescent and star-shaped probe-rich regions at low \( \pi \). At \( \sim 30 \) mN/m, the monolayers containing 50 and 80 mol% cholesterol in DPPC displayed dark fields with low lateral mobility that contained periodic entrapped probe-rich regions. The cholesterol monolayer containing 1 mol% fluorescent probe was similar in appearance to the DPPC/chol/NBD-PC 19:80:1 mixture at all pressures. At a \( \pi \) of \( \sim 40 \) mN/m, elongated fluorescent crystal-like structures were observed in the monolayers of DPPC containing 80 mol% cholesterol and pure cholesterol. In the cholesterol monolayer, these crystallites grew into large opaque flakes with further compression of the collapsed monolayer.

Typical images seen in monolayers of POPC containing 40–80 mol% cholesterol with 1 mol% fluorescent probe NBD-PC are shown in Fig. 4. Pure POPC existed in a LE phase, as indicated by the appearance of a single homoge-

![Typical images of monolayers of POPC:chol:NBD-PC: 59:40:1 (A: \( \pi = 1.1, 3.7, 8.2, \) and 32.9 mN/m), 49:50:1 (B: \( \pi = 2.8, 4.2, 4.9, \) and 40.3 mN/m), 29:70:1 (C: \( \pi = 2.2, 8.0, 12.4, \) and 38.6 mN/m), and 19:80:1 (D: \( \pi = 3.1, 7.6, 15.8, \) and 41.5 mN/m). In monolayers of POPC:NBD-PC (99:1) and POPC:chol:NBD-PC containing 0–30 mol% cholesterol, the fields were homogeneously fluorescent at all surface pressures; the images at different \( \pi \) are not shown. The light areas indicate the phases containing the fluorescent probe NBD-PC. The number in the lower right-hand corner of each image is the surface pressure in mN/m. The scale bar is 25 \( \mu \text{m} \).]
neous fluorescent phase at all surface pressures (images not shown). Increasing the molar concentration of cholesterol from 0 to 30 mol% had no obvious effect on the fluorescent expanded-phase fields of the POPC monolayer at any pressure up to monolayer collapse pressure, indicating that cholesterol at a concentration of \( \leq 30 \) mol% seemed to disperse throughout the fluid POPC phase. In monolayers of POPC containing 40 mol% cholesterol, circular probe-excluded regions were seen at low \( \pi \) (\( \leq 4 \) mN/m), but with increased \( \pi \) (\( \geq 5 \) mN/m) homogeneous fluorescent fields were observed (Fig. 4 A). At 50 mol% cholesterol, the dark regions persisted at higher \( \pi \), but the monolayer eventually converted to a homogeneous fluorescent image at \( \pi \) above 5 mN/m. At low \( \pi \), the appearance and behavior of the POPC films containing 40 and 50 mol% cholesterol were similar to the DPPC films containing 20 and 30 mol% cholesterol, respectively.

A surface texture change similar to that seen in monolayers of DPPC with \( \geq 50 \) mol% cholesterol was observed in POPC monolayers containing \( \geq 70 \) mol% cholesterol. A continuation of probe-excluded domains on a fluorescent background and probe-rich domains on a dark background was visualized (Fig. 4, C and D). With increased \( \pi \) (\( \geq 8 \) mN/m), the monolayers converted to a dark field; however, circular probe-rich areas were observed sporadically.

The total percentage of dark phase (probe-excluding regions in Figs. 3 and 4) plotted as a function of surface pressure for DPPC containing 0–30 mol% cholesterol are shown in Fig. 5. Pure DPPC began to condense at \( \sim 6 \) mN/m, and at \( \sim 26 \) mN/m over 80% of the DPPC monolayer showed dark phase or probe-excluded areas. In monolayers containing DPPC plus 10–30 mol% cholesterol, dark probe-excluding regions were initially observed at very low \( \pi \), especially in those containing 20 and 30 mol% cholesterol, but these dark regions may not be conventional liquid-condensed phase, as seen in pure DPPC monolayers. When the pressure in these DPPC-cholesterol monolayers was raised beyond 5 mN/m, the dark regions disappeared and the monolayer became homogeneous fluorescent, characteristic of the liquid-expanded phase. Further compression of the monolayers containing 10 and 20 mol% cholesterol resulted in the reappearance of dark regions, possibly domains of liquid-condensed phase. Higher pressures were needed for the reappearance of the dark domains, and the total percentage of dark regions was reduced as cholesterol was increased at any given \( \pi \) (see Fig. 5). DPPC monolayers containing 30 mol% cholesterol did not show a reappearance of dark regions up to collapse pressure.

Fig. 6 shows the total percentage of dark regions plotted as a function of surface pressure for DPPC (top) and POPC (bottom) monolayers containing higher mole fractions of cholesterol (\( \geq 20 \) mol%). As noted above, the appearance of dark (probe-excluded) regions at low \( \pi \) in DPPC monolayers containing 20 and 30 mol% cholesterol was dependent on cholesterol content and surface pressure. DPPC monolayers containing 50 mol% cholesterol or more displayed

![FIGURE 5 Total percentage of dark (probe-excluded) regions per frame, plotted as a function of surface pressure for DPPC:chol 99:1 (O), and DPPC:chol:NBD-PC 89:10:1 ( ), 79:20:1 (V), 69:30:1 (W). Error bars indicate \( \pm \) one standard deviation for 10 frames analyzed at each \( \pi \).](image1)

![FIGURE 6 Total percentage of dark (probe-excluded) regions per frame, plotted as a function of surface pressure for DPPC:chol:NBD-PC, 79:20:1 (O), 69:30:1 ( ), 49:50:1 (V), 19:80:1 (W), and chol:NBD-PC, 99:1 ( ) (top); and POPC:chol:NBD-PC, 59:40:1 (O), 49:50:1 ( ), 29:70:1 (V), and 19:80:1 (W), and chol:NBD-PC, 99:1 ( ) (bottom). Error bars indicate \( \pm \) one standard deviation for 10 frames analyzed at each \( \pi \).](image2)
mostly dark regions (70–90%) at all pressures, as did monolayers of cholesterol plus probe. No probe-excluded regions (0% dark) were observed in POPC monolayers containing 0–30 mol% cholesterol. With 40 mol% cholesterol in POPC, dark regions were seen at low \( \pi \), but these regions were reduced in amount as \( \pi \) was increased, as was the case of DPPC monolayers containing 10–30 mol% cholesterol. The percentage of dark regions dropped to 0% at \( \pi > 5 \) mN/m with the appearance of a fluorescent homogeneous field. Increasing the amount of cholesterol to 50 mol% resulted in an increased amount of probe-excluded regions at very low \( \pi \), and higher \( \pi \) was required to convert the monolayers into a single fluorescent phase (0% dark). With 70 and 80 mol% cholesterol in POPC, the amount of probe-excluded regions was high (70–80% dark) at all \( \pi \).

The general pattern of formation of probe-excluded regions as a function of cholesterol concentration in DPPC or POPC monolayers at a surface pressure of 1.5 mN/m is shown in Fig. 7. Both DPPC and POPC showed an increase in the percentage of dark regions as the molar concentration of cholesterol was increased. The two plots show similar shapes, but the POPC plot is shifted to the right of the one for DPPC by 20 mol% cholesterol. These data and those in Fig. 6 suggest that cholesterol affects the fluid phase of DPPC and POPC in a similar manner. At a particular packing density, the amount of probe-excluded phase formed in DPPC and POPC monolayers may be nearly equal, but the miscibility of cholesterol seems to be higher in POPC monolayers than in DPPC monolayers. A molar concentration of 20 mol% was sufficient to produce phase separation in DPPC monolayers at a \( \pi \approx 1.5 \) mN/m, whereas 40 mol% was required in POPC monolayers. Similar patterns were recently observed by Slotte (1995a) in DPPC/cholesterol monolayers.

Fig. 8 shows the phase diagrams defined for DPPC/cholesterol (top) and POPC/cholesterol (bottom) systems. Two boundaries were determined based on data from Fig. 2 (filled circles) using the approach taken by Hirshfeld and Seul (1990), and the third (open circles) was determined from direct visualization of monolayers as done by Subramaniam and McConnell (1987). In the later case, the surface pressure was noted when the initial inhomogeneous texture disappeared in the monolayer field of view. The relative shapes of the two diagrams are similar, but the boundaries for POPC/cholesterol are shifted to higher cholesterol composition in comparison to DPPC/cholesterol.

In the DPPC/cholesterol phase diagram (Fig. 8, top), region I corresponds to an image shown in Fig. 3 C-3. This image shows a web of probe-excluded domains dispersed in a probe-rich area. Region II in the phase diagram appears as large probe-excluded domains (Fig. 3 E-2), and region III gave images containing small probe-excluded domains (Fig. 3 C-I). Region IV may correspond to A-I to A-4 in Fig. 3; these images show a transition from homogeneous fluorescent phase through the classic kidney bean probe-excluded domains to a homogeneous solid-like phase.

![Figure 7](image1.png)  
**FIGURE 7** Total percentage of dark (probe-excluded) regions per frame, plotted as a function of mole fraction of cholesterol. The total percentage of dark regions was analyzed from 10 images at a \( \pi = 1.5 \) mN/m from DPPC (●) and POPC (○). Error bars indicate ± one standard deviation.

![Figure 8](image2.png)  
**FIGURE 8** Surface pressure versus composition phase diagram for DPPC/cholesterol and POPC/cholesterol mixtures. Points were plotted based on data from Fig. 2 (●) and visual observation of the disappearance of inhomogeneous monolayer textures (○).
In the POPC/cholesterol phase diagram (Fig. 8, bottom), images from region I of the phase diagram appear in B-4 of Fig. 4. This shows a homogeneous fluorescent phase. Region II corresponds to Fig. 4 D, and region III to B-2 of Fig. 4.

Region IV corresponds to a homogeneous fluorescent phase seen in POPC monolayers containing \(\leq 30\) mol% cholesterol (images not shown).

DISCUSSION

The pressure-area isotherms indicate that cholesterol condensed the monolayers of DPPC at low surface pressures, and those of POPC at all pressures. The introduction of cholesterol to these monolayers decreased the average molecular area occupied by the PC molecules by inducing order into the acyl chains. Lund-Katz et al. (1988) suggested that cholesterol has a greater affinity for DPPC than for POPC. Our data for average condensation suggests that, at low \(\pi\), cholesterol produces a condensing effect at least as large, if not a larger, on DPPC as it does POPC (Fig. 2). At higher \(\pi\), interpretation of the observed behavior is more complex. Little condensing effect of cholesterol occurs because the DPPC molecules are already densely packed in the liquid-condensed state at high pressure, whereas the POPC molecules remain in the liquid-expanded state and are amenable to further condensation by cholesterol. This effect of cholesterol on the two PCs studied here is consistent with the effect of cholesterol in the system of saturated PC of different chain lengths studied by Slotte (1995b). The acyl chains of DPPC may have a stronger van der Waals attraction with the sterol ring than the acyl chains of POPC, resulting in a greater tendency for cholesterol to compact a monolayer of DPPC than POPC.

Fluorescence microscopy enabled the visualization of the fluidizing effect of cholesterol on the LC domains of DPPC monolayers at pressures greater than 5 mN/m. With increasing concentrations of cholesterol (10–20 mol%) in DPPC, the area occupied by the LC domains diminished until they were not detectable with 30 mol% cholesterol. On the other hand, no visual differences were noticed in the liquid expanded POPC monolayer when cholesterol content was increased from 0 to 30 mol%. Image processing and analysis procedures quantitated the decrease in the LC phase in DPPC/cholesterol monolayers with increased cholesterol concentrations up to 30 mol% (from 89 to 0% at \(\pi > 5\) mN/m).

Many epifluorescence microscopy studies of monolayers at the air-water interface have utilized a fluorescent probe such as NBD-PC, whose structure causes it to preferentially localize in an expanded fluid phase. Any phase that excludes the probe might be considered a condensed region with an ordered structure. However, recent studies of phospholipid monolayers (DMP and DPPC) containing critical amounts of cholesterol (\(\geq 30\) mol%) have shown that these systems show a probe-excluded phase that does not fit the classical description of the LC phase (Benvegnu and McConnell, 1993; Hirshfeld and Seul, 1990; McConnell et al., 1990; Rice and McConnell, 1989; Subramaniam and McConnell, 1987). These new phases exist at low surface pressures, and their domains show circular shapes with unstable phase boundaries that can only be accounted for by a fluid phase (Lee and McConnell, 1993; Subramaniam and McConnell, 1987). The exclusion of the probe from these regions implies that the structure of these domains is more ordered than that of the LE phase. We shall call this phase "liquid-ordered" (lo), a term that was previously used to describe a fluid phase with restricted acyl chain motion and high diffusivity parallel to the membrane plane in bilayers containing cholesterol (Thewalt and Bloom, 1992; Vist and Davis, 1990). The current observations do not allow for determination of whether the observed liquid-ordered phases are analogous to one or both of the liquid-ordered (\(\alpha\)) and liquid-ordered (\(\beta\)) phases proposed for bilayers of DPPC plus cholesterol by McMullen and McElhaney (1995). The laterally separated dark phase observed at low surface pressures in monolayers of DPPC and POPC containing \(\geq 10\) mol% and \(\geq 40\) mol% cholesterol, respectively, may be a liquid-ordered or lo phase. The nucleation of the LC domains of pure DPPC at 21 ± 2°C did not occur until \(\sim 7\) mN/m; therefore the lo phase, which occurs at low surface pressures, may be a consequence of segregation of a cholesterol-rich phase. With POPC monolayers at low \(\pi\), the phase separation observed with 40 mol% cholesterol may have been the segregation of cholesterol-rich domains existing in an ordered fluid phase similar to that seen in the DPPC/cholesterol monolayers but requiring higher cholesterol contents for its production. At all temperatures and pressures above its transition temperature of \(-2^\circ\text{C}\), POPC exists in a liquid expanded phase, thus supporting the notion that the probe-excluded areas in POPC were a result of the segregation of cholesterol into a lo phase. During compression, the probe-excluded domains in the binary lipid mixtures would convert into a homogeneous fluid phase at intermediate pressures, and this mixing was dependent on cholesterol content and surface pressure. With increasing molar concentrations of cholesterol, the cholesterol-rich domains seemed to persist at somewhat higher pressures before the formation of a homogeneous fluorescent phase (see Figs. 3–6).

In monolayers of DMPC/cholesterol containing 10–60 mol% cholesterol at temperatures above the transition temperature of DMPC, the coexistence of fluid cholesterol-rich domains and fluid DMPC-rich domains has been documented (Benvegnu and McConnell, 1993; Hirshfeld and Seul, 1990; Lee and McConnell, 1993; Lee et al., 1994; Subramaniam and McConnell, 1987). The phase diagrams of DMPC/cholesterol monolayers indicate that there are critical surface pressures and compositions over which cholesterol-rich domains coexisting with fluid DMPC-rich ones are observed (Hirshfeld and Seul, 1990; Lee et al., 1994). The critical \(\pi\) for such domain coexistence has been found to be 10 mN/m or less over a composition range of 10–60.
mol% cholesterol, and above these composition ranges and critical $\pi$, the domains merge into a homogeneous phase (Hirshfeld and Seul, 1990; Lee et al., 1994). The domain wall fluctuations and instabilities have been attributed to the competition between the repulsive dipolar interactions and attractive van der Waals interactions of molecules in the domains. During isothermal compression, the balance of these competitive forces initially shifts in favor of the repulsive dipolar interactions, and, as a consequence, the domain wall energy vanishes. This results in a change in monolayer texture from heterogeneous dark and fluorescent phases to a homogeneous fluorescent phase (Seul and Sammon, 1990). Our observations of POPC and cholesterol mixing in monolayers seemed to follow a pattern similar to that observed for the DMPC/cholesterol systems, except the concentration ranges over which such domains were seen differed. POPC monolayers containing $\leq 30$ mol% cholesterol showed, at all $\pi$, homogeneous fluorescent or expanded phase. When $\geq 40$ mol% cholesterol was included, a dark probe-excluded phase was observed at low $\pi$ (Fig. 4), which converted into a single homogeneous phase with increasing $\pi$. These dark domains are probably cholesterol-rich, because larger amounts of this dark phase were seen in the POPC monolayers with increasing cholesterol concentration. Similarly, segregation of cholesterol into probe-excluded domains also occurred at low pressures with DPPC/cholesterol/12H NMR and 12NMR mixtures when DPPC was in the LE phase ($\pi < 3$ mN/m) (Fig. 3), and the amounts of these cholesterol-rich domains increased with increasing amounts of cholesterol in POPC or DPPC monolayers in a similar fashion (Fig. 6).

At low $\pi$, a different surface texture was observed in DPPC and POPC monolayers containing 50 and 70 mol% cholesterol, respectively. Dark domains on fluorescent background and fluorescent domains on dark background were visualized in the same field of view. The packing density of the PC may be a determinant in its interaction with cholesterol in monolayers. With the DPPC/cholesterol monolayers at $\pi$ above 7 mN/m, it was uncertain whether these dark probe-excluded domains were typically cholesterol-rich, because the condensing of DPPC into LC phase, which normally occurs at this $\pi$ in pure DPPC monolayers, may have played a factor in the monolayer texture. The dark phase observed in DPPC monolayers containing $\geq 50$ mol% cholesterol at a $\pi$ above 7 mN/m may be explained as a possible combination of the cholesterol condensed phase with that of the DPPC-rich LC phase. At pressures above 12 mN/m, DPPC monolayers containing $\geq 50$ mol% cholesterol may have formed a condensed phase, as visualization of the monolayer showed predominantly dark regions, with the exception of some entrapped probe. At high $\pi$, the formation of this poorly fluorescent phase in DPPC monolayers of high cholesterol content may be different from the homogeneous phases seen in POPC monolayers containing $\geq 70$ mol% cholesterol at high $\pi$ (Fig. 4). The homogeneous phases observed in POPC monolayers containing 70 mol% or more cholesterol at high $\pi$ appeared darker than the highly fluorescent phase associated with LE seen at low $\pi$ in this system. Furthermore, their motion as determined by monolayer flow was minimal. Intensity estimation (data not shown) implied that this “low-intensity” phase was closer in intensity to the liquid expanded phase than the liquid condensed phase, yet the minimal lateral motion of the monolayer suggested that a relatively ordered condensed phase was present. Increasing surface pressures would possibly overcome the line tensions of the unstable domain phase boundaries between the dark and light domains, and “melt” them into a homogeneous phase in which the probe becomes locked.

The packing density of the acyl chains may have been a factor in the interaction of cholesterol with PC in monolayers. The relevant changes in surface texture of the monolayer occurred at similar packing densities when DPPC and POPC isotherms were compared (see Fig. 1), and the amounts of the probe-excluded phases at these packing densities were similar. The acyl chains of DPPC and POPC occupied nearly the same molecular area with 20 and 40 mol% cholesterol, respectively. Similar molecular areas were also noted in lipid mixtures containing DPPC/cholesterol/NBD-PC (69:30:1) and POPC/cholesterol/NBD-PC (49:50:1), as well as DPPC/cholesterol/NBD-PC (49:50:1) and POPC/cholesterol/NBD-PC (29:70:1). Because of the double bond in the oleoyl chain of POPC, its area per molecule is larger than the saturated chain DPPC. However, the incremental difference in the packing densities of these PCs appears to be eliminated by the addition of 20 mol% cholesterol to the POPC monolayer.

We note that the phase diagrams for DPPC and POPC (Fig. 8) are similar in shape. However, the POPC phase boundaries are shifted to higher cholesterol concentrations. This also suggests that the interactions of cholesterol with DPPC and POPC are similar, but the lower packing density of the unsaturated PC requires more cholesterol to achieve similar phases.

$^3$H NMR and differential scanning calorimetry (DSC) have been used to map the phase boundaries that exist in bilayers of cholesterol and DPPC or POPC (Huang et al., 1993; McMullen and McElhaney, 1995; Thewalt and Bloom, 1992; Vist and Davis, 1990). Comparison of the phase diagrams of bilayers of DPPC and POPC in the presence of cholesterol shows that these PCs interact very similarly with cholesterol. The phase diagrams we defined based on the monolayer characteristics (Fig. 8) of these lipid mixtures also support this notion. The bilayer studies indicate that over a wide temperature range, a fluid but highly ordered phase(s) exists in bilayers of DPPC and POPC with a cholesterol content greater than $\sim 22$ and 25 mol%, respectively, and it may exist with other phases at lower cholesterol contents. This phase has been depicted as liquid ordered (lo). The lo phase has been described as having highly ordered acyl chains that possess little conformational freedom. In contrast to the gel phase, however, the molecules in the lo phase undergo rapid rotational diffusion in the bilayer and are not restricted by the formation of lattice
arrangements (Thewalt and Bloom, 1992; Vist and Davis, 1990).

The phase diagram obtained with monolayers of DPPC/cholesterol is similar to parts of the phase diagram described by Albrecht et al. (1981). These authors suggest that region I represents a coexistence of DPPC-cholesterol with 2–33 mol% cholesterol and pure DPPC. This is consistent with the images seen in Fig. 3 C-3 (10 mol% in our case), where the probe-excluded regions could be nearly pure DPPC domains. Region II of this phase diagram would be expected to consist of DPPC-cholesterol containing domains of excess cholesterol, as suggested by Albrecht et al. (1981). The images seen in Fig. 3 E-2 are also consistent with this, but they do suggest that the DPPC-cholesterol domain is in an ordered state, because the probe is excluded from these domains.

Albrecht et al. (1981) were unable to determine whether region III of their phase diagram was indicative of homogeneous or heterogeneous mixing. In this study, the epifluorescence microscope shows the coexistence of ordered domains in a fluorescent background. We could ascribe these ordered domains to DPPC-cholesterol that may be more ordered than the pure DPPC domains at these low surface pressures. This is supported by the observation that the total area of the probe-excluded domains increased as more cholesterol was added to the monolayer (Fig. 7). This is also in agreement with the suggestion of Hirshfeld and Seul (1990).

Our observations do not allow us to unequivocally describe the monolayer in region IV of the phase diagram. Hirshfeld and Seul (1990) proposed that in this region, monolayers contain coexisting domains of PC/cholesterol and nearly pure PC. They report that these monolayers appear optically homogeneous. We observe with DPPC-cholesterol monolayers that probe-excluded (LC) domains were present in this region. This is consistent with the coexistence of two phases in this region of the phase diagram.

In POPC monolayers, region I of the phase diagram corresponds to the image in Fig. 4 B-3, which shows a homogeneous fluorescent phase. This could be the case if these monolayers contained coexisting domains of POPC-cholesterol and POPC. Unlike the case of DPPC-cholesterol monolayers, in which the nearly pure DPPC domains are more ordered at higher pressures than the PC-cholesterol areas, it appears from the images in Fig. 4 B-3 that the POPC-cholesterol domains and the POPC domains have similar degrees of disorder and remain homogeneous fluorescent with compression.

Region II of the phase diagram for POPC-cholesterol monolayers gave images shown in Fig. 4, C-2 and D-1. These images confirm the coexistence of an ordered phase (lo) (likely POPC-cholesterol) and a fluorescent (LE) POPC phase. The image in Fig. 4 B-1 corresponds to region III of the phase diagram of POPC-cholesterol monolayers. There is clear evidence that there are two coexisting phases in these monolayers. It is difficult to determine the composition of these phases, but the increase in the ordered phase at a higher mole percentage of cholesterol suggests that the probe is excluded from the ordered POPC-cholesterol domains.

In POPC-cholesterol monolayers, the fluorescence microscopy images obtained from region IV of the phase diagram did not show evidence of phase separation. This is similar to the observations of Hirshfeld and Seul (1990) for DMPC-cholesterol monolayers. One possible explanation may be that the difference in physical properties between POPC-cholesterol domains and pure POPC domains might be too small to induce lateral segregation of the probe. A similar situation might be true for the monolayers of mixtures of cholesterol and DMPC studied by Hirshfeld and Seul (1990).

As seen previously in phospholipid-cholesterol monolayers (Slotte and Mattjus, 1995), highly fluorescent probe aggregates persisted until the monolayer collapsed. Subramaniam and McConnell (1987) suggest that NBD-PC is poorly soluble in the cholesterol-rich regions, yet the area occupied by these fluorescent regions is greater than the 1% expected for NBD-PC in the monolayers. It has been postulated that the probe-rich regions impede the condensation of neighboring cholesterol molecules (Möhwald, 1990), and the fluorescent regions observed may be probe and cholesterol in some sort of expanded phase. The phenomena of crystal formation in the monolayers of DPPC/chol/NBD-PC (19:80:1) and cholesterol/NBD-PC (99:1) are of interest. The crystals are highly fluorescent and might be crystals of the probe, yet the relative size of the crystals and their absence from DPPC or POPC monolayers containing 1 mol% NBD-PC argue against the notion that they arise because of the crystallization of NBD-PC. We postulate that the aggregates may be cholesterol crystallites containing some trapped probe that has precipitated out of the monolayer and which can be excluded at the collapse $\pi$ of the molecule. This observation may lead to some insights about the deposition of cholesterol crystals in the extracellular spaces of the arterial wall during the onset of atherosclerosis.

Although some of the notable features observed in our monolayers occurred at a lower surface pressure than that suggested to occur in membrane bilayers, the basic patterns of phase transition in both mono- and bilayer systems show some similarities. In bilayers of DPPC undergoing gel to liquid-crystalline phase transitions, cholesterol modulates acyl chain mobility of the lipids up to a critical concentration of $\sim 30$ mol% (Ladbrooke et al., 1968), beyond which the phase transition is no longer detectable by conventional low-sensitivity DSC. In DPPC monolayers containing 30 mol% cholesterol, the absence of a liquid-condensed phase gives further evidence of the fluidizing effect of cholesterol on gel-phase phospholipids. $\textsuperscript{2}$H NMR studies have indicated that within and beyond this cholesterol concentration range, a preponderance of liquid-ordered and gel phases may arise (Vist and Davis, 1990). The appearance of an immiscible fluid phase in the monolayer may be caused by the segregation of cholesterol and may be similar to this liquid-ordered phase.
Membrane cholesterol may not be distributed uniformly in the bilayer, but segregate into cholesterol-rich domains whose size and distribution may depend on factors such as phospholipid chain unsaturation and sterol carrier protein expression (Schroeder et al., 1995). Our studies suggest that cholesterol-rich and phospholipid-rich domains phase-separate in the monolayers, depending on cholesterol content and pressure. At the present time, it is not fully understood how cholesterol disperses in a lipid bilayer or monolayer containing more than 30 mol% cholesterol, but high amounts of cholesterol (>40 mol%) could be incorporated into the monounsaturated lipid monolayers (POPC). If this situation can be applied to the cholesterol-rich membranes of the lamellar bodies found in atherosclerotic lesions, it may explain how cholesterol is carried in the lamellar body and later expelled into the extracellular space of the atherosclerotic plaque.

This work was supported by the Heart and Stroke Foundation of New Brunswick and the Medical Research Council of Canada.

REFERENCES


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