Closed-loop fluid-fluid immiscibility in binary lipid-sterol membranes

by

SHABEEB P K

Thesis submitted to the
Jawaharlal Nehru University
for the degree of
Doctor of Philosophy

2024

Raman Research Institute

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DECLARATION

I hereby declare that this thesis is composed independently by me at Raman Research Institute, Bangalore, under the supervision of Prof. Pramod A Pullarkat and Prof. V. A. Raghunathan. The subject matter presented in this thesis has not previously formed the basis of the award of any degree, diploma, associateship, fellowship or any other similar title. I also declare that I have run it through the DrillBit plagiarism detection software.

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CERTIFICATE

This is to certify that the thesis entitled “Closed-loop fluid-fluid immiscibility in binary lipid-sterol membranes" submitted by SHABEEB P K for the award of degree DOCTOR OF PHILOSOPHY of Jawaharlal Nehru University is his original work. This has not been published or submitted to any other university for any other degree or diploma.

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Synopsis

Thesis title: Closed-loop fluid-fluid immiscibility in binary lipid-sterol membranes

Cell membranes are two-dimensional fluids in which membrane proteins and other embedded molecules are free to diffuse. They facilitate selective transport of materials and signals that are essential for various cellular activities. The lipid bilayer is the structural basis of all biological membranes. Sterols, such as cholesterol, are essential components of the plasma membrane, which separates the interior of the cell from the external environment. Sterol-induced compositional heterogeneities in plasma membranes, called lipid rafts, have been implicated in a variety of cellular functions such as signaling and trafficking.

Experiments with cell membranes are extremely challenging because of their structural complexity. Model membranes containing a few lipids and sterols offer a much simpler platform to study the physical properties and phase behavior of membranes. It is easy to prepare model membranes since lipid molecules self-assemble in water to form bilayers. The lipid raft hypothesis, proposed more than two decades ago, has motivated a large number of studies on fluid-fluid coexistence in model lipid membranes, as the membrane rafts can be looked upon as fluid domains dispersed in a fluid membrane.

Interestingly, even before the advent of the lipid raft hypothesis, fluid-fluid phase separation in binary lipid cholesterol membranes had been suggested by some spectroscopy experiments and supported by theoretical models. However, in spite of a large number of subsequent experimental investigations, no binary lipid-sterol membrane has been unambiguously shown to exhibit fluid-fluid coexistence. As a result, ternary mixtures consisting of two lipids (with widely different chain melting transition temperatures) and cholesterol are considered as the simplest “raft mixtures” that exhibit fluid-fluid coexistence.

In this thesis, we present the first examples of binary lipid-sterol membranes that exhibit true fluid-fluid coexistence. In these systems fluid-fluid immiscibility is observed within a closed-loop region, characterized by an upper critical solution temperature and a lower critical solution temperature, with the latter lying slightly above the chain melting transition temperature

of the membrane. This thesis describes an extensive fluorescence microscopy investigation of the phase behavior of binary mixtures of oxygenated derivatives of cholesterol, called oxysterols, with a variety of lipids. This study identifies structural features of the lipid and the sterol molecules that are essential for the occurrence of fluid-fluid coexistence. We also propose a novel mechanism that is responsible for the induction of this phase behavior in these membranes.

This thesis consists of 6 chapters. In Chapter 1, we give a brief introduction to the self-assembly of lipid molecules in water and to earlier studies on the phase behavior of binary and ternary lipid-sterol membranes. Different experimental techniques used to probe the phase behavior of these systems, and the conclusions drawn from them, are critically reviewed. It is shown that different techniques are sensitive to different types of compositional heterogeneities in the membrane and that many reports of fluid-fluid phase separation in the literature are untenable. Experimental protocols used to prepare giant unilamellar vesicles (GUV) and supported multilayers are described here. The main experimental technique employed in the present study is fluorescence microscopy, and hence a detailed discussion of this technique is also presented.

In Chapter 2, we describe fluorescence microscopy studies of binary mixtures of the zwitterionic lipid, 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), with either 25-hydroxycholesterol (25HCH) or 27-hydroxycholesterol (27HCH). Fluorescence images clearly show phase separation over an extensive temperature range above the main transition temperature (T_m) in DMPC-25HCH and DMPC-27HCH multilayers prepared on solid substrates and kept in a high humidity chamber. Furthermore, domains formed on phase separation are found to coalescence, confirming their fluid nature. Partial phase diagrams of DMPC-25HCH and DMPC-27HCH have been determined from the microscopy data. We confirm the occurrence of fluid-fluid phase separation using GUVs made from DMPC-25HCH and DMPC-25HCH membranes. Observation of phase separation in GUVs indicates that interactions within each bilayer are responsible for this phase behavior and that inter-bilayer interactions are irrelevant. Moreover, these observations show that fluid-fluid coexistence is present under excess water conditions, which are relevant for cell membranes. Results of some recent computer simulation studies of DMPC-27HCH membranes are also discussed, which suggest that the two-phase

coexistence observed in these membranes is due to the ability of the oxysterol molecule to take different orientations in the membrane depending on the temperature.

In chapter 3, we investigate the influence of the chain length of the lipid molecule on the two-phase coexistence. Mixtures of 25HCH/27HCH with phosphocholine (PC) lipids having saturated hydrocarbon chains containing 12 to 16 carbon atoms are used in these studies. Binary mixtures of 25HCH/27HCH and, 1,2-ditridecanoyl-sn-glycero-3-phosphocholine (13:0 PC), 1,2-dipentadecanoyl-sn-glycero-3-phosphocholine (15:0 PC) exhibit fluid-fluid immiscibility over a wide temperature range. However, 1,2-dilauroyl-sn-glycero-3-phosphocholine (DLPC) is found to exhibit this behavior with 25HCH, but not with 27HCH. On the other hand, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) shows this behavior with 27HCH, but not with 25HCH. These observations indicate that the phase behavior is sensitive to the thickness of the lipid membrane, which is determined by the chain length of the lipid molecule and the position of the –OH group on the terminal chain of the oxysterol.

In chapter 4, we investigate the effect of unsaturation of the hydrocarbon chains of the lipid on fluid-fluid coexistence. Fluorescence microscopy observations suggest the occurrence of fluid-fluid coexistence in 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC)-27HCH multilayers, but not in POPC-25HCH multilayers. Influence of the nature of the lipid headgroup is studied using 1,2-dilauroyl-sn-glycero-3-phosphoethanolamine (DLPE) multilayers containing 25HCH/27HCH. These membranes do not exhibit fluid-fluid coexistence. We also studied binary mixtures of 25HCH and 27HCH with lipids whose fatty acid chains are attached to the glycerol backbone with ether linkages, instead of ester linkages. It is found that both ester-linked and ether-linked PCs with saturated chains show fluid-fluid immiscibility.

In Chapter 5, we present studies on the influence of two other oxysterols, namely, 27-hydroxy cholestenone and cholestenoic acid on the phase behavior of saturated PC multilayers. Structures of these two sterols are very similar to that of 27HCH, but with the difference that the former has a keto group instead of the OH group in the sterol ring, and the latter has a COOH group instead of the OH group in the side chain. It is found that 27-hydroxy cholestenone does not show fluid-fluid coexistence, whereas cholestenoic acid shows a stronger ability to induce fluid-fluid immiscibility compared to 27HCH. These experimental results provide important insights into the mechanism responsible for the observed phase behavior.

Chapter 6 gives a brief summary of the main results of this thesis. Their relevance to the functioning of cell membranes is discussed. We also indicate some directions for future research.

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Publication

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Contents

CHAPTER 1

Introduction

Membranes are the most common cellular structure present in both animals and plants. Biological membranes are complex in their composition and function. According to the fluid mosaic model, a cell membrane is a two dimensional oriented solution of integral proteins in a viscous lipid bilayer solvent [Fig. 1.1] [1]. Lipid bilayers are considered as the universal basis for cell membrane structure. This model does not contain the membrane associated structures such as cytoskeletal elements and extra cellular matrix. Moreover, organization of membranes in to various domains of different compositions, mobilities, life times and functions is not included. Adding the later insights, plasma membrane is considered as an adaptable fluid mosaic having compositional and functional susceptibility in response to interactions beyond the plane of the membrane [2, 3].

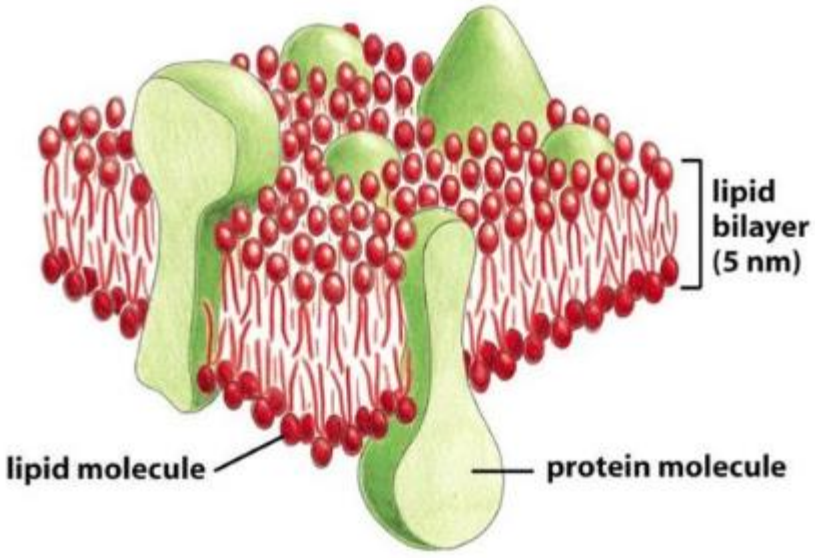

Fig. 1.1: A cartoon of the three dimensional view of a cell membrane proposed by the Fluid Mosaic Model [4]

1.1 Self-assembly of amphiphiles

Membrane lipids are amphiphilic in nature, i.e., they have a polar head (hydrophilic) and one or more non polar tails (hydrophobic). Phospholipids are the most abundant class of membrane lipids [Fig. 1.2] [4]. Amphiphilic molecules are dispersed in water as monomers at

very low concentrations. Self-assembly of amphiphiles above a critical micellar concentration (CMC) is well understood both theoretically and experimentally. Above CMC, phospholipids spontaneously form stable bilayers with their polar head groups exposed to water and non-polar hydrocarbon chains buried in the interior of the bilayer [5].

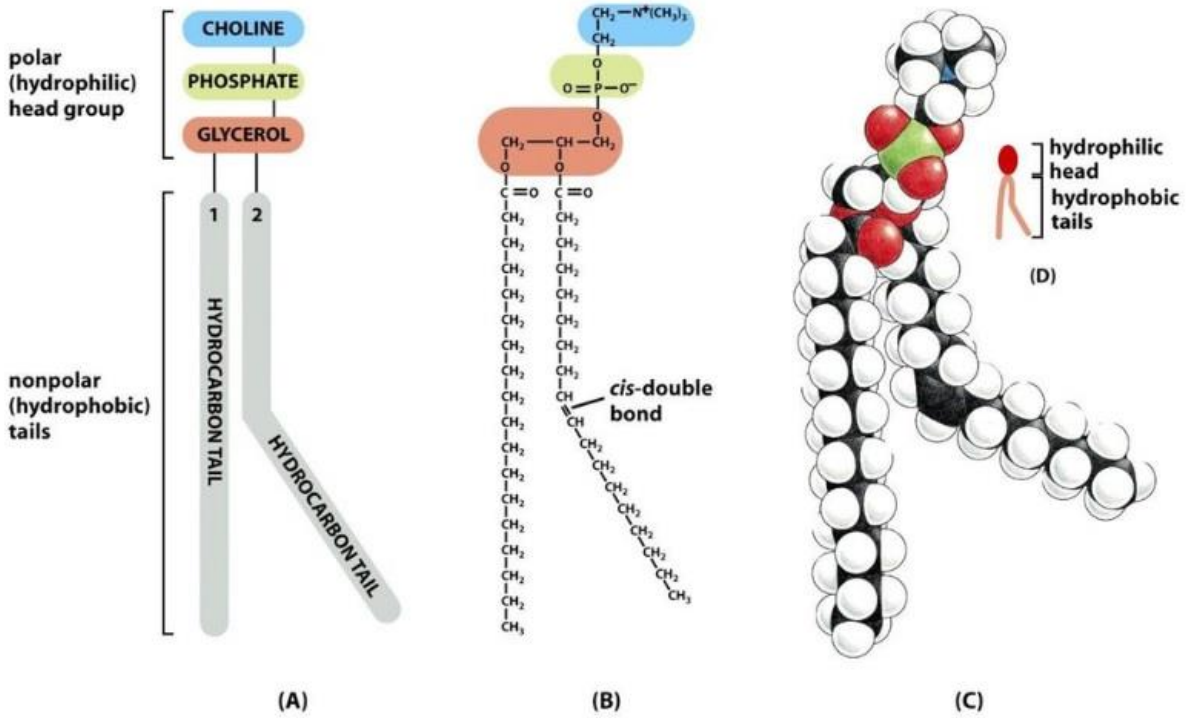

Fig. 1.2: Different parts of a phospholipid molecule. The example is phosphatidylcholine, represented by (A) schematically (B) by a formula (C) as a space filling model and (D) as a cartoon [4].

Organization of constituent lipids in to a bilayer is basically due to the hydrophobic effect. Water molecules are envisaged to be more ordered around a hydrophobic molecule than in the bulk liquid. This is entropically unfavorable and results in an effective attraction between hydrophobic molecules in water. This hydrophobic attraction between amphiphiles leads to their spontaneous self-assembly into structures, such as micelles and membranes [4, 6]. This process is energetically favorable as the entropy gain of water molecule outweighs the enthalpy penalty of demixing (of water and amphiphile) and the loss of configurational entropy of amphiphiles due to aggregation.

Shape of the constituent amphiphiles determines the shape of the aggregate formed above the CMC. A dimensionless quantity called the packing parameter or shape parameter ($\mathbf{v/a_0\, l_c}$) is

used to describe the shape of the molecule; where **v** is the volume of the hydrocarbon chain, $\mathbf{a_0}$ is the optimal head group area and $\mathbf{l_c}$ is the critical chain length, which is the maximum chain length in the fluid state. Values of the Packing parameter and the corresponding structures formed are shown in Fig. 1.3.

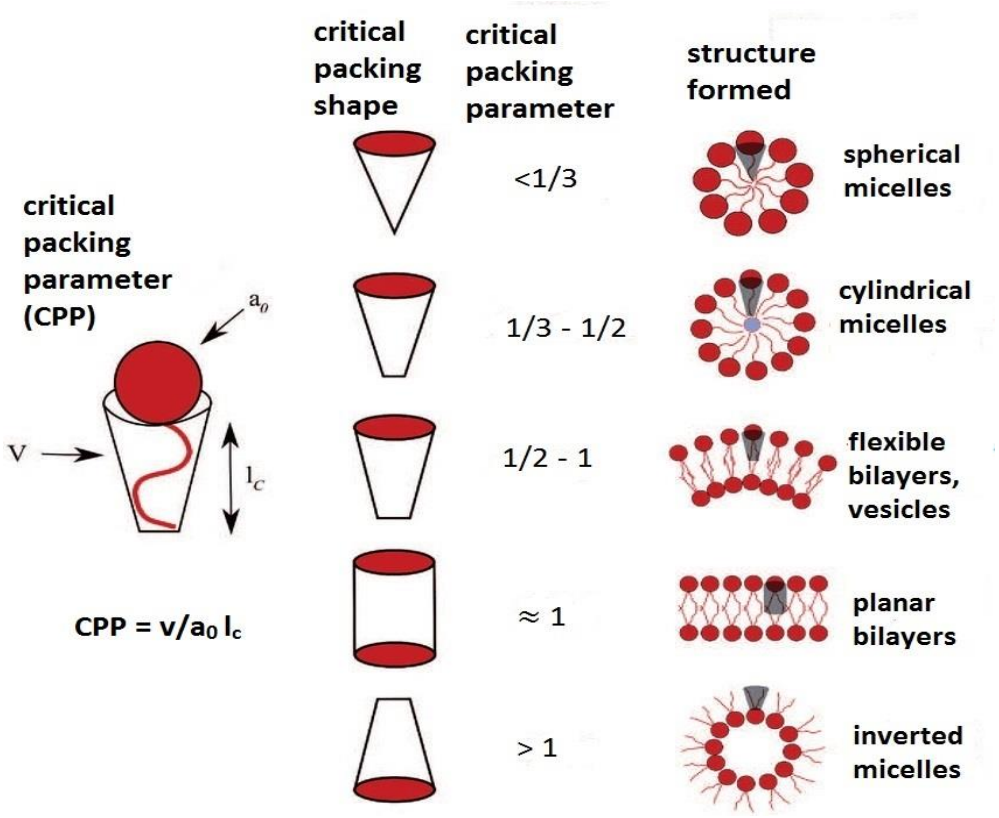

Fig. 1.3: Mean packing shapes of lipids and the structures they form [5]

Double-chained lipids have a cylindrical packing shape and hence form bilayers above CMC. The two hydrocarbon chains of these lipids increase their hydrophobicity and hence drastically lower their CMC (10^{-6} – 10^{-10} M). As a result the membranes remain intact even if there is a gross depletion of lipids from the bathing medium.

Based on the chemical structure of head groups, phospholipids are classified in to several groups, such as phosphatidyl choline (PC), phosphatidyl ethanolamine (PE), phosphatidyl serine (PS) and phosphatidyl glycerol (PG) [Fig. 1.4]. The head group moiety of the lipid can be either electrically charged or neutral. Neutral lipids which possess dipole moments in the aqueous medium are called zwitterionic. PCs and PEs are zwitterionic whereas PSs and PGs are electrically charged.

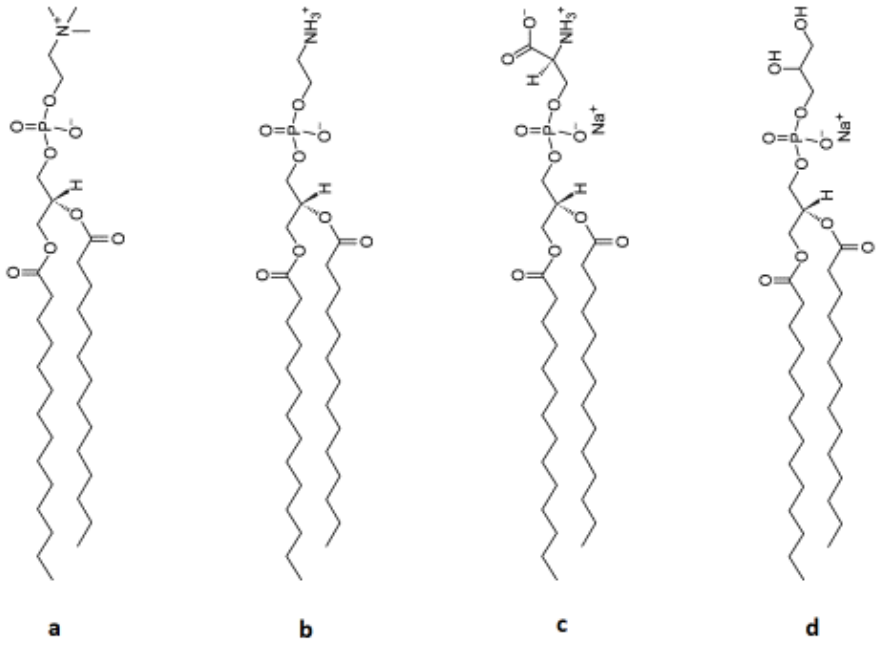

Fig. 1.4: Structure of (a) PC, (b) PE, (c) PS and (d) PG with two saturated hydrocarbon chains of 14 carbon each

1.2 Phase behaviour of lipid-water systems

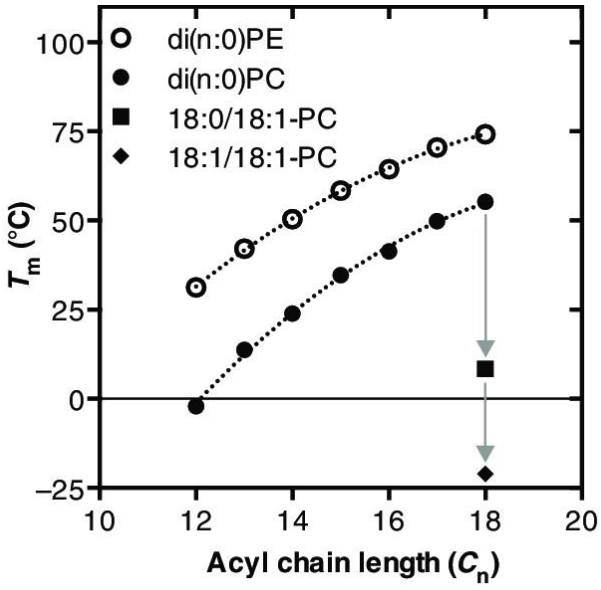

Fig. 1.5: Effect of acyl chain length and unsaturation on T_m of PC and PE lipids; n represents the number of carbon atoms in the chain [8].

Self-assembly of lipids in water results in the formation of periodic stacks of bilayers called lamellar phases. Depending upon the structure of lipid molecules involved and the temperature, they exhibit a variety of lamellar phases [7]. The temperature above which the lipid bilayers are in the fluid phase is called the main transition temperature or chain melting temperature T_m. T_m depends on the nature of the head group, hydrocarbon chain length and the degree of unsaturation. Variation of T_m of phospholipids with their acyl chain length and the number of double bonds is shown in Fig. 1.5 [8].

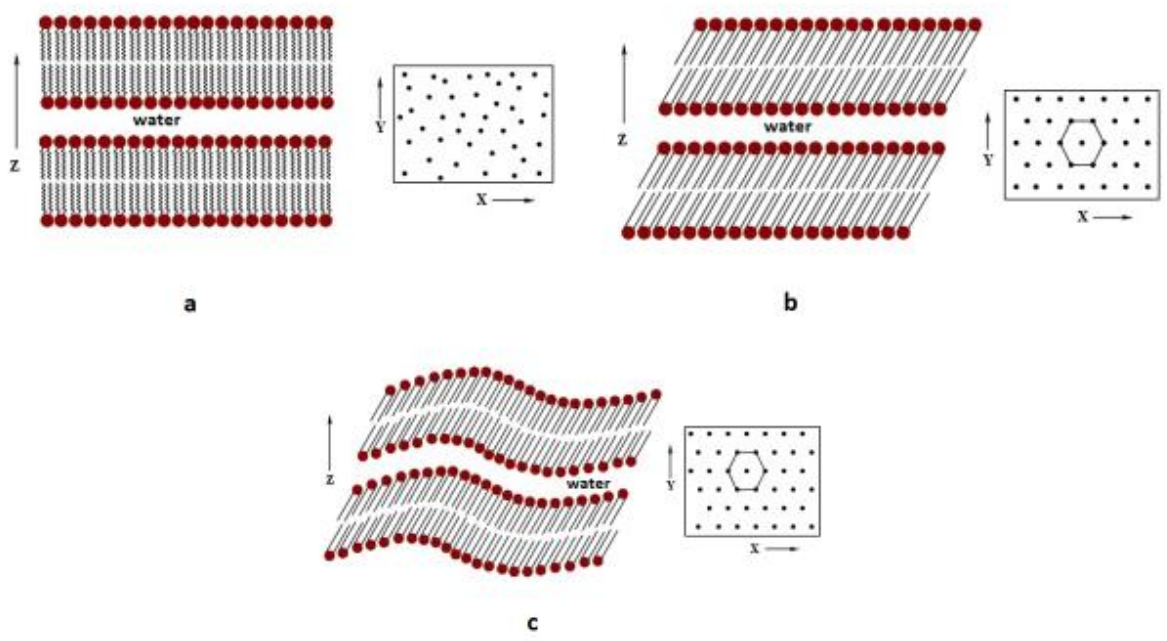

Fig. 1.6: Schematic representation of (a) L_α phase, (b) $L_{\beta'}$ phase and (c) $P_{\beta'}$ phase. Dots in the figure represent positions of chains of the lipid molecules in the plane of the bilayer [9].

Above T_m lipid bilayers are in the fluid state and the chains are completely molten and disordered. The corresponding lamellar phase is called the L_α phase. Below T_m, hydrocarbon chains are predominantly in the fully stretched all-trans conformation. This more ordered phase is called the gel phase. Depending on the size of the head group, there may be a chain tilt with respect to the bilayer normal. . If the hydrocarbon chains are parallel to the bilayer normal (zero tilt) as in PEs, the gel phase is called the L_β phase. Gel phases of lipids with larger head groups, such as PCs, have a non-zero chain tilt with respect to the bilayer normal. This is called the $L_{\beta'}$ phase. Some lipids exhibit an intermediate phase called the ripple phase ($P_{\beta'}$ phase) in between the L_α and $L_{\beta'}$ phases. $L_{\beta'}$ to $P_{\beta'}$ transition is called the pre-transition and $P_{\beta'}$ to L_α transition is

referred as the main or chain melting transition. The $P_{\beta'}$ phase is characterized by a two dimensional oblique lattice formed by height modulated bilayers. Schematic representations of different lamellar phases are shown in Fig. 1.6.

1.3 Sterols in membranes

Sterols are lipids belong to a class of polycyclic organic compounds. In a sterol molecule a small hydrocarbon chain is attached to the bulky steroid core. Cholesterol is an extremely important sterol present in eukaryotic membranes. Approximate lipid compositions of different plasma membranes are compared in table 1.1.

Percentage of total lipid by weight

Lipid	Liver Cell Plasma Membrane	Red Blood Cell Plasma Membrane	Myelin	Mitochondrion (Inner and Outer Membranes)	Endoplasmic Reticulum	E Coli Bacterium
Cholesterol	17	23	22	3	6	0
PE	7	18	15	25	17	70
PS	4	7	9	2	5	trace
PC	24	17	10	39	40	0
Sphingomyelin	19	18	8	0	5	0
Glycolipids	7	3	28	trace	trace	0
Others	22	13	8	21	27	30

Table 1.1: Approximate lipid compositions in different cell membranes [4]

The structure of cholesterol molecule is shown in Fig.1.7. It has a very small polar head in the form of a hydroxyl (-OH) group. The rigid steroid ring structure and the attached hydrocarbon chain together contribute its hydrophobic part. Cholesterol molecules orient themselves in a bilayer with their hydroxyl group at the membrane water interface and their plate like steroid ring and the hydrocarbon chains interact with the hydrocarbon chains of the phospholipids [Fig. 1.7]. Ergosterol, Sitosterol and Stigmasterol are also biologically important sterols with structural features similar to cholesterol.

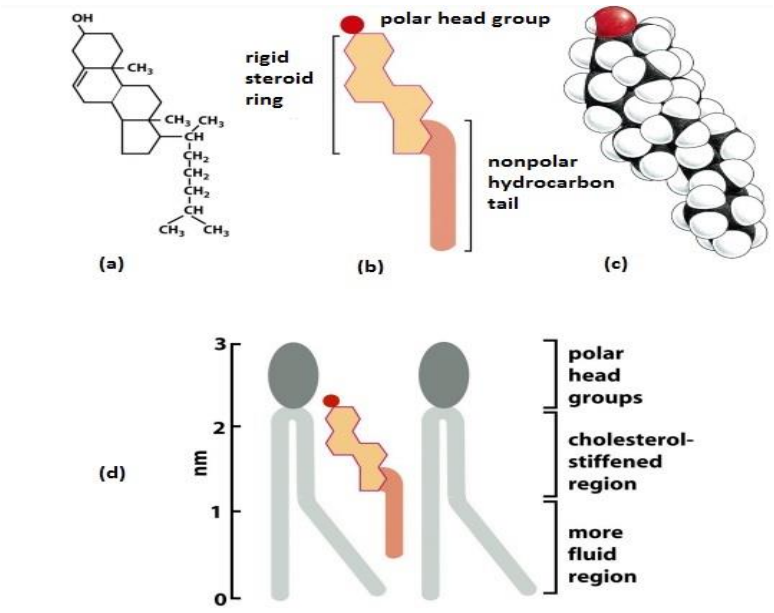

Fig. 1.7: Cholesterol structure (a) by formula, (b) by schematic diagram and (c) as a space filling model. (d) Schematic drawing of cholesterol interaction with two phospholipid molecules in a bilayer [2].

Cholesterol is known to influence membrane organization and function. Addition of cholesterol introduces various structural changes in the lipid bilayer; hence there are changes in physical properties and phase behavior. Cholesterol rich sub-micron domains called lipid rafts are hypothesized to be the functional platform for various cellular activities such as intracellular trafficking and signal transduction [10-12]. Model membrane systems including giant unilamellar vesicles (GUVs) are found to show optically resolvable coexisting fluid phases. Giant plasma membrane vesicles (GPMVs) derived from biological cells is found to segregate into micrometer scale domains [13]. Nano -sized domains in cell membranes have also been observed using super-resolution microscopy [14, 15]. However, formation and function of membrane rafts on unperturbed living cells are not yet fully established. An overarching conclusion that may be derived from all these studies is that nano scale liquid ordered domains are present in live cell membranes that influence cellular physiology, signaling and trafficking [16].

Micron sized domains are observed in model membranes consisting of ternary mixtures of cholesterol, a saturated lipid and an unsaturated lipid. This microscopic phase separation has been proposed to mimic the cholesterol-rich ‘rafts’ proposed to exist in cell membranes [17]. The above fluid- fluid coexistence is observed only below the chain melting temperature T_m of the

saturated lipid. Fluorescence microscopy images of domains observed in giant unilamellar vesicles (GUVs) are shown in Fig 1.8.

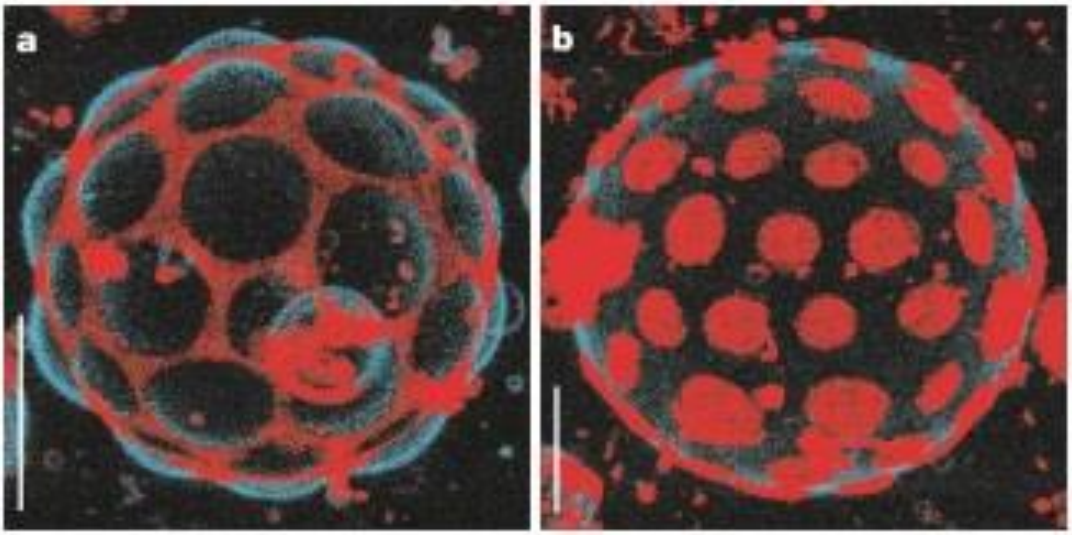

Fig 1.8: Two photon confocal fluorescence microscopy images of GUVs made from lipid mixtures composed of sphingomyelin, DOPC and cholesterol with $l_o - l_d$ coexistence labelled with perylene (blue) and with rho-DPPE (red) at 25^oC at two different compositions (a) and (b) [17].

1.4 Phase behavior of binary lipid-cholesterol membranes

Phase behavior of binary mixtures of phosphatidylcholine (PC) and cholesterol has been studied experimentally and theoretically. Experimental techniques include differential scanning calorimetry (DSC), spectroscopic techniques (E.g.: NMR, ESR, fluorescence), diffraction techniques (E.g.: X-ray, neutron scattering) and microscopy (E.g.: AFM, fluorescence, freeze fracture electron microscopy) [18-23]. Partial phase diagrams constructed using various experimental techniques and theoretical models are shown in Fig. 1.9.

Spectroscopic studies suggest the coexistence of a cholesterol-rich liquid ordered (l_o) phase and a cholesterol-poor liquid disordered (l_d) phase above chain melting temperature of the lipid [18]. Coexistence of two lamellar phases is observed only below the chain melting temperature in diffraction experiments and cholesterol is found to be miscible above T_m [20]. A detailed investigation on aligned bilayers of binary mixtures of PC- cholesterol systems using x-ray diffraction has confirmed the absence of phase separation above the chain melting temperature [25-28].

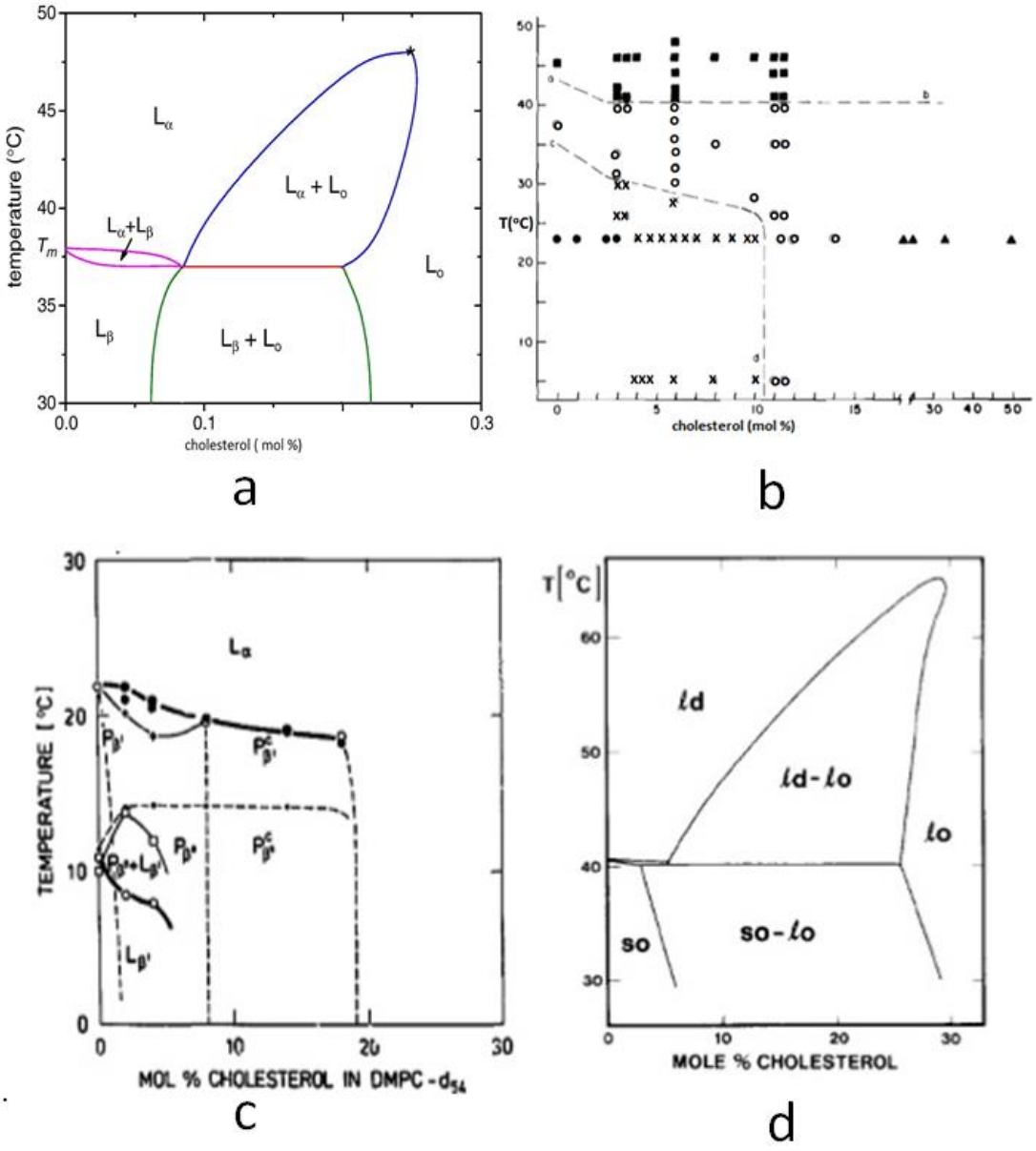

Fig. 1.9: Phase diagrams of PC- Cholesterol mixtures obtained from (a) NMR studies [24], (b) x ray scattering [20], (c) neutron scattering [22] and (d) theoretical model [23]. L_α or L_d represents cholesterol poor fluid phase, L_o represents cholesterol rich fluid phase, $L_{\beta'}$ or S_o represents gel phase and $P_{\beta'}$ represents ripple phase. In figure (b) 'x' represents coexistence region.

An important result obtained from the x-ray diffraction studies of saturated PC-cholesterol model membranes is the observation of a modulated phase called P_β phase at intermediate cholesterol concentrations [25]. This phase is found to be different from the normal

ripple phase ($P_{\beta'}$) exhibited between the gel phase and fluid phase. It is also observed that chain tilt is necessary to form the P_β phase. Concentration of cholesterol is found to be spatially non-uniform in the modulated phase. Phase diagram constructed for a binary system with modulated phase is shown in Fig. 1.10.

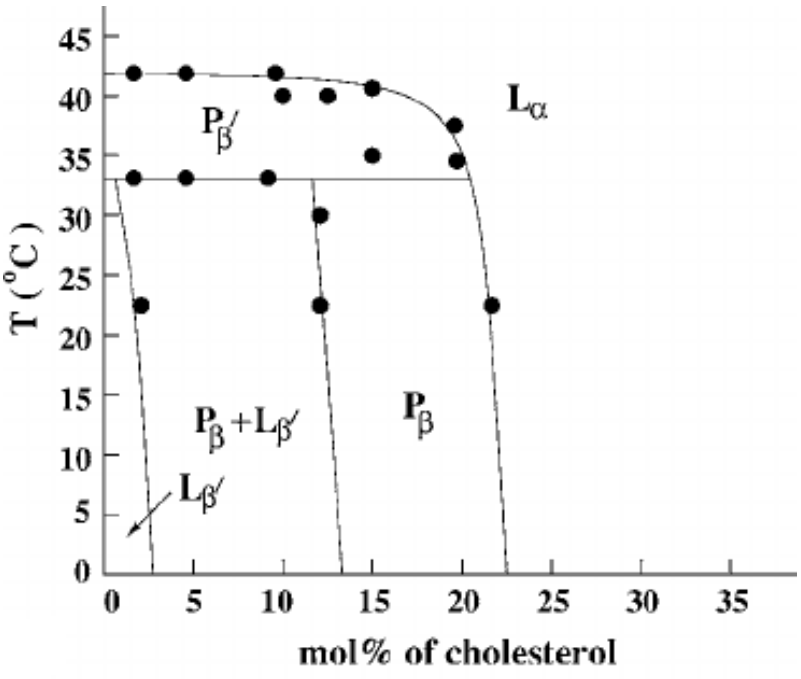

Fig 1.10: Partial phase diagram of binary mixture of DPPC and cholesterol obtained from x-ray scattering studies on aligned multilayers [25].

The difference in the phase behavior of binary lipid-cholesterol membranes deduced from spectroscopic studies and scattering experiments might be related to the spatially non-uniform concentration of cholesterol found in the P_β phase. It is very likely that this non-uniform distribution of cholesterol might persist as concentration fluctuations at higher temperatures which can be detected as two chain conformations over spectroscopic time scales. Diffraction experiments probe the system at much longer length and time scales and can detect only macroscopic phase separation leading to the formation of two lamellar phases. However number of subsequent investigations has clearly shown that no fluid- fluid coexistence above T_m exist in binary membrane systems of PC and cholesterol [25-28].

1.5 Phase behaviour of ternary raft mixtures

Fluid- fluid coexistence is observed in model membranes consisting of ternary mixtures of cholesterol, a saturated lipid and an unsaturated lipid, which leads to the formation of micron-sized membrane domains. These domains disappear when the temperature is above the chain melting temperature of the saturated lipid. A number of ternary lipid mixtures have been used to mimic the raft domains proposed to exist in biological membranes. The main role of cholesterol is to convert the fluid- gel phase separation observed in binary lipid mixtures [29] to a fluid-fluid coexistence.

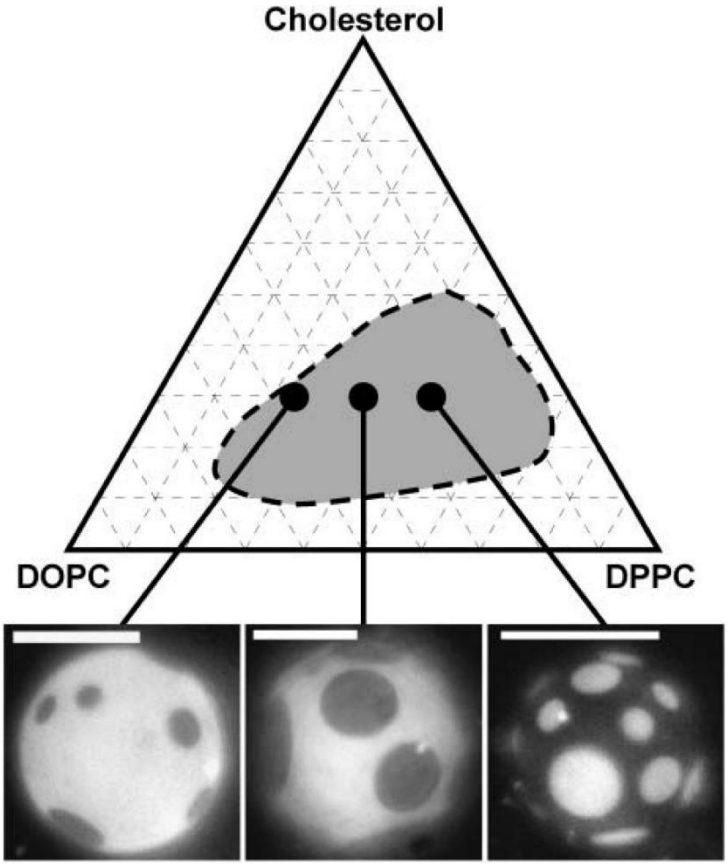

Fig. 1.11: Miscibility phase boundary of a ternary membrane system of DOPC, DPPC and cholesterol (top) and fluorescence microscopy images of GUVs at 25^{o}C for a cholesterol concentration of 30 mol% in lipid mixtures with DOPC:DPPC in the ratio 2:1, 1:1 and 1:2 (from left to right) [30].

Fluid- fluid coexistence in ternary mixtures has been studied extensively using a variety of experimental techniques [9, 17, 30-34]. Phase diagram of DOPC-DPPC-cholesterol ternary mixtures determined using fluorescent microscopy technique at 25^{o}C is shown in Fig. 1.11 [30].

The preferential partitioning of fluorescent dye into one of the two fluid phases has been used to visualize phase separation. Fig. 1.12 shows x-ray diffraction pattern from aligned multilayers of equimolar DPPC, DOPC and cholesterol with fluid – fluid coexistence at 10^{o}C and a single fluid phase at 35^{o}C [9]. Two sets of d-spacing corresponding to l_o-l_d coexistence are observed at the lower temperature.

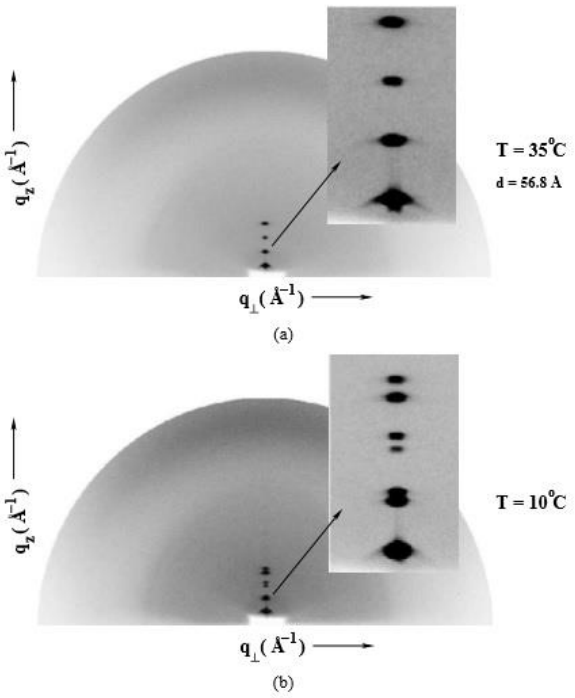

Fig. 1.12: Diffraction pattern from aligned multilayers of equimolar DPPC,DOPC and cholesterol mixture showing (a) single fluid phase at 35^{o}C and (b) fluid-fluid coexistence at 10^{o}C [9].

1.6 Influence of other sterols on membranes

Apart from cholesterol there are many other biologically relevant sterols. The major sterol found in the plasma membrane of almost all higher order eukaryotes is cholesterol, whereas ergosterol is found in the membranes of lower order eukaryotes like yeasts and fungi [35]. Phytosterols are important structural components of plant membranes. β- Sitosterol and stigmasterol are common phytosterols [36]. Lanosterol is the common precursor of cholesterol and ergosterol in their biochemical synthesis pathways [37]. Structures of these sterols are shown in Fig. 1.13. Influence of these sterols on the phase behavior of model membranes has also been studied using a variety of techniques.

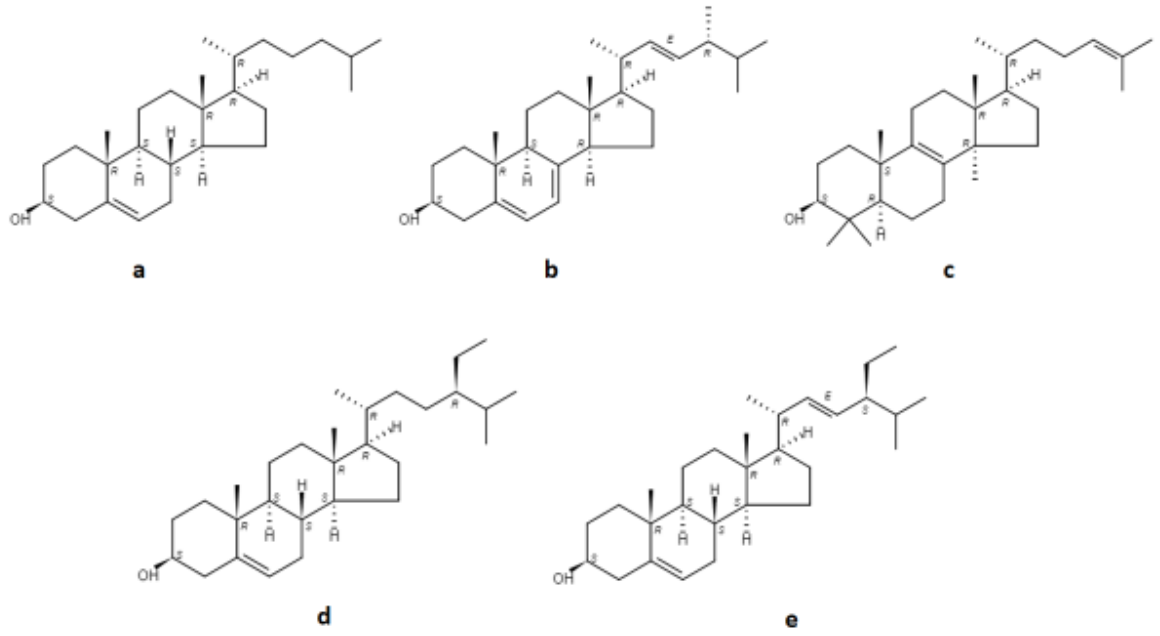

Fig. 1.13 : Chemical structure of (a) cholesterol, (b) ergosterol, (c) lanosterol, (d) β- sitosterol and (e) stigmasterol.

A partial phase diagram of DPPC – ergosterol mixtures constructed using NMR and calorimetric studies shows two phase coexistence above the chain melting temperature [38]. X-ray diffraction studies on aligned multilayers of DPPC and ergosterol do not show two phase coexistence above T_m [39] and the corresponding phase diagram is shown in Fig.1.14. The discrepancy arises due to the difference in the length and time scales at which the two techniques (spectroscopic and scattering) probe the system as explained in the case of DPPC- cholesterol model membranes. Lanosterol is found to show similar phase behavior with DPPC as cholesterol [39]. Phase behavior of binary mixtures of phytosterols with DPPC is also observed to be similar to that of DPPC- cholesterol membranes [37]. It can be concluded that binary mixtures of the above sterols and PC do not show fluid- fluid coexistence above the chain melting temperature.

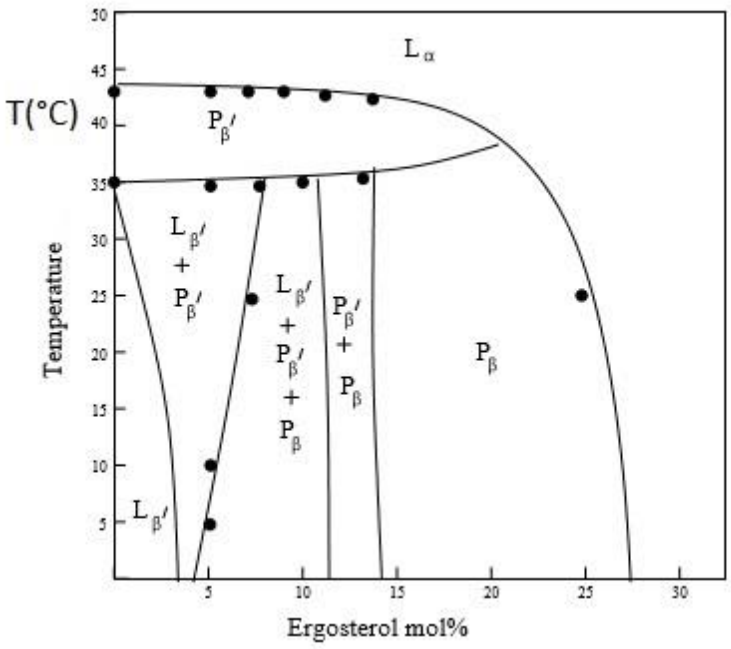

Fig. 1.14: Partial phase diagram of DPPC- ergosterol mixtures at 98% RH obtained from x-ray scattering studies on aligned multilayers [39].

1.7 Oxysterols in membrane

Oxysterols are oxygenated derivatives of cholesterol. They are either intermediates or end products of cholesterol excretion pathways. They are found only in trace amounts in biological systems, accompanied with bulk amount of cholesterol (10^4 to 10^6 fold). But in certain cases, relative cholesterol concentration may be lower than 10^3 [40].

Compared to cholesterol, oxysterols contain an additional oxygen moiety which makes them more hydrophilic. Oxysterols have poorer membrane packing ability compared to cholesterol due to significant changes in their 3D shape due to oxygen substitution. They are produced by oxidation of cholesterol either on the steroid ring or on the lateral chain. The mechanism of oxidation is either enzymatic or non-enzymatic (auto oxidation). Fig. 1.15 represents production of oxysterols by cholesterol oxygenation reactions enzymatically by different cytochrome P450 species and non-enzymatically in the presence of a reactive oxygen species (ROS) [41]. As a result oxysterols are transferred between membranes at rates orders of magnitude faster than cholesterol [42, 43].

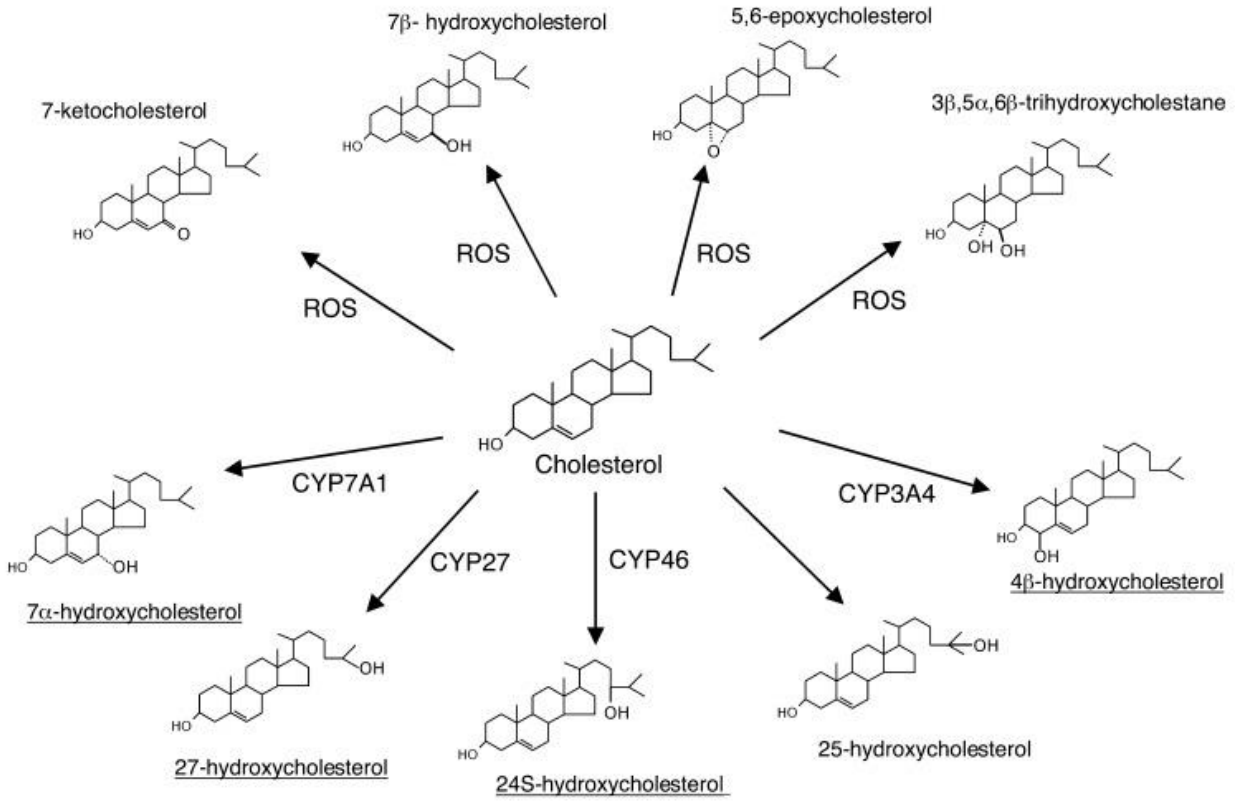

Fig. 1.15: Production of oxysterols by oxygenation reactions mediated by different cytochrome P-450 species or non-enzymatically in the presence of reactive oxygen species (ROS) [41].

Formation of oxysterol is the mechanism of elimination of excess cholesterol in some of the cells and hence oxysterols act as transport forms of cholesterol. Oxysterols can also function as regulators of cholesterol homoeostasis. Role of oxysterols in patho-physiological mechanisms including atherosclerosis, lung diseases, degenerative diseases such as Alzheimer's diseases etc. are also well documented [44-47]. Recently it has been shown that 25-hydroxycholesterol is a potent inhibitor of SARS-Cov-2 [48].

Oxysterol can function as modifiers of biophysical properties of membranes depending upon their conformation in the membrane [49]. Depending on the chemical nature and the location of the oxygen substitution, they can either promote or inhibit 'raft' formation [49]. Effect of ring substituted oxysterols on the phase behavior of DPPC model membranes is found to be similar to that of cholesterol [50]. Orientation dependent domain formation in membranes containing side chain substituted sterols (25-hydroxysterol or 27-hydroxysterol) is identified as the underlying mechanism for oxysterol induced cellular apoptosis [51]. Coexistence of two fluid

phases above the chain melting temperature has been observed in DMPC-25hydroxycholesterol, DMPC-27hydroxycholesterol and DPPC-27hydroxycholesterol membranes in x-ray scattering studies [52]. Computer simulations on POPC-25hydroxycholesterol, DMPC-27hydroxycholesterol and DPPC-25hydroxycholesterol also show three different possible orientations of oxysterols in the membrane with comparable energies [53-56]. A detailed study of different lipid-oxysterol binary membrane systems is presented in this thesis.

1.8 Experimental Techniques

1.8.1 Vesicle Preparation

Self-assembled structures in the form of single bilayer shells of diameter 10- 100 μm are called Giant Unilamellar Vesicles (GUVs).

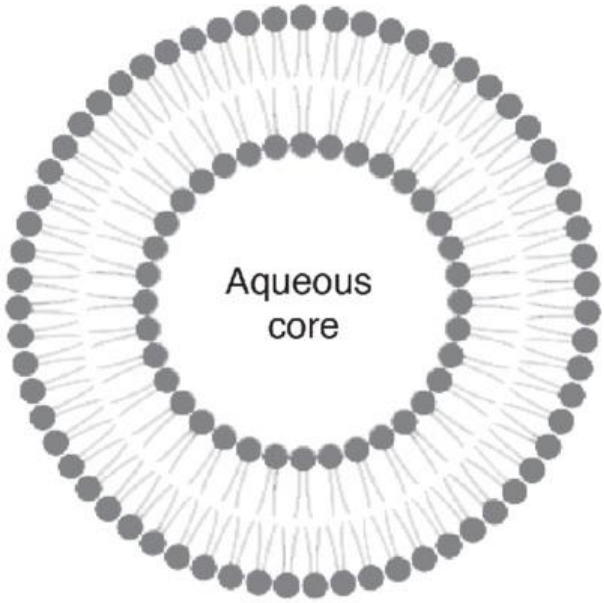

Fig. 1.16: Schematic diagram of a unilamellar vesicle

There are various methods for GUV preparation [57-59] and the most common method is electro formation introduced by Angelova et al [59-61]. In this method a dry lipid film is allowed to swell in excess water under an AC electric field. During this process temperature of the sample should be above the chain melting temperature of the lipid, since the process involves bending of

the membrane to form vesicles; so the lipid bilayers should be in the fluid phase, where the bilayers are very flexible.

In the electro formation method, a small AC voltage of 1-3 volt at a frequency of about 10 Hz is applied for 2-3 hours on a lipid film in excess water. We have designed an electro formation chamber whose schematic diagram is shown in Fig. 1.17. It consists of two platinum wires of diameter 0.5 mm each electrically isolated by a separation of about 1 mm. The chamber consists of a hollow metallic block made of copper with an inlet and outlet for water circulation. The whole chamber is kept in a Teflon casing for better thermal stability.

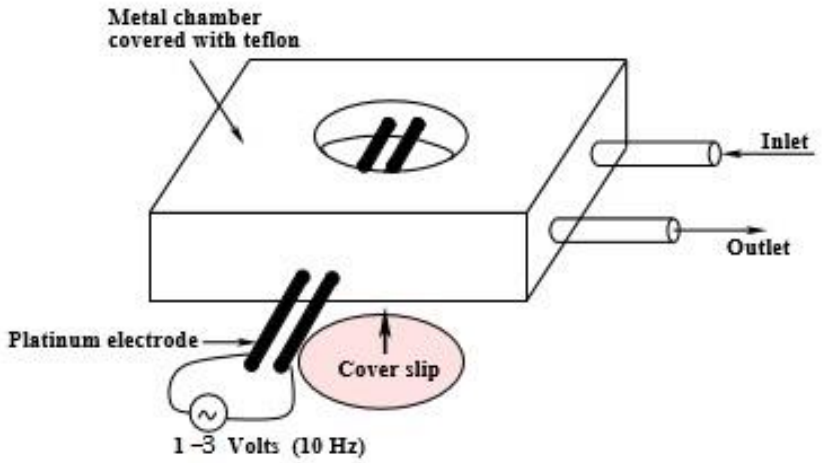

Fig. 1.17: A schematic diagram of electro formation chamber used for GUV preparation [9].The inlet and outlet are for controlling the temperature through water circulation.

Stock solutions of lipid- sterol mixtures are made at 0.5 mg/ml in chloroform and 0.1 mol% of a fluorescent dye is added. 1-2 μL of the stock solution is coated uniformly on each platinum electrode using a Hamilton syringe. The dried lipid film is hydrated by 0.5 ml of Millipore water and then an AC field is applied by maintaining the temperature of the water above T_m. After 2 hours the AC field is switched off. Vesicles formed near the platinum wires detach from the wires and coalesce to form GUVs. GUVs are transferred to an observation chamber for observation under a fluorescence microscope.

1.8.2 Multilayer Preparation

Homogeneous and substrate aligned multilayers of lipid- sterol mixtures are prepared for x-ray studies and microscopy observations by spreading the mixture on a solid surface [62,63]. The solution in chloroform is deposited on the substrate using a micro dispenser, allowing it to spread spontaneously and the solvent to evaporate. Multilayers are formed when the completely dried film is kept in a 100% relative humid environment above the chain melting transition temperature of the lipid. A slow and gradual hydration leads to the formation of well oriented multilayers.

Stock solutions of lipids and sterols are prepared in chloroform at a concentration of 5 mg/ml. For microscopy observations a fluorescent dye is added at a concentration of 0.1 mol% of the total lipid plus sterol concentration. 1-3 μL of the stock solution is spread on a freshly cleaned glass slide and chloroform is allowed to evaporate. Incubation of the dry plaques of the sample in a sealed chamber containing a water reservoir at a temperature above the chain melting temperature of the lipid for two days leads to multilayer formation. The multilayers so formed are taken for observations under a fluorescence microscope.

1.8.3 Fluorescence Microscopy

Fluorophores are a class of molecules which emit longer wavelength radiation than the excitation wavelength. This fluorescence emission is the underlying principle of fluorescence microscopy. Here the sample is labelled by a suitable fluorophore. Typical fluorescence life time is about 10 ns. There is a non-radiative transition before coming to ground state resulting in a lower energy (longer wavelength) of emitted photons. Emitted photons are detected using a charge coupled device (CCD), photodiode or photo multiplier tube (PMT). Mercury lamps or metal halide lamps are used as excitation sources [64, 65].

A schematic diagram of a fluorescence microscope is shown Fig. 1.18. Here the sample is illuminated by a band of exciting wavelengths isolated by an excitation filter. Subsequently the excitation radiation is removed from the image forming radiation by a barrier filter (emission filter). Only fluorescent radiation is allowed to form the image. Fluorescent radiation is of very

low intensity; hence the excitation radiation has to be sufficiently intense and has to be effectively excluded before observation.

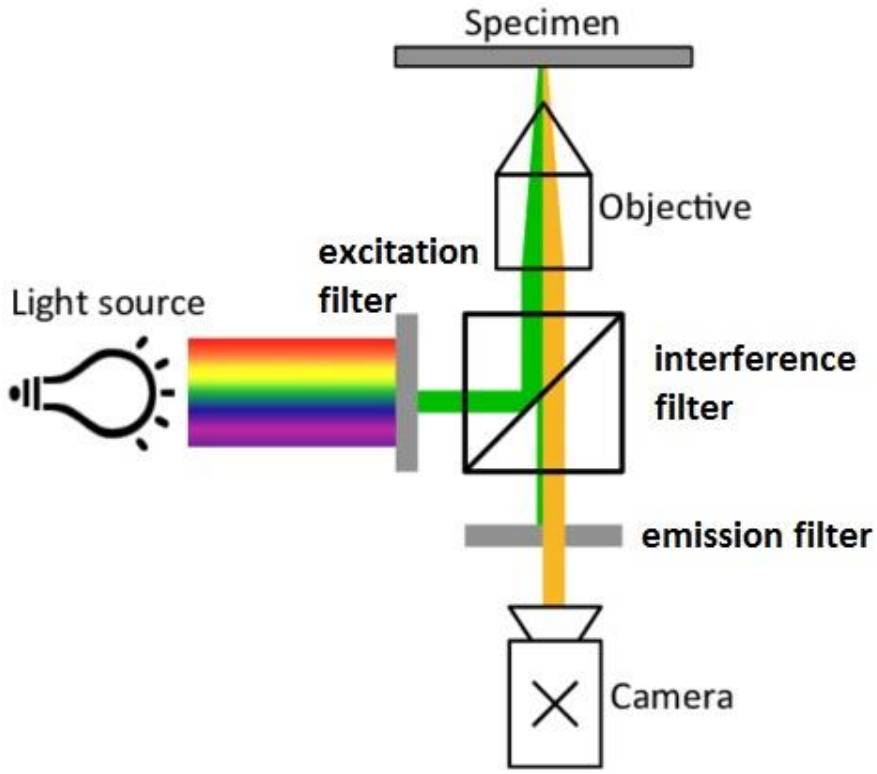

Fig. 1.18: Schematic diagram of fluorescence microscope

In a typical fluorescence microscope illumination of the sample and the collection of the emitted light from the sample happens through the same objective. This arrangement is known as epi-fluorescence. A chromatic beam splitter (interference filter) is used for this purpose to transmit fluorescent wavelength and reflect the excited wavelength selectively.

Fluorophores are selected depending upon the nature of the sample and the availability of the excitation source and filters. Rhodamine DHPE is a head group labelled lipid probe which partitions effectively into Liquid disordered (L_d) phase. We have used rhodamine DHPE fluorescent dye for our experiments whose structure and spectra are shown in Fig. 1.19.

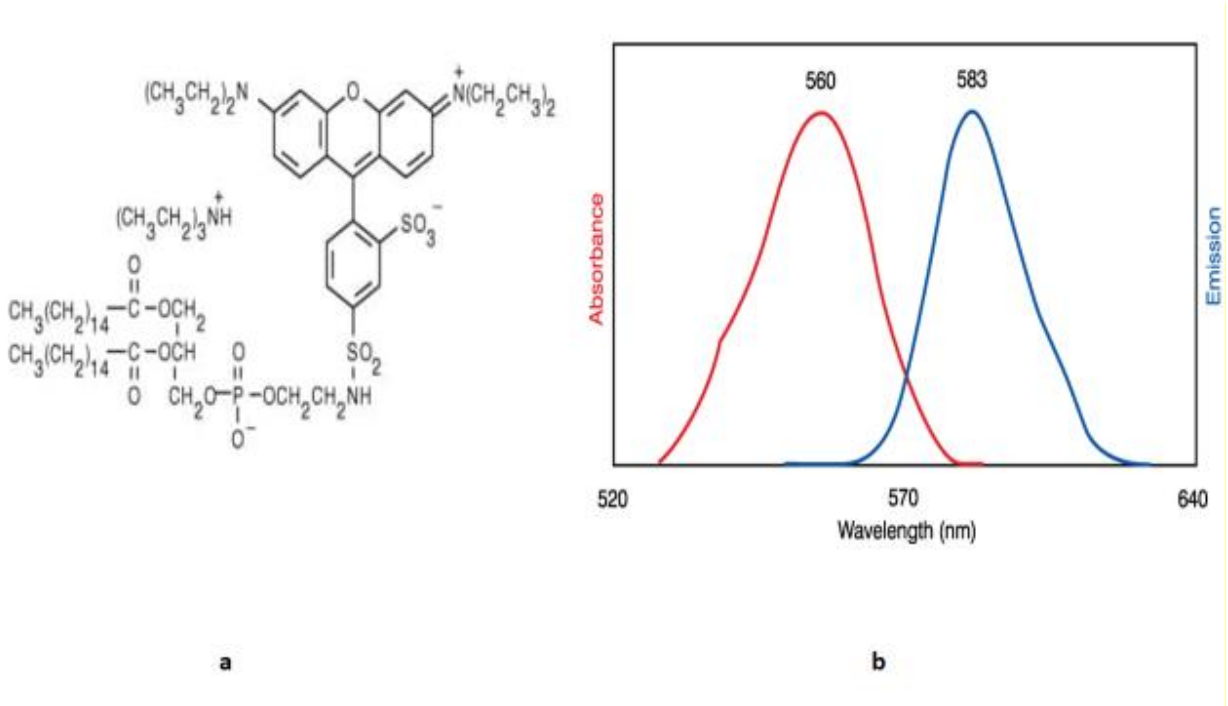

Fig. 1.19: (a) chemical structure of rhodamine DHPE and (b) and its spectrum

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CHAPTER 2

Influence of 25-hydroxycholesterol and 27-hydroxycholesterol on the phase behaviour of DMPC model membranes

2.1 Introduction

Dimyristoylphosphatidylcholine (DMPC) is a zwitterionic glycerophospholipid composed of saturated and symmetric hydrocarbon tails with 14 carbon atoms. As the main transition temperature of DMPC (T_m= 24^{o}C) is close to room temperature, it is a very convenient system to investigate the phase behavior of lipid-sterol binary mixtures. Pure DMPC exhibits three different lamellar phases consisting of stacks of bilayers separated by water at high hydration: The fluid phase (L_α) above the main transition temperature T_m, the gel phase ($L_{\beta'}$) below the pre-transition temperature (around 15^{o}C) and the ripple phase ($P_{\beta'}$) in between [1]. These phases can be easily identified based on their characteristic X-ray diffraction patterns [2].

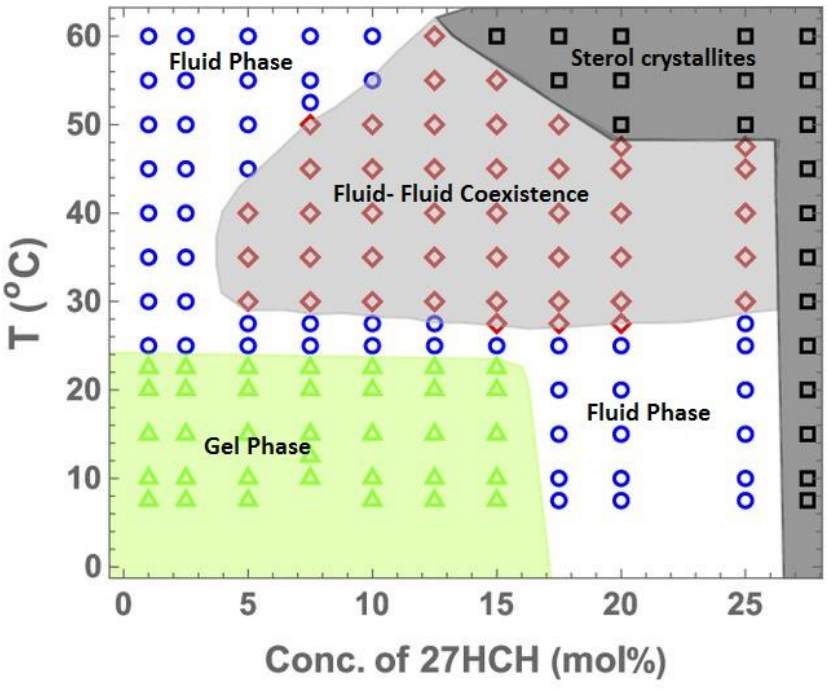


Fig 2.1: Partial phase diagram of DMPC-27HCH binary membrane at 99% RH determined from SAXS data from aligned multilayer samples [11].

Phase behavior of binary mixtures of DMPC with 25-hydroxycholesterol (25HCH) and 27-hydroxycholesterol (27HCH) has been determined from small-angle and wide angle X-ray scattering (SAXS, WAXS) experiments on aligned multilayers [3]. . A partial temperature-composition phase diagram of aligned DMPC-27HCH multilayers deduced from SAXS and WAXS data at 98 ±2% relative humidity is shown in Fig 2.1. Here we shall confine our discussion to the phase behavior at temperatures above T_m. Up to about 15 mol% of 27HCH, T_m remains almost constant, but it drops very sharply slightly above this concentration. The most interesting part of the phase diagram is the two phase co-existence region seen above T_m, the right boundary of which is preempted by the solubility limit of 27HCH.

The lamellar phase is made up of a periodic stack of bilayers separated by water, and hence it has a one-dimensional spatial periodicity along the local normal to the bilayer. The lamellar periodicity (d) is the sum of the thickness of the bilayer and that of the water layer separating adjacent bilayers. The SAXS pattern of such a one-dimensional crystal consists of a set of peaks, which corresponds to different orders of Bragg reflections arising from the lamellar periodicity, d. SAXS patterns shown in Fig 2.2(a) and 2.2(c) depict this situation. In the case of two coexisting lamellar phases, two sets of reflections are observed due to their distinct lamellar periodicities. The SAXS pattern shown in Fig 2.2(b) corresponds to this scenario.

WAXS pattern of the lamellar phase has information regarding the organization of the lipid chains in the plane of the bilayer. In the case of the fluid lamellar phase, the in-plane order within the bilayer is liquid-like, and the WAXS pattern contains a broad peak. In the gel phase, which occurs below the chain-melting transition temperature, the in-plane order is crystalline and the WAXS pattern of this phase contains one to three sharp peaks; the number of peaks depending on the magnitude and direction of the tilt of the lipid chains with respect the chain lattice. We have a diffuse peak which is the characteristic of a fluid phase. Hence within this region two fluid lamellar phases coexist.

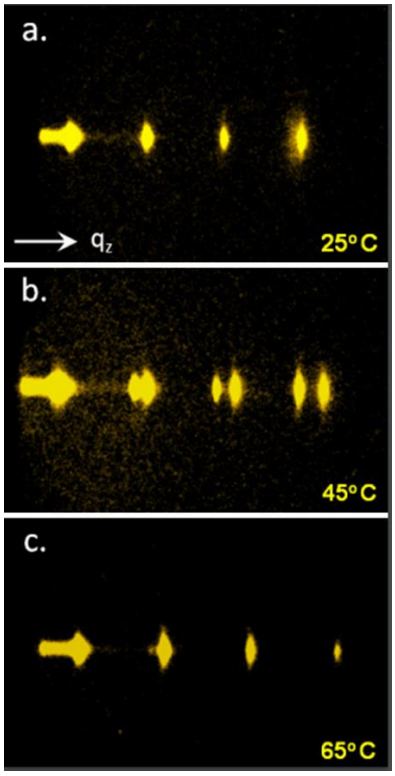


Fig 2.2: SAXS patterns of aligned DMPC-multilayers with 10 mol % 27HCH, (a) in the low-temperature single phase at 25^{o}C, (b) in the Intermediate two-phase region at 45^{o}C showing two sets of lamellar peaks and (c) in the high temperature single phase at 65^{o}C; bilayer normal is along z indicated by the arrow in (a) [3].

The low temperature boundary of the co-existence region is rather flat and has a minimum at around 17.5 mol% of 27HCH and at T=27.5^{o}C, which is slightly above T_m of the membrane. Hence there is a narrow region of the fluid phase intervening between the fluid-fluid coexistence region and the gel phase of the membrane. Influence of 25HCH on DMPC membranes is very similar [Fig. 2.3].

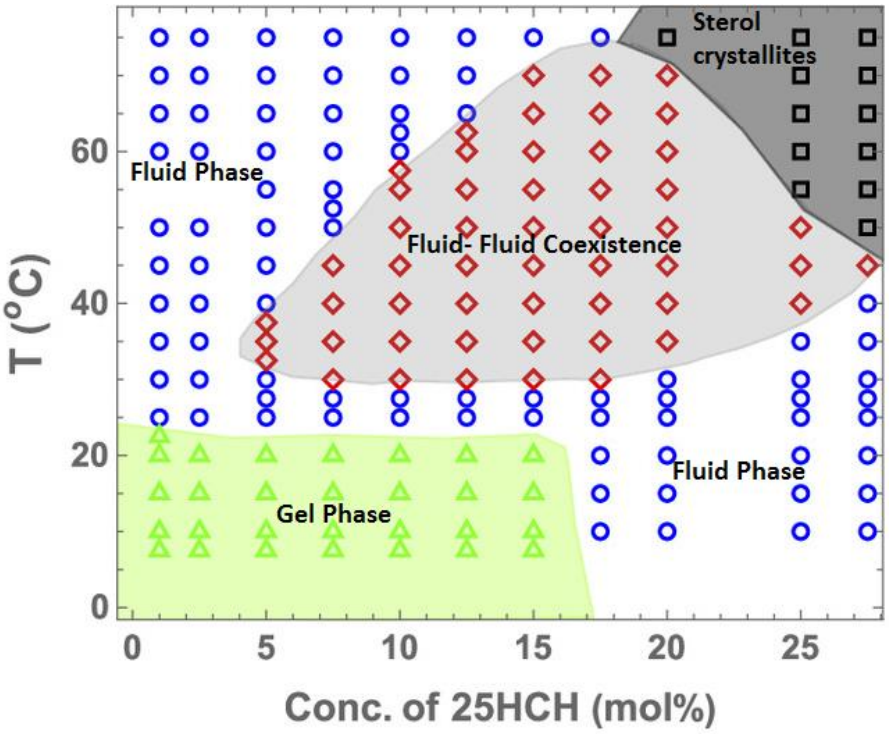


Fig 2.3: Partial phase diagram of DMPC-25HCH binary membrane at 99% RH determined from SAXS data from aligned multilayer samples [11].

The lamellar phases exhibited by lipid-water systems have a one dimensional periodicity along the bilayer normal. The lamellar periodicity (d) is the sum of the bilayer thickness and the water layer thickness. The two sets of d-spacing correspond to the coexistence of two fluid phases. One of the fluid phases in the coexistence region has a lamellar periodicity (d) comparable to that of the fluid phase observed at higher sterol concentrations, at the same temperature. The sterol-rich phase has a higher lamellar periodicity in comparison to the other coexisting phase. On cooling from high temperature fluid phase, the sterol-rich phase appears within the coexistence region. The difference in d-spacing of the two coexisting phases is about 0.5 nm. This difference is found to be almost constant irrespective of the sterol content of the membrane. Trans bilayer electron density profiles of the two coexisting fluid phases, deduced from the diffraction data using methods described in the literature [4] show that the bilayer thickness (d_{pp}) of the two phases also differ by a similar amount, with the sterol-rich phase being thicker.

On increasing the sterol concentration, the second lamellar phase with a higher d appears on crossing the low concentration boundary of the coexistence region. The amount of this lamellar phase increases gradually on increasing the sterol concentration. It is not always possible to reach the single fluid phase at high sterol content, since 27HCH often precipitates out of the membranes giving rise to additional sharp peaks in the diffraction patterns. Analogous behavior is observed in the case of DMPC-25HCH membranes.

X ray diffraction gives structural information of correlated bilayers whereas fluorescence microscopy provides a method of direct visualization of phase separation in GUVs or multilayers. We can track the real time features of the system by labeling the coexisting phases of the sample with fluorescent markers.

In this chapter we describe fluorescence microscopy studies on binary mixtures of DMPC with 25HCH and 27HCH. Fluorescence microscopy observations on GUVs made from DMPC-27HCH and DMPC-25HCH membranes confirm the presence of a two phase region above T_m. Even though GUVs are very convenient model membrane morphology, there is a possible non-uniformity in their composition. So phase boundaries are found from supported multilayers. Multilayers provide uniformity in the composition. DMPC-27HCH and DMPC-25HCH multilayers clearly show fluid-fluid coexistence above T_m. Furthermore, the domains observed in this temperature range are found to coalesce, confirming their fluid nature.

2.2 Materials of Study

1, 2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 25-hydroxycholesterol (25HCH) and 27-hydroxycholesterol (27HCH) were obtained from Avanti Polar Lipids in powder form. Stock solutions of lipid and oxysterols were prepared in chloroform (HPLC grade) obtained from Sigma-Aldrich. Rhodamine-DHPE was obtained from Invitrogen bioservices.

2.3 Experimental Results

A systematic investigation on mixtures of 25HCH or 27HCH with DMPC was carried out using fluorescence microscopy. Presence of the two phase region above T_m is confirmed by fluorescence microscopy observations on GUVs made from DMPC-25HCH and DMPC-27HCH membranes. In order to avoid possible non uniformity in the composition of GUVs prepared from binary lipid mixtures, we have used supported multilayers for detecting phase boundaries.

Mixtures with sterol concentrations of 2.5, 5, 7.5, 10, 15, 20, 25, 27.5 and 30 mol% were studied. Fluorescence images clearly show phase separation above T_m. Domains are found to coalescence, confirming their fluid nature. On the basis of fluorescence microscopy observations partial phase diagrams of the two binary systems have been drawn. Phase boundaries were determined both on heating and cooling, but always maintaining the samples at temperatures above T_m.

2.3.1 Phase behavior of DMPC-25HCH multilayer systems

At low sterol concentration (2.5 mol%), there is no significant change in the phase behavior of supported multilayers of DMPC, compared to that of the pure lipid. At 5 mol% concentration of 25HCH, we observe the two-phase coexistence region for a temperature range of 13^{o}C above the main transition temperature. At 10 mol% the fluid-fluid coexistence is observed from 30^{o}C to 49^{o}C [Fig 2.4].

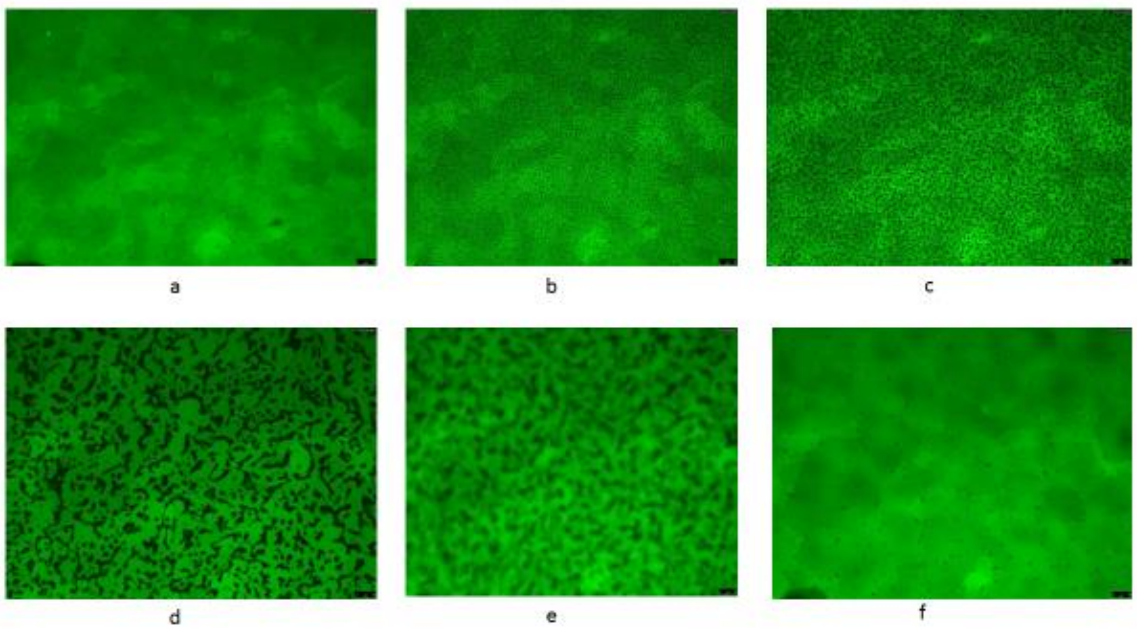


Fig 2.4: Fluorescence microscopy images of supported multilayers of DMPC containing 10 mol% 25HCH at 100% relative humidity: fluid phase at 28^{o}C (a), appearance and coalescence of fluid-fluid coexistence at 30^{o}C (b, c, d), at 48^{o}C (e) and fluid phase at 49^{o}C (f) (image size: 1.2 mm x 0.9 mm). In (b) to (e), the bright region corresponds to the sterol-poor phase and the dark region to the sterol-rich phase. Scale bar is 100 µm.

Appearance and coalescence of fluid-fluid domains in supported multilayers of DMPC with 20 mol% of 25HCH are shown in Fig 2.5. Coalescence of domains with respect to time is also studied by keeping temperature constant [Fig 2.6].

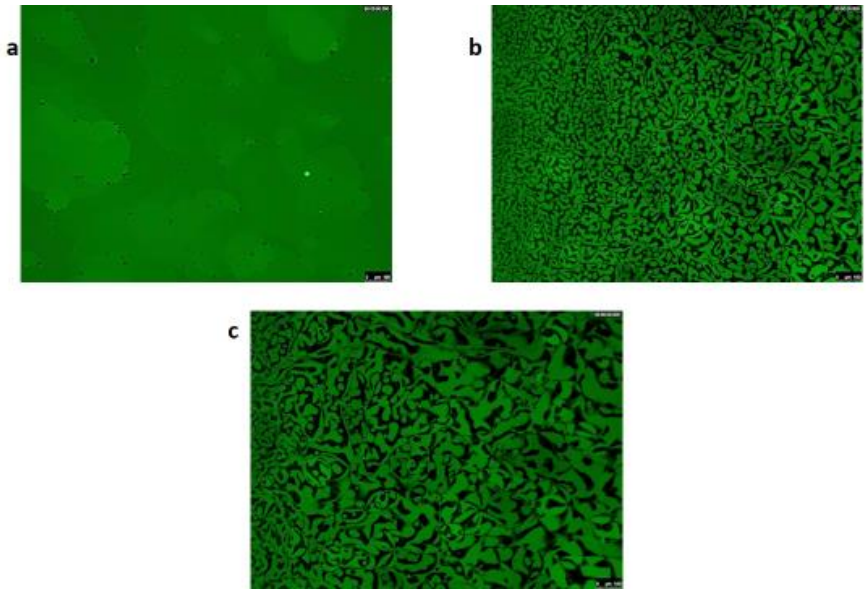


Fig 2.5: Fluorescence microscopy images of supported multilayers of DMPC containing 20 mol% 25HCH at 100% relative humidity: fluid phase at 28^{o}C (a), appearance and coalescence of fluid-fluid coexistence at 30^{o}C (b, c) (image size: 1.2 mm x 0.9 mm).Scale bar is 100 µm.

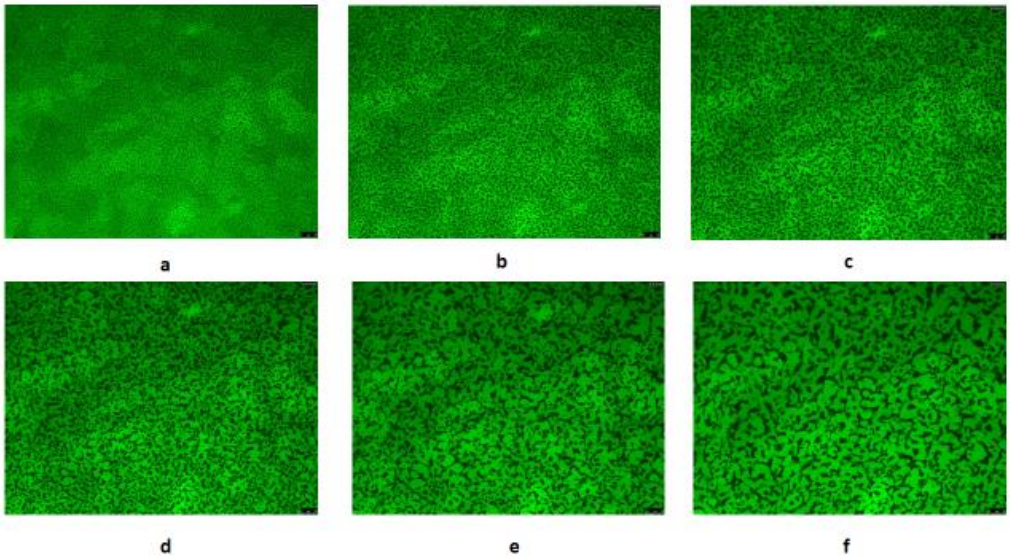
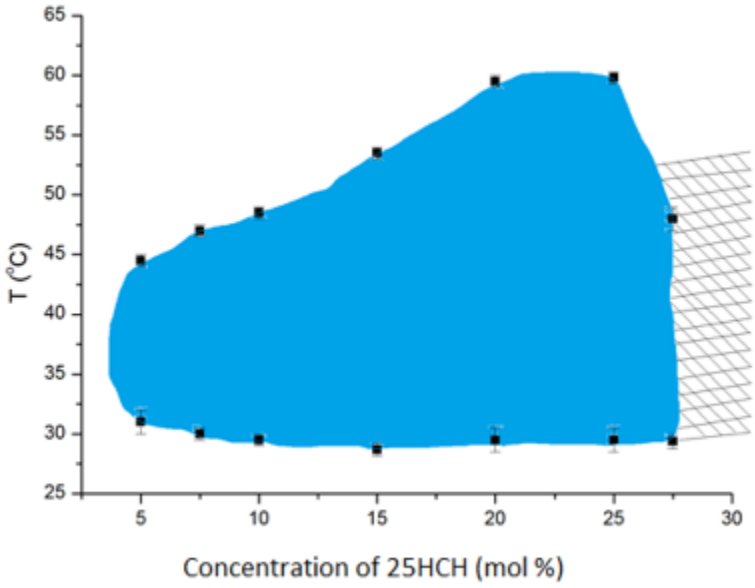


Fig 2.6: Coalescence of domains of DMPC multilayer with 10 mol% of 25HCH at 31^{o}C (a) t=0 s, (b) t= 15 s, (c) t= 31 s, (d) t= 63 s, (e) 143 s and (f) 284 s (image size: 1.2 mm x 0.9 mm). Scale bar is 100 µm.

A partial phase diagram has been constructed from the experimental data [Fig 2.7]. At higher temperatures the regime of coexistence ends, giving a single fluid phase. The extent of the two-phase region is observed to initially increase gradually with sterol concentration, with the high temperature single phase appearing only at about 60^{o}C at a sterol concentration of 25 mol %. Further increase in the sterol concentration to 27.5 mol% results in diminishing of the temperature range of the two-phase coexistence region. Measurements could not be made beyond 27.5 mol% due to the formation of crystallites of 25HCH. The low temperature boundary of the coexistence region is almost flat from 5 mol % to 27.5 mol % of sterol concentration.



CONCENTRATION OF 25HCH (mol %)	T min (°C)	T max (°C)
5	31.0±1.0	44.5±0.5
7.5	30.0±0.5	47.0±0.5
10	29.5±0.5	48.5±0.5
15	28.7±0.5	53.5±0.5
20	29.5±1.0	59.5±0.5
25	29.5±1.0	59.8±0.5
27.5	29.4±0.6	48.0±1.0

Fig 2.7: Partial phase diagram of DMPC-25HCH constructed from the fluorescence Microscopy observations on supported multilayers. The fluid-fluid coexistence region is shaded in blue.

At high temperature, the single phase appears bright since the dye is uniformly dispersed in it. On cooling across the high temperature boundary, the sterol rich phase nucleates as dark domains. With time these domains coalesce. After a long time we see dark domains (minority phase) dispensed in a bright background (majority phase) at lower sterol concentrations and bright domains (minority phase) dispensed in a dark background (majority phase) at high sterol concentrations, as shown in Fig. 2.8.

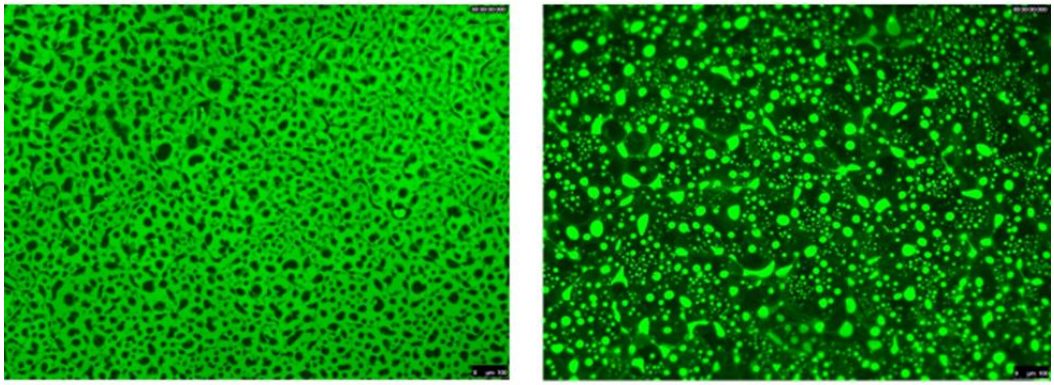


Fig. 2.8: Fluorescence microscopy images of supported multilayers of DMPC at 40^{o}C containing (a) 15 mol% of 25HCH showing dark domains in bright background and (b)27.5 mol% of 25HCH showing bright domains in dark background (scale bar : 100 μm).

2.3.2 Phase behavior of DMPC-27HCH multilayer systems

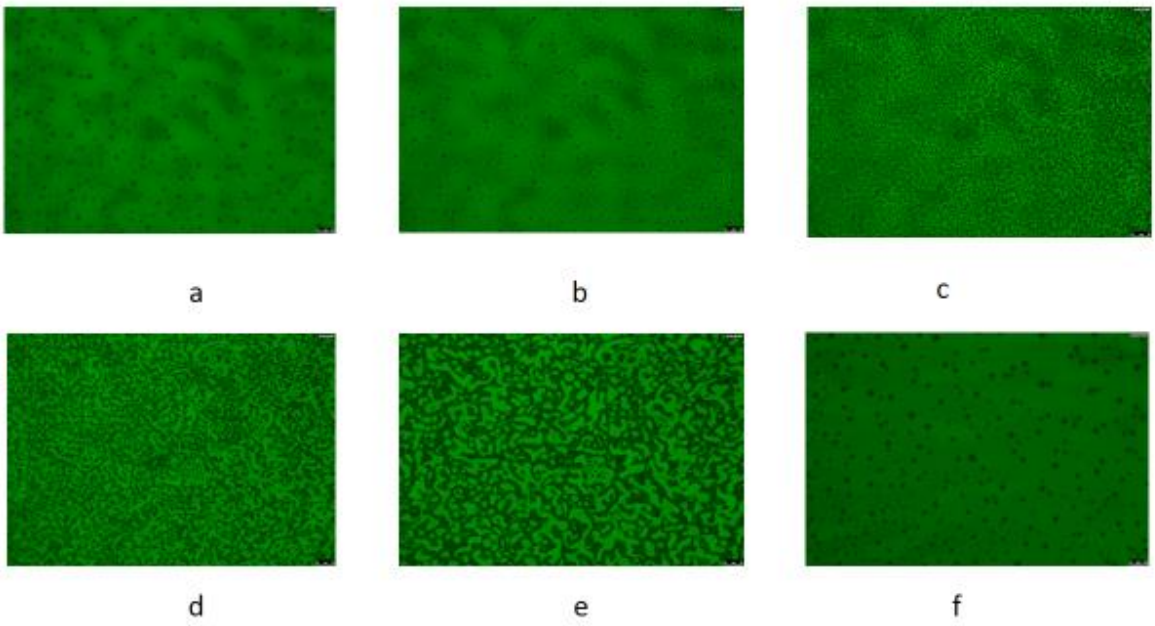


Fig 2.9: Fluorescence microscopy images of supported multilayers of DMPC containing 10 mol% 27HCH at 100% relative humidity: fluid phase at 29^{o}C (a), appearance and coalescence of fluid-fluid coexistence at 30^{o}C (b, c, d, e) and fluid phase at 70^{o}C (f) (image size: 1.2 mm x 0.9 mm). Scale bar is 100 μm.

Supported multilayers of DMPC with 10 mol% 27HCH show fluid-fluid coexistence between 29^{o}C and 70^{o}C [Fig 2.9]. Appearance and coalescence of domains in supported multilayers of DMPC with 15 mol % of 27HCH is shown in Fig 2.10.

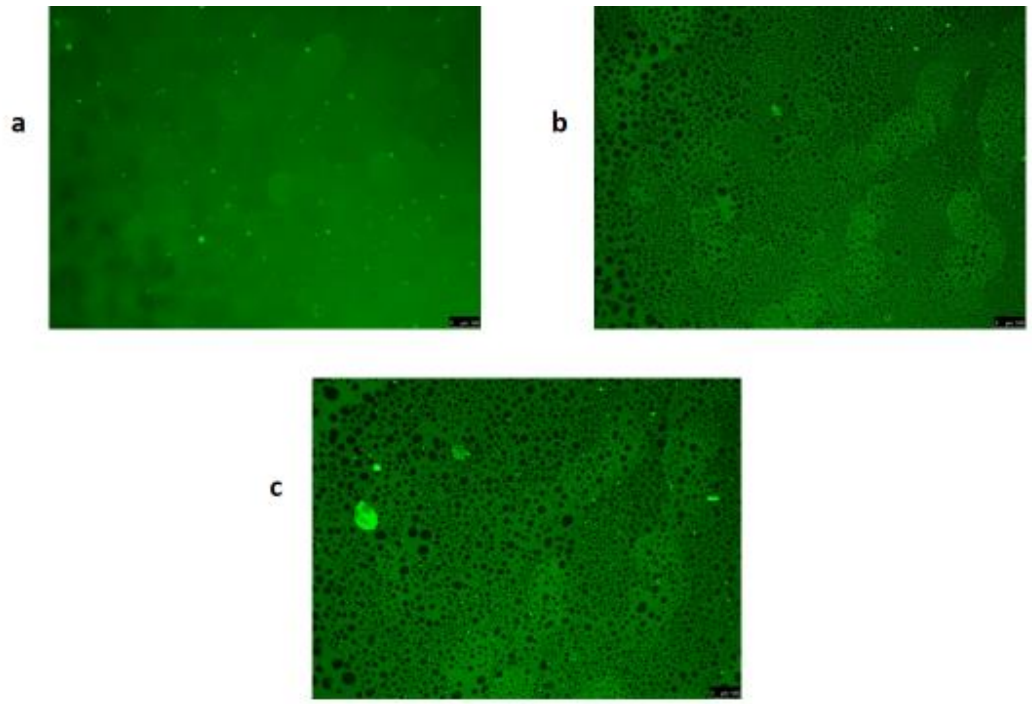
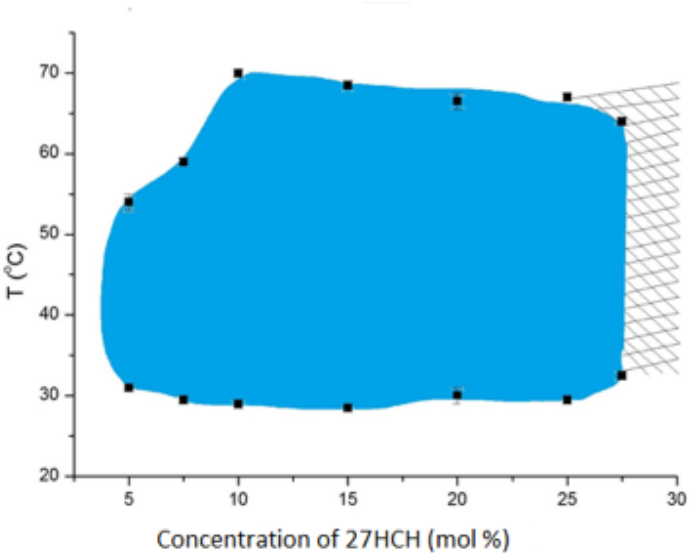


Fig 2.10: Fluorescence microscopy images of supported multilayers of DMPC containing 15 mol% 27HCH at 100% relative humidity: fluid phase at 28^{o}C (a), appearance and coalescence of fluid-fluid coexistence at 29^{o}C (b, c) (image size: 1.2 mm x 0.9 mm). Scale bar is 100 µm.

Phase diagram of DMPC-27HCH mixtures [Fig 2.11] is very similar to that of the DMPC-25HCH system. Phase separation is observed over an extensive temperature range above the main transition temperature for sterol concentrations varying from 5 mol% to 27.5%. No phase separation is observed at a sterol concentration of 2.5 mol % and crystallites of 27HCH are observed beyond 27.5 mol%. The low temperature boundary of the coexistence region remains almost flat whereas the high temperature boundary increases till 10 mol% sterol concentration and then decreases slightly till 27.5 mol%.



CONCENTRATION OF 27 HCH (mol %)	T min (^{0}C)	T max (^{0}C)
5	31.0±0.5	54.0±1.0
7.5	29.5±0.5	59.0±0.5
10	29.0±0.5	70.0±0.5
15	28.5±0.5	68.5±0.5
20	30.0±1.0	66.5±1.0
25	29.5±0.5	67.0±0.5
27.5	32.5±0.5	64.0±0.5

Fig 2.11: Partial phase diagram of DMPC-27HCH constructed from the fluorescence Microscopy observations on supported multilayers. The fluid-fluid coexistence region is shaded in blue.

2.3.3 GUVs of DMPC-25HCH and DMPC-27HCH

GUVs prepared from both DMPC-25HCH membranes and DMPC-27HCH membranes show two-phase coexistence above the main transition temperature of DMPC. DMPC vesicles containing 15 mol% of 27HCH show two-phase coexistence between 29^{o}C and 44^{o}C [Fig 2.12]. The domains are found to coalesce over time (of the order of few minutes) confirming the fluid nature of the two phases [Fig 2.13].

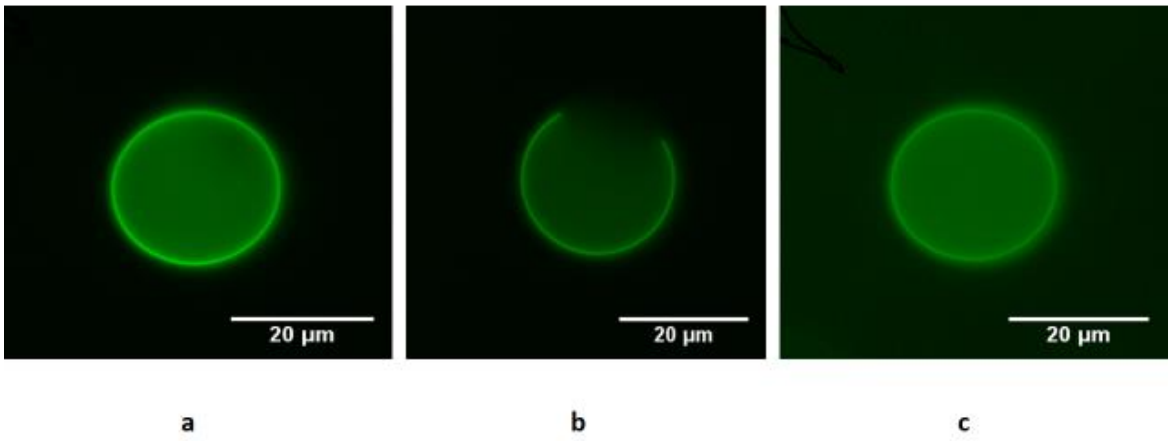


Fig 2.12: Fluorescence microscopy images of a GUV made from DMPC containing 15 mol% 27HCH in low temperature fluid phase at 28^{o}C (a), in the intermediate two-phase region at 35^{o}C (b) and the high temperature fluid phase at 45^{o}C (c).

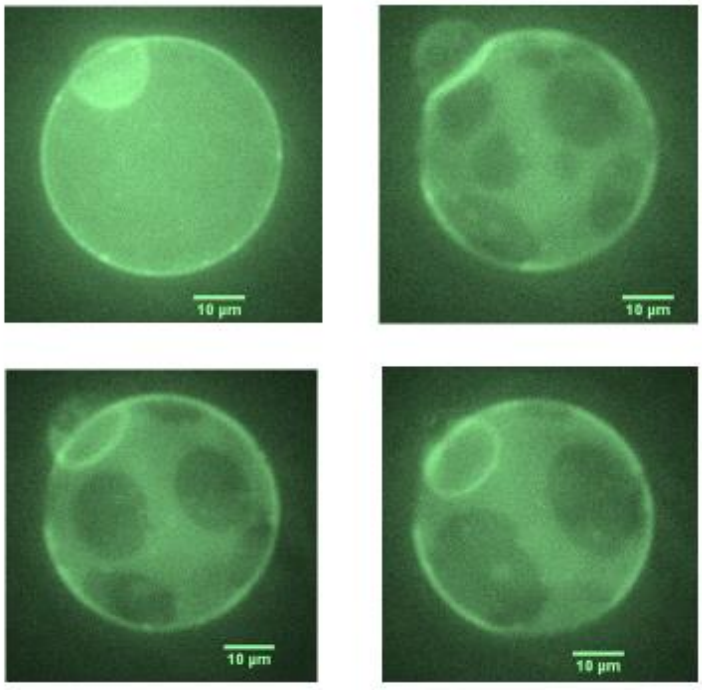


Fig 2.13: Coalescence of circular domains of DMPC vesicles with 20 mol% 27HCH; the elapsed time between the second and the last frames is of the order of a few minutes.

At 25 mol% of 27HCH concentration, DMPC vesicles show fluid-fluid coexistence between 29^{o}C and 59^{o}C [Fig 2.14].

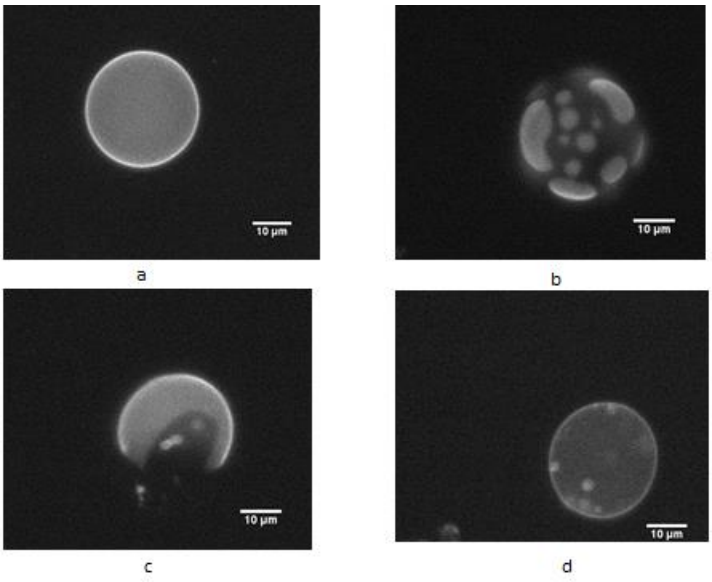


Fig 2.14: DMPC Vesicles with 25 mol% of 27HCH at T=25^{o}C (a), at 30^{o}C (b), at 40^{o}C (c) and at 60^{o}C (d) Scale bar: 10 µm.

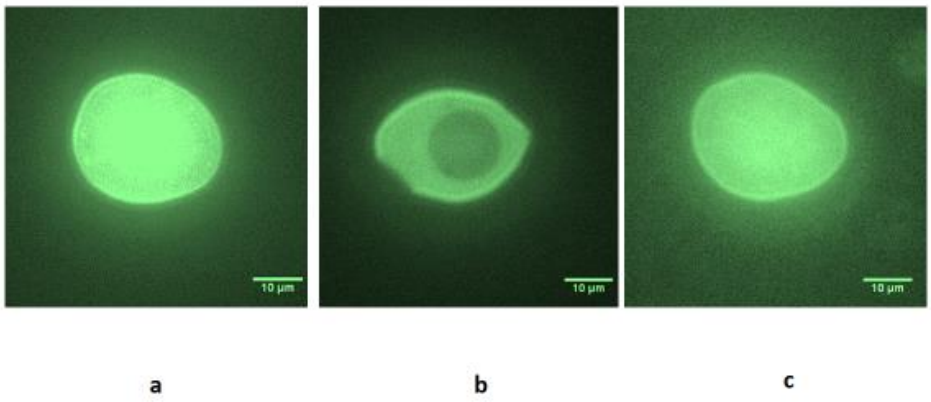


Fig 2.15: Fluorescence microscopy images of a GUV made from DMPC containing 20 mol% 25HCH in low temperature fluid phase at 30^{o}C (a), in the intermediate two-phase region at 35^{o}C (b) and the high temperature fluid phase at 49^{o}C (c).

Vesicles of DMPC with 20 mol% 25HCH shows two phase coexistence region between 33^{o}C and 49^{o}C [Fig 2.15].

Observations of phase separation in GUVs confirm that interactions within each bilayer are responsible for the phase behavior, and that interlayer interactions are not important.

2.4 Discussion

Suppression of the gel phase of DMPC at high cholesterol concentrations (about 20 mol%) has been reported in earlier studies [5]. Similar behavior has been reported with 25HCH and 27HCH [6]. However the phase behavior above T_m reported here has not been observed with any other sterol.

Coexistence of two fluid phases in DMPC-25HCH and DMPC-27HCH membranes is observed in SAXS studies [3]. Fluorescence microscopy studies on multilayer systems and GUVs confirm this closed-loop fluid-fluid immiscibility above T_m. Phase boundary at high sterol content is not accessible due to the crystallization of sterols out of the membrane and the low temperature boundary is almost flat. Binary mixtures exhibiting closed loop immiscibility regions are characterized by upper and lower critical points. Upper critical points of DMPC-27HCH and DMPC-25HCH membranes occur around 70^{o}C, 10 mol% and 60^{o}C, 22.5 mol% respectively. We have not attempted an accurate determination of the location of the critical points in the present study.

Location of the high temperature phase boundaries in the phase diagrams from fluorescence microscopy is found to be different from corresponding SAXS data. This may be due to the longer time scales of SAXS data collection. The reason may be also that the two techniques are inherently sensitive to different modes of phase separation. In fluorescence microscopy preferential partitioning of a dye in to one of the coexisting phases occurs to reveal domains in individual bilayers. In X-ray diffraction, formation of lamellar phases with two different periodicities by stacking up of domains in different bilayers is the indication of two-phase coexistence. Observation of phase separation in GUVs indicates that interactions within each bilayer are responsible for this phase behaviour and the inter-bilayer interactions are irrelevant. Moreover, these observations establish that fluid-fluid coexistence occurs under

excess water condition, which is relevant for cell membranes. However, GUVs formed from a multicomponent system are known to have compositional inhomogeneity [7], resulting in variations in the phase transition temperature from GUV to GUV. Therefore, the phase behaviour is better studied using supported multilayers, which do not suffer from the above drawback. Fluorescence microscopy studies using supported multilayers provide information on the phase behaviour comparable to those obtained from x-ray diffraction studies on aligned multilayers. Samples used in these two techniques are almost identical, the only difference being that those used for microscopy studies contain a small amount of a fluorescent dye, which partitions unequally between the two coexisting phases. But the microscopy experiments are much faster compared to the x-ray experiments.

Earlier computer simulation studies on POPC- 25HCH and DPPC- 25HCH membranes have shown that the 25HCH molecules can take three different orientations of comparable energies in the bilayer [8-10]. At lower 25HCH concentrations POPC-25HCH membranes prefer interfacial orientation whereas cholesterol like orientation is preferred in DPPC- 25HCH membranes. A schematic of three different orientations of the oxysterols in phospholipid membranes is shown in Fig. 2.16.

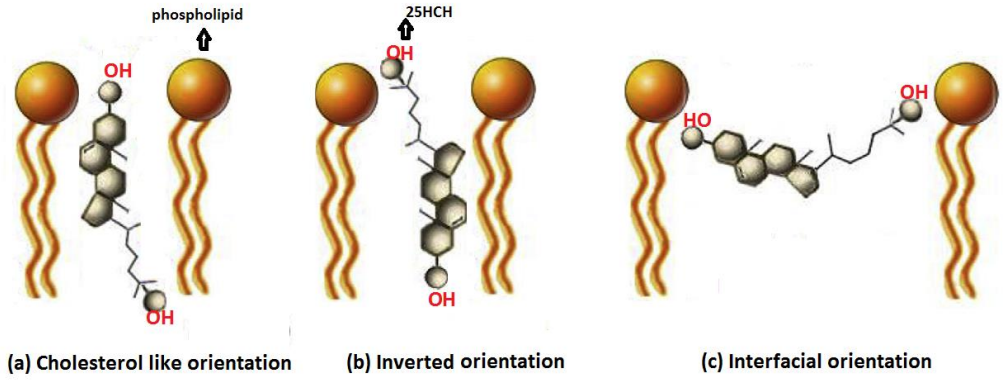


Fig. 2.16: A schematic of the three different orientations of 25HCH in a phospholipid membrane.

Computer simulations of DMPC-cholesterol and DMPC-27HCH membranes have been carried out to gain some insights into the mechanism responsible for the observed fluid-fluid coexistence [11]. In both cases the sterol concentration was 20 mol%. Simulations were carried out at 25^{o}C, 35^{o}C and 70^{o}C. Water to lipid ratio was chosen as 32:1 to ensure the complete

hydration of the lipid bilayer membrane. GROMOS force field parameters were used. Both electrostatic interactions and Lennard-Jones interactions were incorporated. Orientational order parameter is defined as cosθ, where θ is the tilt angle of the long axis of the 27HCH molecule, directed from the O-atom at the tip of the chain to the O-atom attached to the sterol core, with respect to bilayer normal.

Spatial distribution of DMPC number density (ρ_l), sterol number density (ρ_s), bilayer thickness (d) and the orientational order parameter (cosθ) within the membrane patch were obtained from computer simulations. These special distributions were found to be non-uniform in DMPC-27HCH membrane at 35^{o}C, as shown in Fig. 2.17.

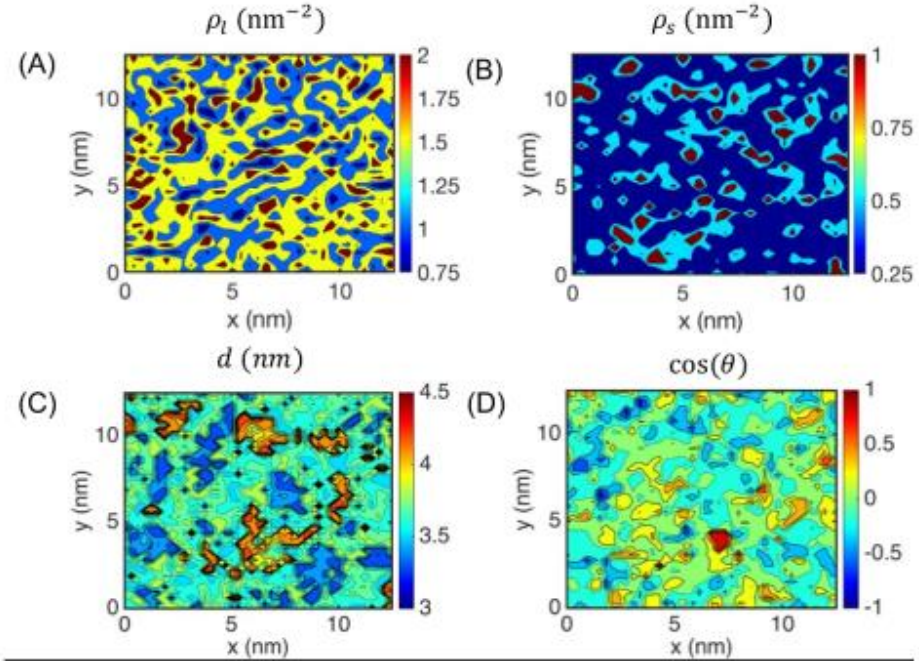


Fig 2.17 : Spatial distribution of (A) DMPC number density (ρ_l), (B) 27HCH number density (ρ_s), (C) bilayer thickness (d) and (D) the orientational order parameter (cosθ) at 35^{o}C in one leaflet of a symmetric DMPC bilayer containing 20 mol% of 27HCH obtained from computer simulations [11].

In order to find out the correlation between different values the above parameters, the joint probability distribution (JPD) of bilayer thickness d_{pp} was calculated with each of the other three parameters, ρ_l, ρ_s and cosθ [Fig 2.18]. The results correctly reproduce the occurrence of fluid-fluid coexistence over a temperature range above T_m .Two phases in the coexistence region are sterol-poor phase with interfacial orientation and sterol-rich phase with cholesterol like and inverted orientations. Thus computer simulations clearly suggest that the observed phase separation is associated with different orientations of sterol molecules in the bilayer [10].

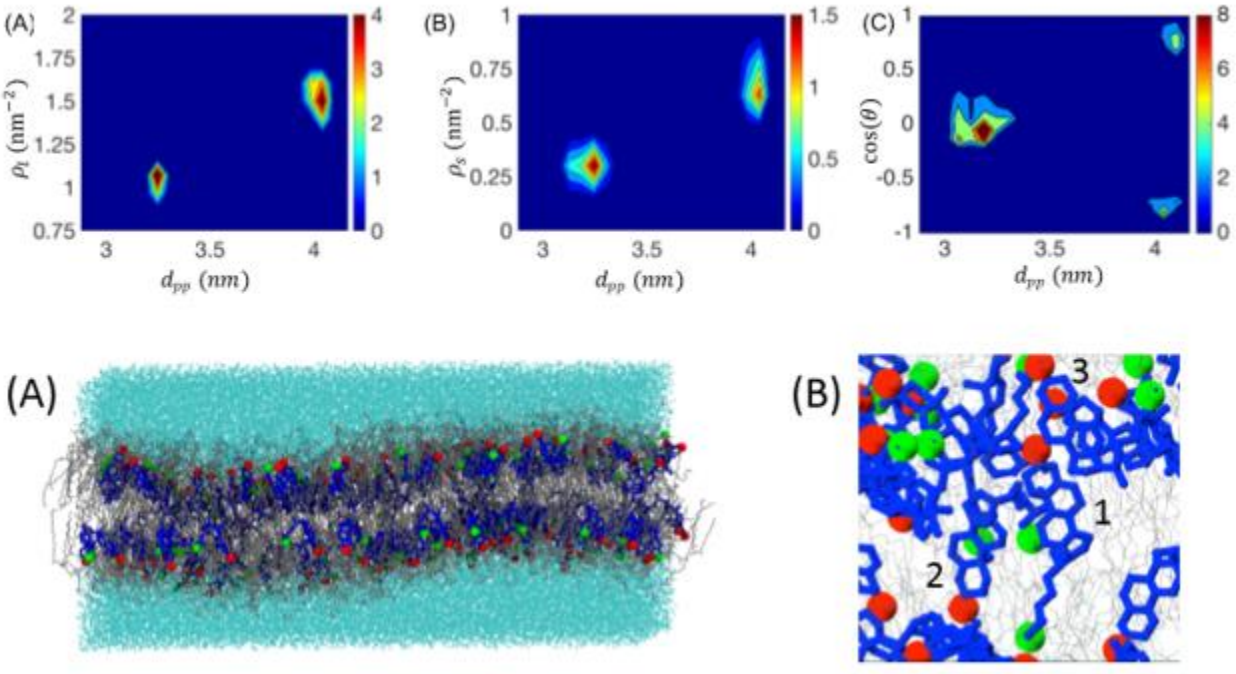


Fig 2.18 : (Top) Joint Probability distributions (JPD) of bilayer thickness, d_{pp} with (A)DMPC number density, ρ_l , (B)27HCH number density, ρ_s and (C) the orientational order parameter, cosθ (Bottom) (A) A snapshot of equilibrated bilayer at 35^{o}C. DMPC molecules are shown in grey and water molecules in cyan. The 27HCH molecules are shown in blue, with the O at carbon 3 in red and that at carbon 27 in green. (B) An enlarged view of bilayer to highlight the different orientations of 27HCH molecules in the bilayer. Cholesterol-like (1), inverted (2), and interfacial (3) orientations of the sterol can be distinguished [11].

27HCH molecules take interfacial orientation at temperatures above the two-phase region, whereas below they take the cholesterol like and inverted orientations [Fig 2.19].

Occurrence of two orientations at lower temperatures indicates that their energies are comparable. Computer simulations have shown that orientation of cholesterol in DMPC membranes does not change with temperature, consistent with the observation that this system does not show a two phase region above T_m [11].

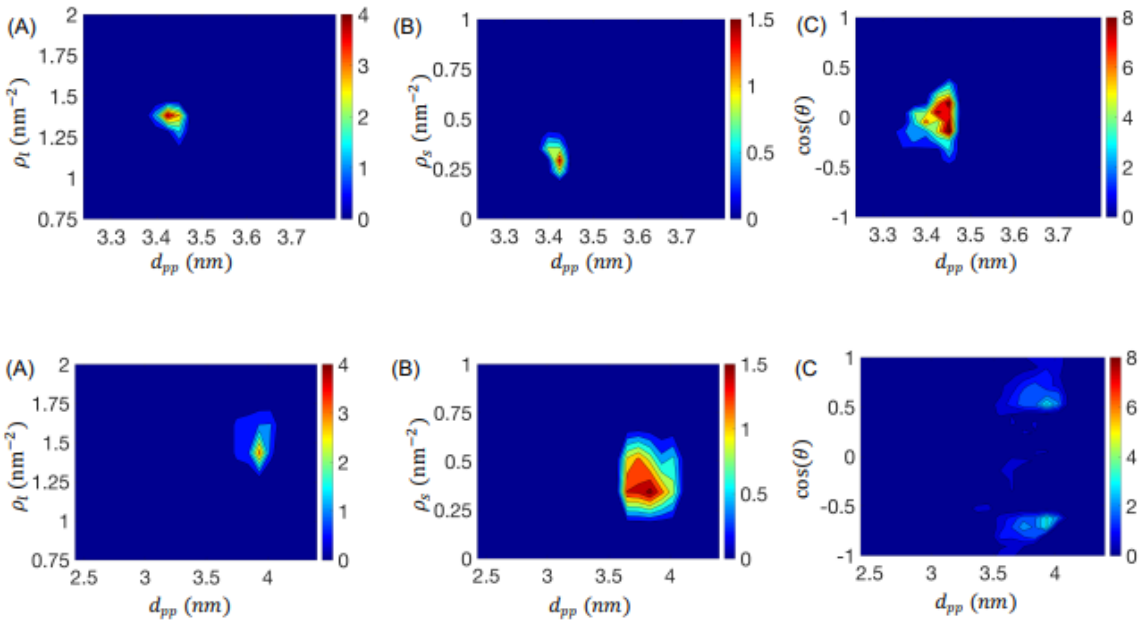


Fig 2.19 : Joint Probability distributions (JPD) of bilayer thickness, dpp with (A)DMPC number density, ρ_l , (B)27HCH number density, ρ_s and (C) the orientational order parameter, cosθ at 70^{o}C (top) and at 25^{o}C (Bottom) [11].

The closed-loop fluid-fluid immiscibility observed in DMPC-27HCH and DMPC-25HCH systems seems to originate from the ability of the sterol molecules to adopt different orientations in the bilayer as a function of temperature. Different orientations have different thicknesses and the difference in thicknesses causes interfacial energy which drives into phase separation.

The energies corresponding to these different orientations are determined by various interactions of the sterol molecule with the lipid and water molecules, including hydrogen bonding.

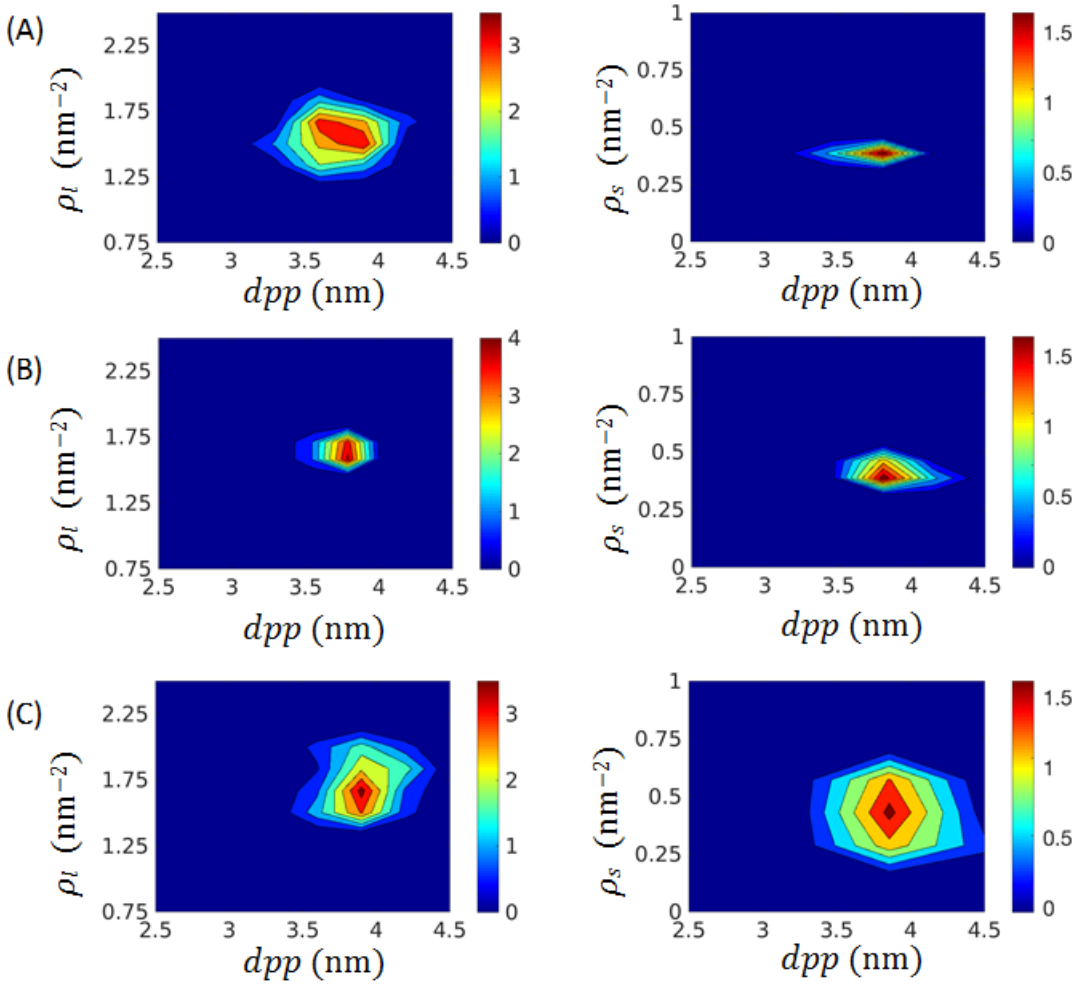


Fig 2.19: Joint Probability distributions (JPD) for DMPC-cholesterol membranes at 70^{o}C (A), 35^{o}C (B) and at 25^{o}C (C) of local lipid concentration ρ_l and bilayer thickness d_{pp} (left) and the local cholesterol concentration ρ_s and d_{pp} (right) [10]. Note the presence of a single phase at all the three temperatures [11].

2.5 Conclusion

Here we present the first fluorescence microscopy observations of fluid-fluid coexistence in lipid sterol membranes. The closed-loop fluid-fluid immiscibility observed in the phase diagrams of DMPC-27HCH and DMPC-25HCH membranes above the chain melting transition temperature is consistent with earlier small-angle X-ray scattering studies. Computer simulations of DMPC-27HCH membrane suggest that the observed phase separation arises from the ability of oxysterol molecules to take different orientations in the lipid membrane depending on the temperature. These results suggest a novel mechanism for the induction of fluid-fluid coexistence in lipid membranes.

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CHAPTER 3

Influence of 25-hydroxycholesterol and 27-hydroxycholesterol on the phase behaviour of saturated PC model membranes

3.1 Introduction

In chapter 2, we presented two binary lipid-sterol membrane systems (DMPC–25HCH and DMPC–27HCH) showing fluid-fluid coexistence above the chain melting transition temperature T_m. In this chapter, we investigate the influence of the chain length of the lipid molecule on the two phase coexistence. Model membranes of saturated PCs, having hydrocarbon chains containing 12 to 16 carbon atoms are used for these investigations.

A partial temperature – composition phase diagram of DPPC (16 carbon atoms in the chain) membranes containing 25HCH has been determined at 98±2% relative humidity using SAXS data from aligned multilayers [1]. Above T_m, the fluid phase (Lα) was found at all concentrations of 25HCH [Fig. 3.1], and no fluid-fluid coexistence was observed.

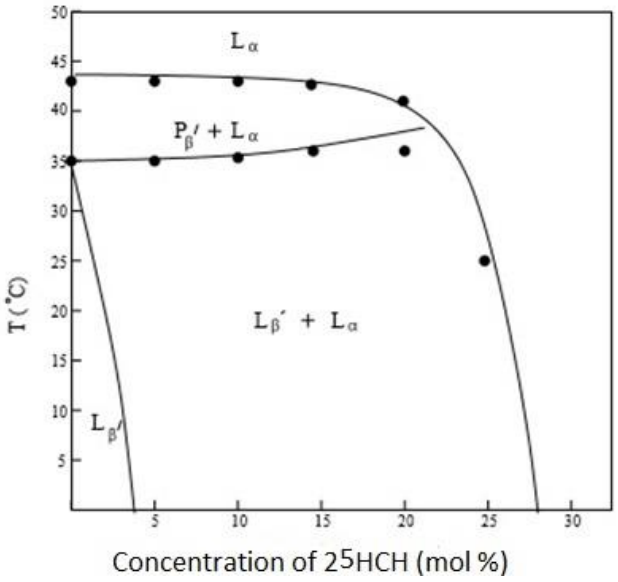


Fig 3.1: A partial phase diagram of DPPC-25HCH binary membranes at 98±2% RH determined from SAXS data from aligned multilayer samples [1].

The effect of 27HCH on the phase behavior of DPPC model membrane was found to be different from that of 25HCH [2]. Partial phase diagram of DPPC – 27HCH binary mixtures shows the coexistence of two fluid phases above the chain melting temperature (T_m) for a concentration range of 5 – 35 mol% of 27HCH [Fig 3.2]. This is inferred from the presence of two sets of lamellar peaks in the small angle region of the diffraction pattern. A diffuse peak is observed in the wide angle region, characteristic of the fluid phase. The difference in the d-spacing between the two coexisting fluid phases was found to be constant irrespective of the sterol content in the membrane.

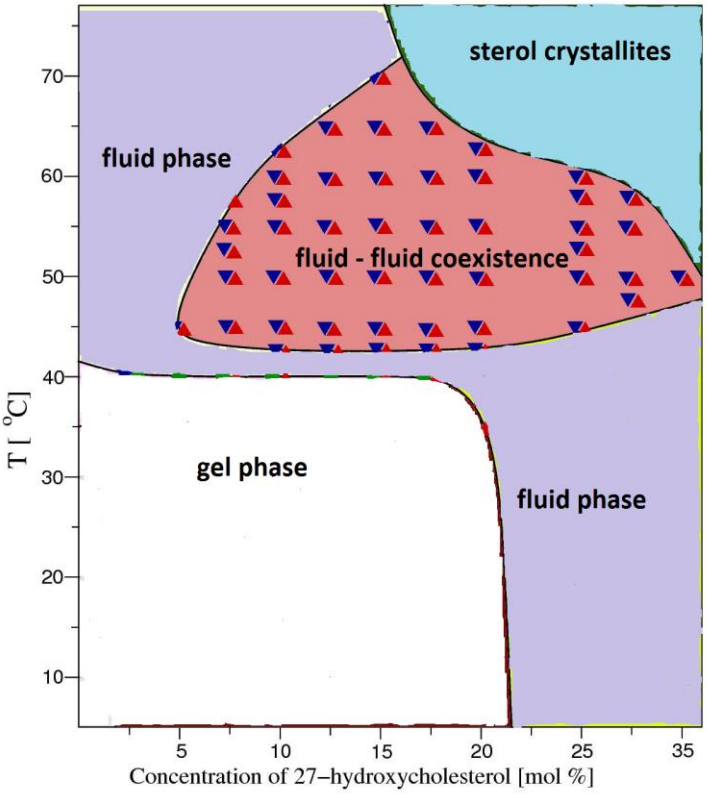


Fig 3.2: A partial phase diagram of DPPC-27HCH binary membrane at 98±2% RH determined from SAXS data from aligned multilayer samples [2]. The fluid-fluid coexistence region is shaded in pink.

The high temperature boundary of the coexistence region shows a gradual increase till 15 mol% of 27HCH and beyond that crystallization of the oxysterol is observed. The low

temperature boundary of the coexistence region is almost flat till 20 mol% of 27HCH and it is found to increase slightly beyond that. There is a very narrow region of the single fluid phase between the chain melting temperature (T_m) and the low temperature boundary of the coexistence region.

A partial phase diagram of binary mixtures of 25HCH with DLPC, which has two 12-carbon chains, has also been established using SAXS methods [3]. Two sets of lamellar peaks were observed for a range of sterol concentrations from 10 mol% to 20 mol% and the corresponding phase diagram showing coexistence of two fluid phases is given in Fig 3.3.

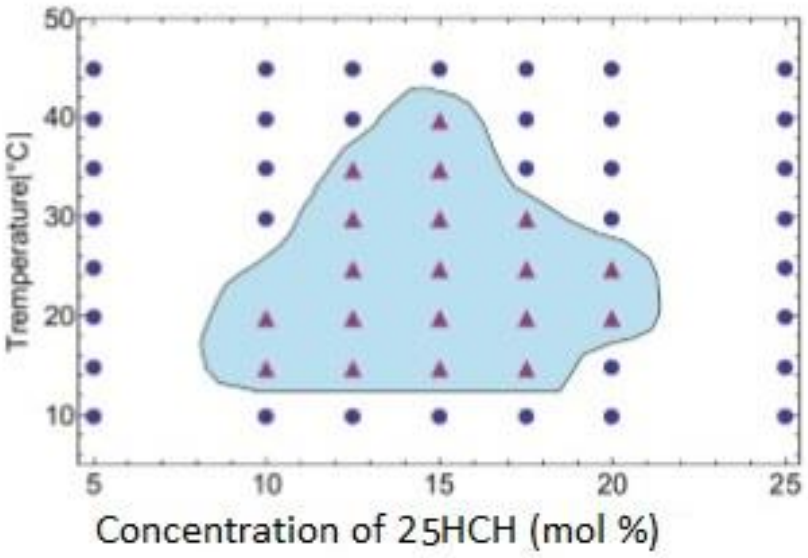


Fig 3.3 : Partial phase diagram of 25HCH – DLPC bilayers. Coexistence of two fluid phases is seen over the shaded region and the single lamellar phase over the rest [3].

The extent of the two phase region is found to increase gradually with increase in the concentration of 25HCH and reaches its maximum at 15 mol%, then decreases. Characteristic X-ray diffraction patterns of DLPC multilayers with 15 mol% of 25HCH are shown in Fig 3.4. Single uniform fluid phase is observed at low sterol concentration of 5 mol% and at high sterol concentration of 25 mol%.

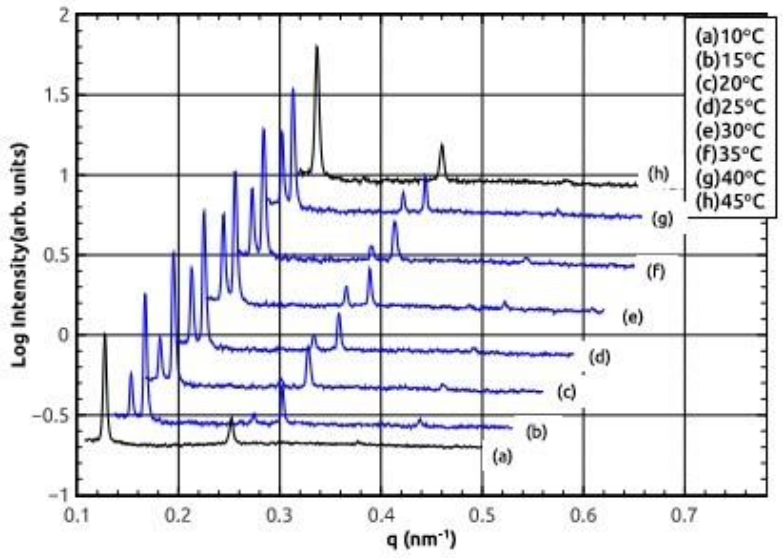


Fig 3.4: Characteristic x ray diffraction pattern of DLPC bilayers containing 15 mol% of 25HCH at different temperatures [3]. Note the presence of two lamellar phases in (b) to (g).

In this chapter we describe fluorescence microscopy studies on binary mixtures of 25HCH and 27HCH with disaturated lipids with carbonyl chains of 12 (DLPC) to 16 (DPPC) carbon atoms. Supported multilayers of each system are prepared and observed under a fluorescence microscope. These studies reveal the existence of fluid-fluid immiscibility for a wide range of chain lengths and its chain length dependence. Presence of fluid-fluid coexistence is also verified using GUVs made from DPPC – 27HCH membranes.

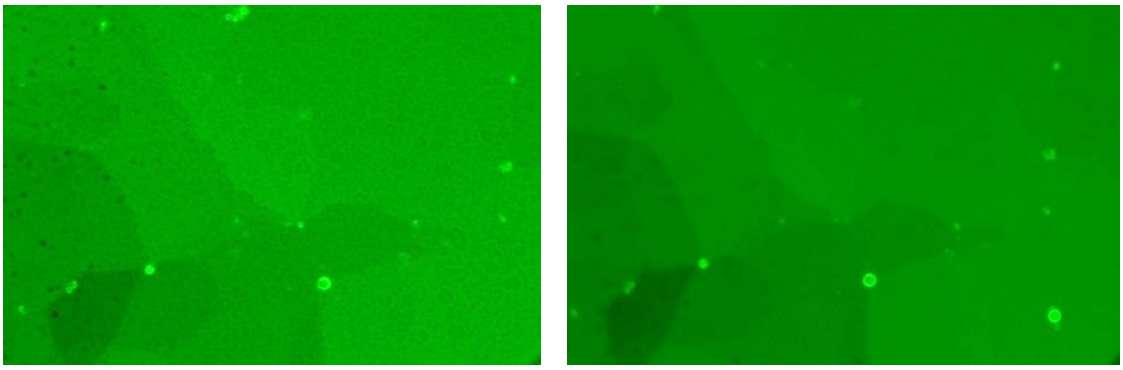
3.2 Materials of Study

1, 2-dilauroyl-sn-glycero-3-phosphocholine (DLPC), 1, 2-ditridecanoyl-sn-glycero-3-phosphocholine (13:0 PC), 1, 2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1, 2-dipentadecanoyl-sn-glycero-3-phosphocholine (15:0 PC), 1, 2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 25-hydroxycholesterol (25HCH) and 27-hydroxycholesterol (27HCH) were obtained from Avanti Polar Lipids in powder form. Stock solutions are prepared in chloroform (HPLC grade) obtained from Sigma-Aldrich. Rhodamine-DHPE dye was obtained from Invitrogen bioservices.

3.3 Experimental Results

A systematic investigation on mixtures of 25HCH or 27HCH with saturated PCs (DLPC, 13:0 PC, DMPC, 15:0 PC and DPPC) was carried out using fluorescence microscopy. Fluorescence images show that saturated PCs of carbonyl chains varying from 12 to 15 carbon atoms show fluid-fluid coexistence with 25HCH above the chain melting temperature Tm. Similar behaviour is observed with 27HCH for PCs having carbonyl chains varying from 13 to 16 carbon atoms. Presence of the two phase region is confirmed by fluorescence microscopy observations on GUVs made from DPPC-27HCH membranes. On the basis of fluorescence microscopy observations partial phase diagram of the DPPC-27HCH system has been drawn. Phase boundaries were measured both on heating and cooling, but always maintaining the sample temperatures above T_m.

3.3.1 Phase behavior of DPPC-27HCH multilayers



a b

Fig 3.5: Fluorescence microscopy images of supported multilayers of DPPC containing 5 mol% 27HCH at 100 % relative humidity: fluid phase at 44^{o}C (a) and at 52^{o}C (b) (image size: 0.2 mm x 0.15 mm). Patches of different intensities are due to differences in the thickness of the lipid film, resulting in varying concentrations of the dye molecule.

There is no significant change in the phase behavior of DPPC multilayers up to 5 mol% of sterol concentration [Fig 3.5]. In the concentration range between 7.5 mol% to 30 mol% we observe the coexistence of two fluid phases. At 7.5 mol% fluid-fluid coexistence is observed

from 42^{o}C to 51^{o}C, where the low temperature boundary is just above the chain melting temperature of DPPC (41^{o}C).

Crystallites of 27HCH are observed beyond a sterol concentration of 30 mol%. The low temperature boundary of the two phase region is found to be almost flat whereas the high temperature boundary has a maximum for a sterol concentration of 20 mol%. At 20 mol% of 27HCH the fluid-fluid coexistence is observed from 43^{o}C to 62.5^{o}C, in form of dark domains on a bright background. These domains are found to coalesce over time, which confirms the fluid nature of both phases [Fig 3.6].

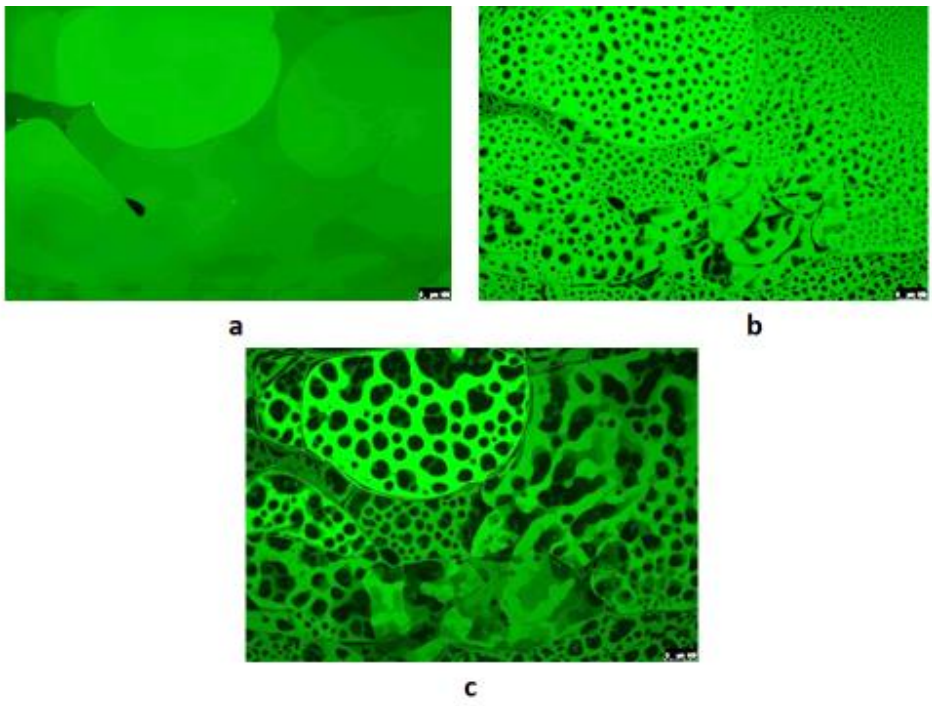
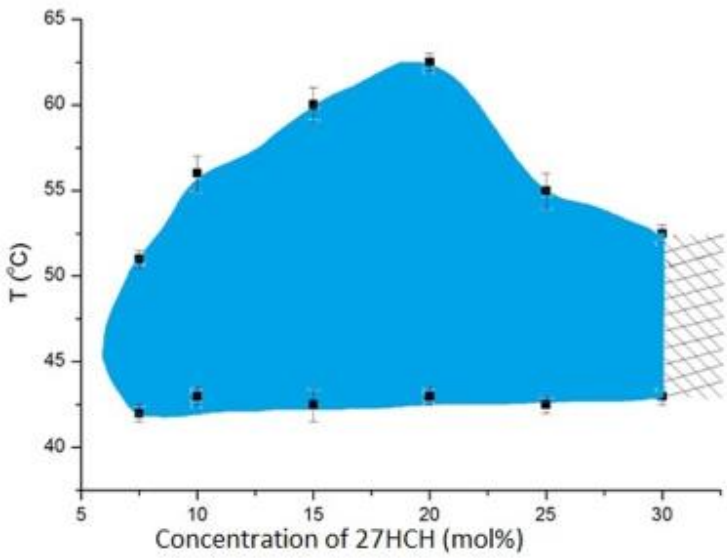


Fig 3.6: Fluorescence microscopy images of DPPC multilayers containing 15 mol % of 27HCH at T = 62^{o}C (a), 53^{o}C (b), and 45^{o}C (c) (image size: 1.2 mm x 0.9 mm). Scale bar is 100 µm.



CONCENTRATION OF 25HCH (mol %)	T min (°C)	T max (°C)
5	NIL	NIL
7.5	42.0±0.5	51.0±0.5
10	43.0±0.5	56.0±1.0
15	42.5±1.0	60.0±1.0
20	43.0±0.5	62.5±0.5
25	42.5±0.5	55.0±1.0
30	43.0±0.5	52.5±0.5

Fig 3.7: Partial phase diagram of DPPC-27HCH constructed from the fluorescence Microscopy observations on supported multilayers. The two phase region is shaded blue.

A partial phase diagram of DPPC- 27HCH binary mixtures across the composition temperature plane deduced from the fluorescence microscopy data is shown in Fig 3.7.

3.3.2 GUVs of DPPC-27HCH

GUVs prepared from DPPC-27HCH membranes show fluid-fluid coexistence above T_m (41^{o}C) of DPPC. DPPC vesicles containing 25 mol% of 27HCH starts showing two fluid phases above 48^{o}C while heating from 41^{o}C and it is found to disappear while cooling below 48^{o}C [Fig 8]. Observation of phase separation in GUVs tells us that the interaction within the bilayer is responsible for this phase behaviour.

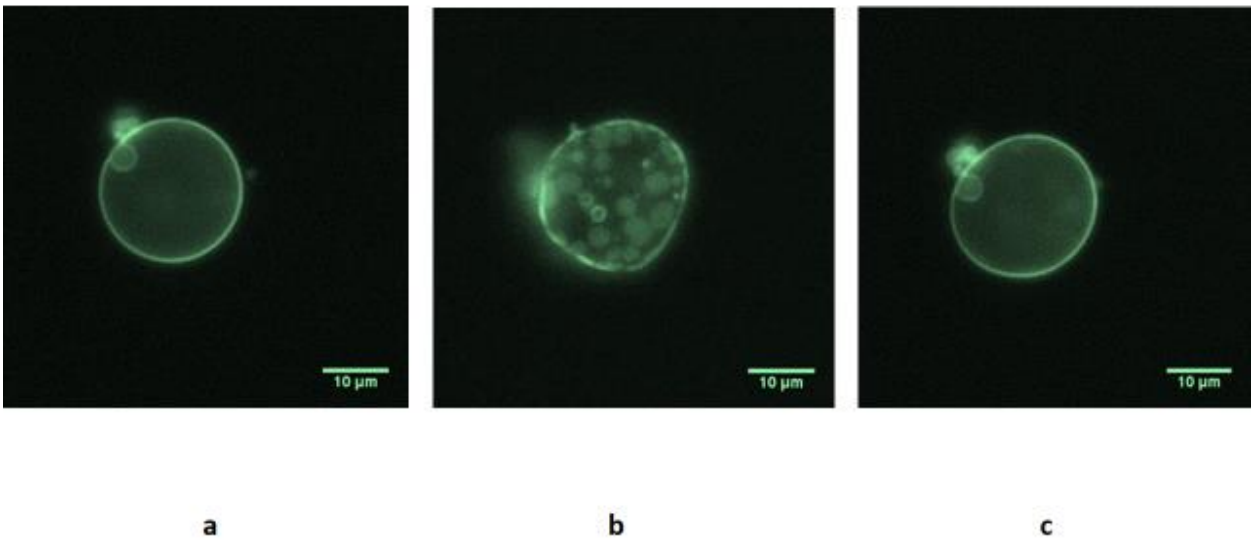


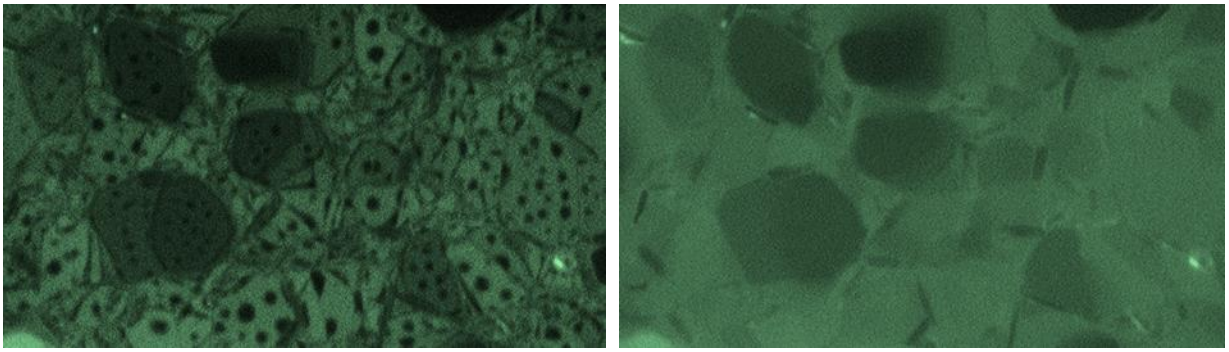
Fig 3.8: Fluorescence microscopy images of a GUV made from DPPC containing 25 mol % 27HCH in the fluid phase at 45^{o}C on heating (a), in the two-phase region at 51^{o}C (b) and in the fluid phase at 37^{o}C on cooling (c).

3.3.3 Effect of 25HCH on the phase behavior of DPPC multilayers

We have carried out experiments on multilayers of binary mixtures of DPPC – 25HCH for various concentrations. It is found that 25HCH does not induce fluid-fluid coexistence above the main transition temperature of DPPC.

3.3.4 15:0 PC Multilayers with 25HCH and 27HCH

Multilayers of disaturated lipid with 15 carbon atom chains having chain melting transition temperature of 35^{o}C show fluid-fluid coexistence above T_m with 25HCH and 27HCH. For 15:0 PC multilayers with 25HCH phase separation is observed between 39^{o}C and 45^{o}C [Fig 9].



a b

Fig 3.9: Fluorescence microscopy images of supported multilayers of 15:0 PC containing 20 mol% 25HCH under 100% relative humidity at 44^{o}C (a) and 39^{o}C (b). (Image size: 0.4 mm x 0.2 mm).

3.3.5 DMPC Multilayers with 25HCH and 27HCH

The closed loop fluid-fluid immiscibility observed in the phase diagrams of DMPC-25HCH and DMPC-27HCH membranes above the chain melting temperature has been described in the previous chapter.

3.3.6 13:0 PC Multilayers with 25HCH and 27HCH

Multilayers of 13:0 PC (T_m =14^{o}C) shows two phase coexistence with 25HCH. At a concentration of 20 mol%, domains are observed at a temperature of 25^{0}C on heating and they are found to coalesce over time [Fig 3.10].

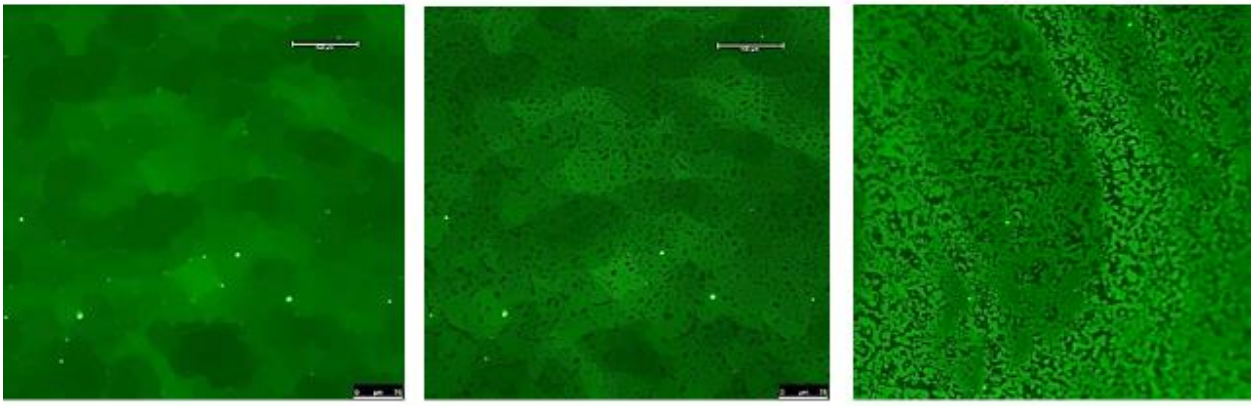


Fig 3.10: Fluorescence microscopy images of 13:0 PC multilayers containing 20 mol % of 25HCH at 21^{o}C (a), at 25^{o}C (b) and at 30^{o}C (c) (image size: 0.4 mm x 0.4 mm).

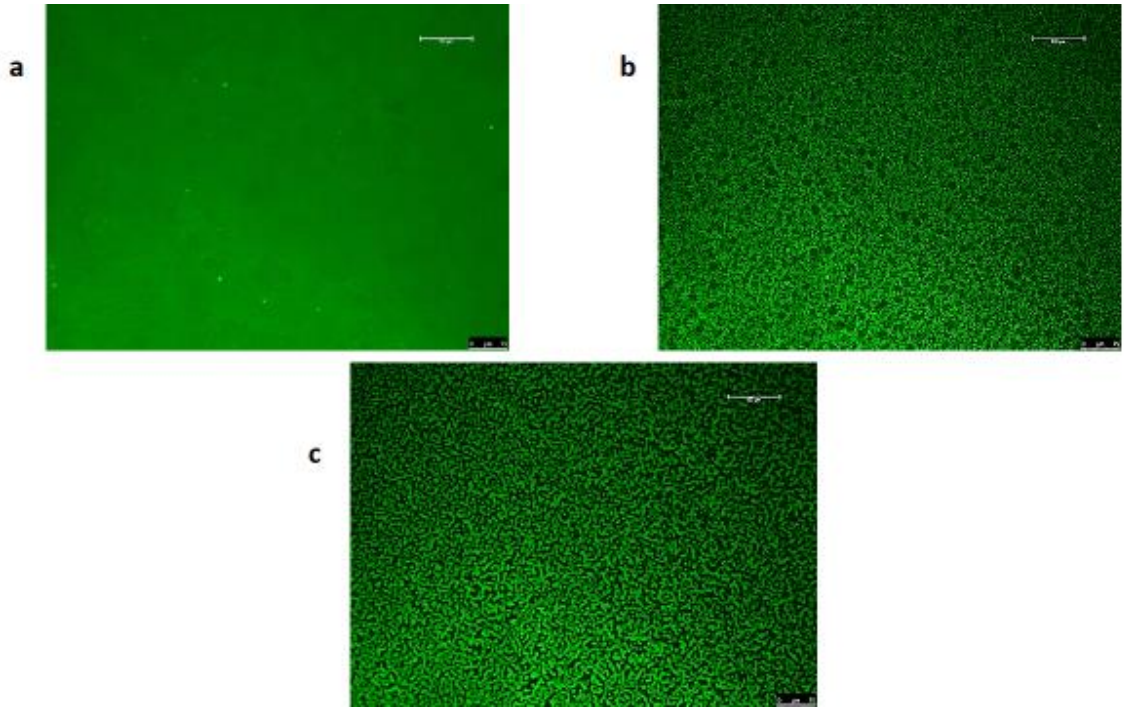


Fig 3.11: Fluorescence microscopy images of 13:0 PC multilayers containing 20 mol % of 27HCH at 19^{o}C (a), at 23^{o}C (b) and at 25^{o}C (c) (image size: 1.2 mm x 0.9 mm).

Supported multilayers of 13:0 PC with 20 mol% of 27HCH also show fluid-fluid coexistence above Tm. The domains are found to appear at a temperature of 23^{o}C on heating. Coalescence of domains clearly indicates its fluid nature [Fig 3.11].

3.3.7 DLPC Multilayers with 25HCH and 27HCH

DLPC- 25HCH multilayers were prepared at room temperature. During observation, these samples were cooled down to 8^{0}C and then heated up. While heating domains are found to appear at a temperature of 14^{0}C, at a concentration of 20 mol% of 25HCH. Domains are found to coalesce confirming their fluid nature. The fluid- fluid coexistence disappears on heating at a temperature around 24^{0}C [Fig 3.12].

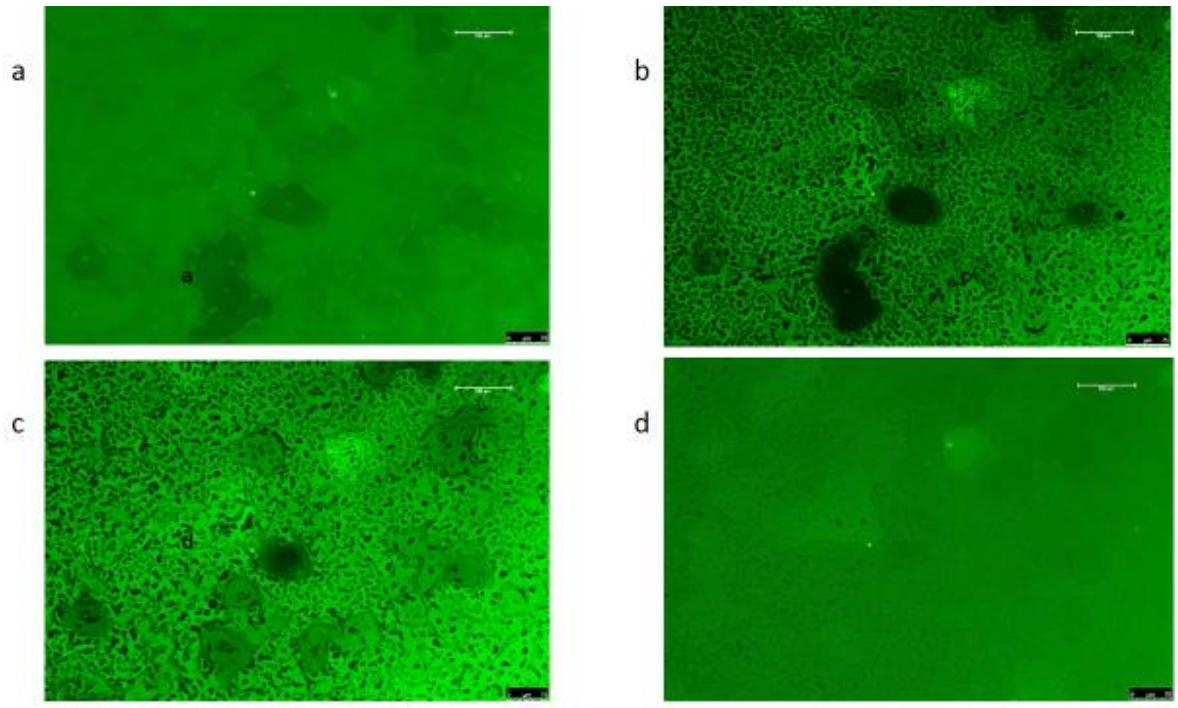


Fig 3.12: Fluorescence microscopy images of DLPC multilayers containing 20 mol % of 25HCH at 12^{o}C (a), 14^{o}C (b and c) and 27^{0}C (d) (image size: 1.2 mm x 0.9 mm).

Multilayers of DLPC do not show two phase coexistence with 27HCH. Observations were carried out from 10^{0}C to 30^{0}C at a sterol concentration of 20 mol%. Crystallites of 27HCH were observed at higher temperatures [Fig 3.13].

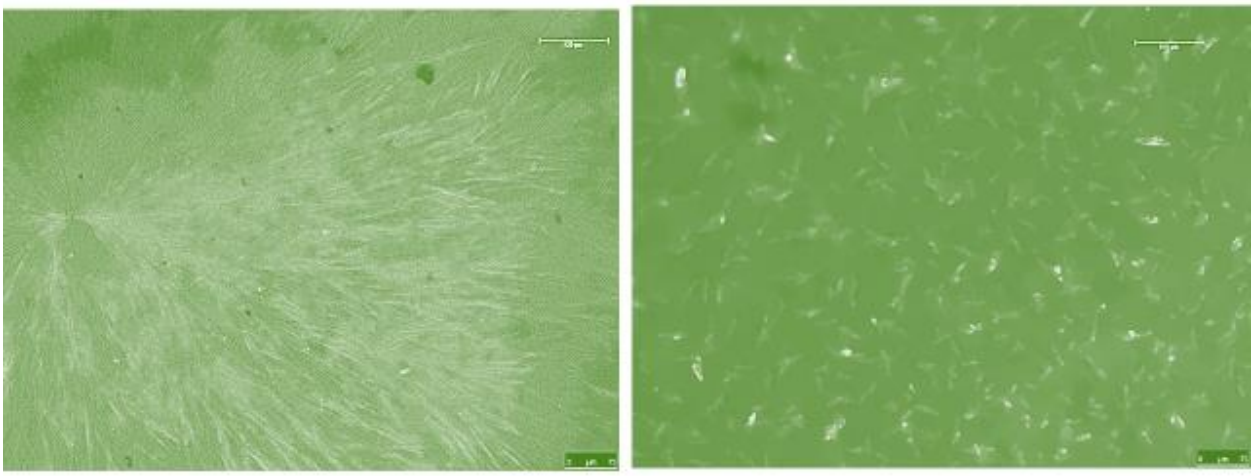


Fig 3.13: Fluorescence microscopy images of DLPC multilayers containing 20mol % of 27HCH at T=10^{o}C (a) and at 30^{o}C (b) (image size: 1.2mm x 0.9mm).

3.4 Discussion

The present results indicate that the acyl chain length of the saturated lipid plays an important role in the fluid- fluid phase separation seen in saturated lipid – oxysterol membranes. Saturated PCs of varying chain length from 12 to 15 show fluid- fluid coexistence with 25HCH above the chain melting temperature T_m. Similar behaviour is observed with 27HCH for chain length varying from 13 to 16. This shows that the occurrence of two phase coexistence depends on the acyl chain length and the position of the –OH group in the side chain of the oxysterol.

The extent of the low temperature fluid region decreases as the acyl chain length increases [Fig. 3.14]. For DLPC – 25HCH membranes, the low temperature boundary of the coexistence region is about 15^{0}C above the chain melting temperature (-2^{0}C) of DLPC. For 13:0 PC – oxysterol membranes, the difference in the low temperature boundary of the fluid – fluid coexistence and the chain melting temperature is about 10^{0}C. The same for the DMPC – oxysterol membranes is about 6^{0}C. It is found that the low temperature boundary of the fluid-fluid coexistence region for 15:0 PC – oxysterol membrane is 39^{0}C, whereas the chain melting

temperature of 15:0 PC is 35^0C. The low temperature boundary differs only by 2^0C with respect to chain melting temperature of DPPC (41^0C) for DPPC – 27HCH membranes.

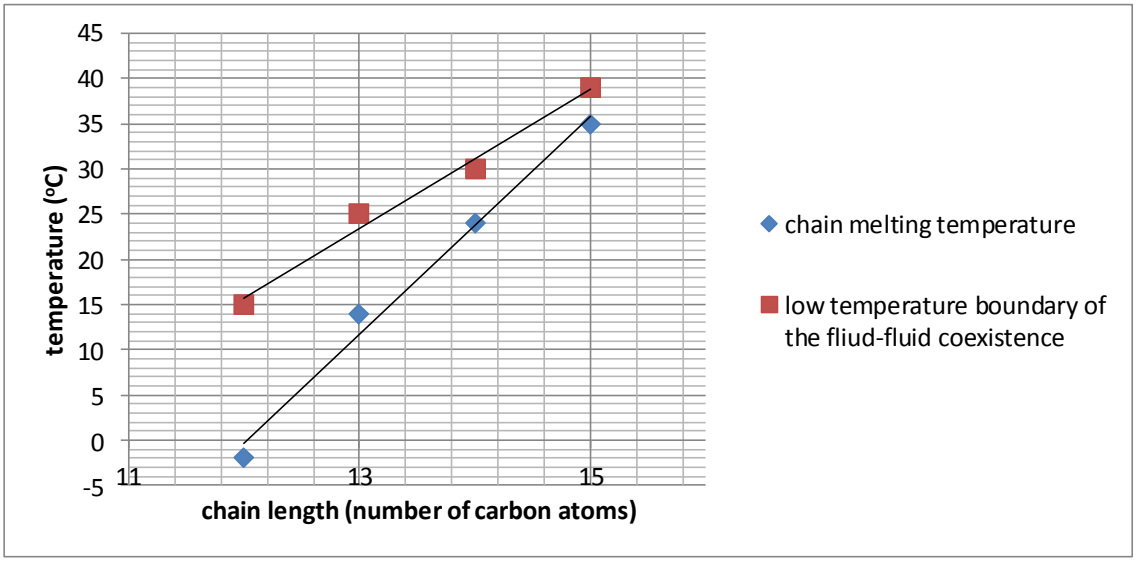


Fig. 3.14: Plot showing variation of chain melting temperature of saturated PC and the low temperature boundary of the fluid-fluid coexistence region of saturated PC-25HCH membranes with the acyl chain length of PC.

The DPPC – 27HCH phase diagram constructed from fluorescence microscopy has a higher critical point at 62.5^0C, 20 mol%. The low temperature boundary of the phase diagram is found to be almost flat. The location of the phase boundaries in the phase diagrams determined from the fluorescence microscopy is found to be different from the same constructed from SAXS data; but both phase diagrams are qualitatively similar. The observed differences are not unexpected since there are inherent differences in the way these two techniques detect phase separation, as discussed in the previous chapter.

It is concluded from the earlier chapter that the fluid-fluid coexistence is driven by temperature dependent orientations of the oxysterols. The three different orientations of 25HCH and 27HCH in a bilayer are based on how tightly they interact with the acyl chains of phospholipids [4]. Cholesterol – like and inverted orientations of sterol molecules below two phase coexistence region and interfacial orientation above two phase region, established by computer simulations, are discussed in chapter 2 [5].

The fluid-fluid coexistence in the symmetric saturated PCs occurs only for a narrow range of the bilayer thickness which depends on the specific oxysterol. The range for 27HCH extends from $16 \geq n \geq 13$, whereas that for 25HCH extends from $15 \geq n \geq 12$, where n is the number of C atoms in the chain. . Thus these observations indicate that fine tuning of the bilayer thickness is required for the occurrence of fluid-fluid coexistence in lipid-sterol membranes. This may be due to the possibility that the ability of sterol molecule to take different orientations might depend on the bilayer thickness. Moreover, this sensitivity to the bilayer thickness might also suggests a possible role of hydrogen bonding between the -OH groups of the oxysterol molecules buried deep in the two apposing leaflets of a membrane, resulting in the formation of dimers. Further studies are required to arrive at a proper explanation of the observed dependence of the phase behaviour on the bilayer thickness.

Closed loop fluid – fluid immiscibility has been observed in some binary liquid mixtures dominated by orientation dependent interactions like hydrogen bonding [6]. In the present system also the energies corresponding to the three different orientations are determined by various interactions including hydrogen bonding. The position of –OH group on the isooctyl side chain of the oxysterol and acyl chain length of the lipid seem to play important role in fixing the energies of different orientations.

3.5 Conclusion

Fluid – fluid coexistence in the binary lipid –sterol membrane depends on the bilayer thickness. It also depends on the position of the oxygen moiety on the isooctyl side chain of oxysterol. It implies either that the ability of the sterol to take different orientations depends on the membrane thickness, or that they are capable of forming dimers through hydrogen bonding. Observation of fluid – fluid immiscibility in many different saturated lipid model membranes containing 25HCH or 27HCH shows the generic nature of this phase behaviour.

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CHAPTER 4

Influence of 25-hydroxycholesterol and 27-hydroxycholesterol on the phase behaviour of unsaturated PC/PE/ether PC model membranes

4.1 Introduction

Influence of the hydrocarbon chain length of saturated PCs on the fluid – fluid coexistence in model membranes of lipid – sterol binary mixtures was described in the previous chapter. In this chapter, we investigate the effect of unsaturation of the hydrocarbon chains of the lipid on fluid – fluid coexistence. For this, we use multilayers of POPC (1-palmitoyl-2-oleoyl-glycero-3-phosphocholine) with 25HCH and 27HCH. Influence of the nature of the lipid head group on the phase behavior of lipid – sterol model membranes is also studied using DLPE (1,2-dilauroyl-sn-glycero-3-phosphoethanolamine) multilayers containing 25HCH and 27HCH. In addition, the effect of chain glycerol linkage of the lipid on fluid – fluid coexistence is investigated using 14:0 diether PC (1, 2-di-O-tetradecyl-sn-glycero-3-phosphocholine) multilayers with 25HCH and 27HCH.

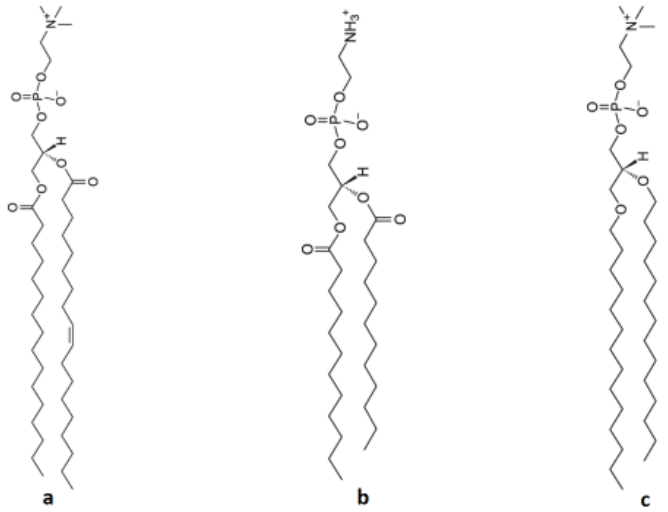


Fig 4.1: Structure of (a) POPC, a mixed acyl lipid with one saturated chain and one unsaturated chain (b) DLPE, a saturated lipid having phosphatidylethanolamine as its head group and (c) 14:0 diether PC, saturated lipid having ether bond between the glycerol backbone and the hydrocarbon chains.

Mixed acyl lipids with one saturated chain and one unsaturated chain, such as POPC, are important components of biological membranes [1]. Earlier X-ray scattering studies on membranes of POPC-25HCH and POPC-27HCH mixtures did not show fluid – fluid coexistence above the chain melting transition temperature T_m [2]. However, tension-induced fluid – fluid coexistence has been observed in GUVs of POPC with 20 mol% of 27HCH (Fig 4.2) [3].

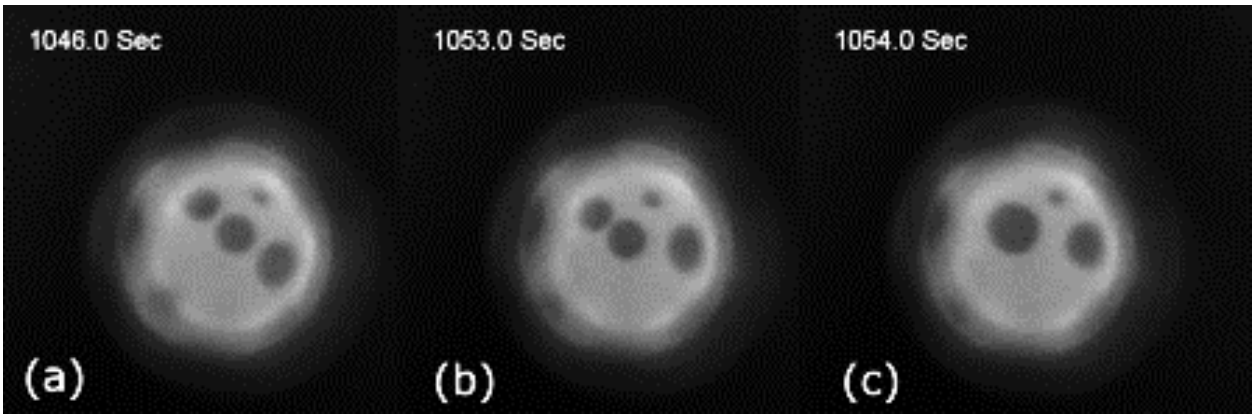


Fig 4.2: Diffusion and coalescence of domains observed in GUVs of POPC containing 20 mol% of 27HCH. This osmotic pressure induced phase separation was observed when the vesicles, prepared in 50 mM sucrose solution, were transferred to water at room temperature [3].

In this chapter, we describe fluorescence microscopy studies on POPC-25HCH and POPC-27HCH multilayers. POPC does not show two phase coexistence with 25HCH, whereas POPC-27HCH multilayers have a tendency to exhibit fluid – fluid coexistence above T_m, as evidenced by the fact that this behaviour is observed occasionally, but not always. To probe the matter in detail, phase behavior of POPC-16:1 PC (3:1) model membranes with 27HCH is examined and two phase coexistence above T_m is observed unambiguously.

PCs and PEs are the most abundant phospholipid head groups present in biological membranes [4]. Nature of the polar head group is known to be important in the phase behavior of lipid – sterol membranes. For example, phase behavior of DLPE – Cholesterol membranes, determined using X-ray diffraction techniques, is found to be different from that of DLPC-Cholesterol membranes (Fig 4.3) [5]. In this chapter we also describe fluorescence microscopy studies on supported multilayers of DLPE with 25HCH and 27HCH.

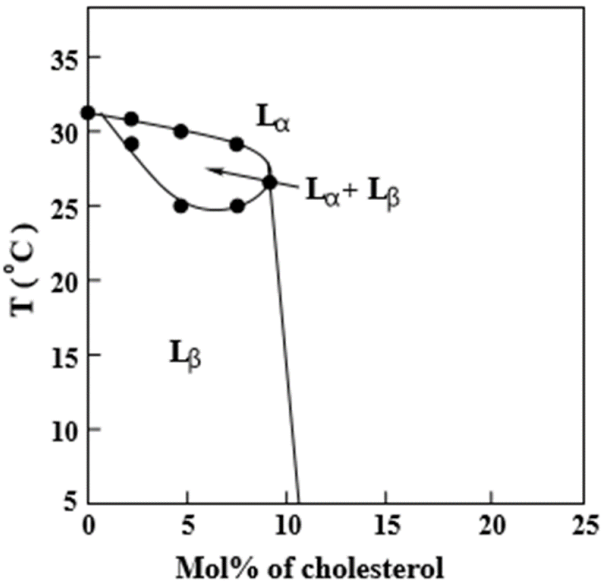


Fig 4.3: Partial phase diagram of DLPE – Cholesterol mixtures at RH 98% determined from SAXS data [5].

Ether-linked lipids are the major constituent of archaea membranes, whereas ester-linked lipids are common in bacterial and eukaryotic membranes [6]. Phase behavior of PC membranes is found to be dependent on the type of linkage between the chain and glycerol backbone of lipid molecules [7, 8, 9]. Effect of cholesterol on the properties of ester and ether linked PC membranes has been studied [10]. In this chapter we report fluorescence microscopy observations on supported multilayers of 14:0 diether PC containing 25HCH and 27HCH. A two-phase region above T_m is observed in both systems.

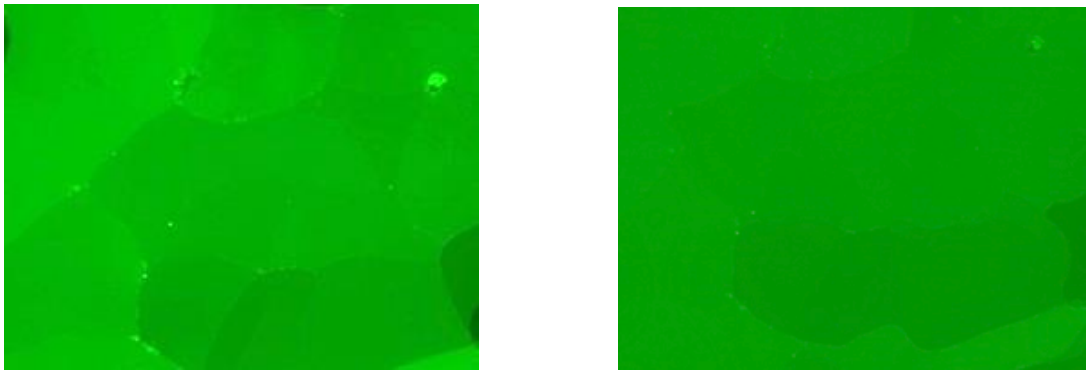
4.2 Materials of Study

1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC), 1,2-dilauroyl-sn-glycero-3-phosphoethanolamine (DLPE), 1,2-di-O-tetradecyl-sn-glycero-3-phosphocholine (14:0 diether PC), 1,2-dipalmitoleoyl-sn-glycero-3-phosphocholine (16:1 (Δ9-Cis) PC), 25-hydroxycholesterol (25HCH) and 27-hydroxycholesterol (27HCH) were obtained from Avanti Polar Lipids in powder form. Stock solutions were prepared in chloroform (HPLC grade) obtained from Sigma-Aldrich. Rhodamine B 1, 2-Dihexadecanoyl-sn-Glycero-3-Phosphoethanolamine, Triethylammonium Salt (Rhodamine-DHPE) dye was obtained from Invitrogen bioservices.

4.3 Experimental Results

4.3.1 POPC multilayers with 25HCH and 27HCH

A systematic investigation on mixtures of 25HCH or 27HCH with POPC was carried out using fluorescence microscopy. Supported multilayers of POPC with 25HCH do not show fluid – fluid coexistence above 10^0C (T_m for POPC = -2^0C). Fluorescence microscopy images of POPC multilayers containing 15 mol % of 25HCH at temperatures 13^0C and 27^0C are shown in Fig. 4.4.



a b

Fig 4.4: Fluorescence microscopy images of supported multilayers of POPC containing 15 mol% 25HCH at 100% relative humidity: fluid phase at 13^oC (a) and 27^oC (b); image size 0.4 mm x 0.35 mm.

Behavior of POPC multilayers with 15 mol% 27HCH is found to be different from that of POPC-25HCH membranes. On heating from 10^oC, domains are observed between 18^oC and 30^oC occasionally in parts of the samples, but not always [Fig.4.5].

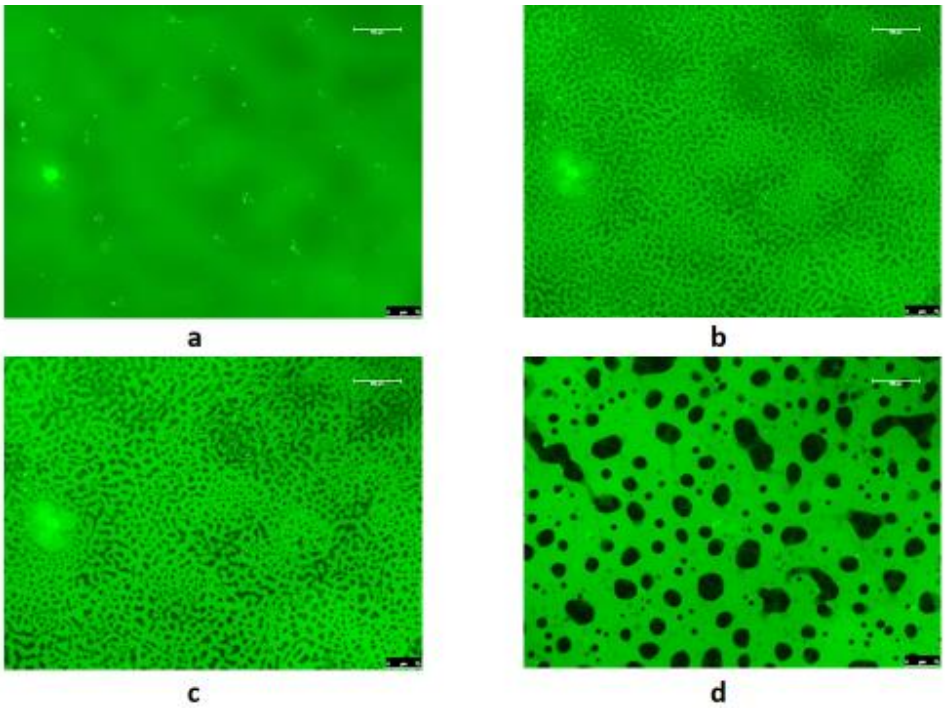


Fig 4.5 : Fluorescence microscopy images of supported multilayers of POPC containing 15 mol% 27HCH at 100 % relative humidity at temperatures 12^{o}C (a), 18^{o}C (b) 19^{o}C (c) and 30^{o}C (d) ; scale bar = 100 μm.

In order to probe this behavior in detail, 20 mol% doubly unsaturated lipid with smaller chain length, 16:1 PC, was mixed with POPC and then supported multilayers of the above mixture were prepared with 20 mol% 27HCH. In these samples domains appear on cooling below 24^{o}C and disappear on heating above this temperature [Fig. 4.6, 4.7]. This behaviour is observed consistently in all the samples studied.

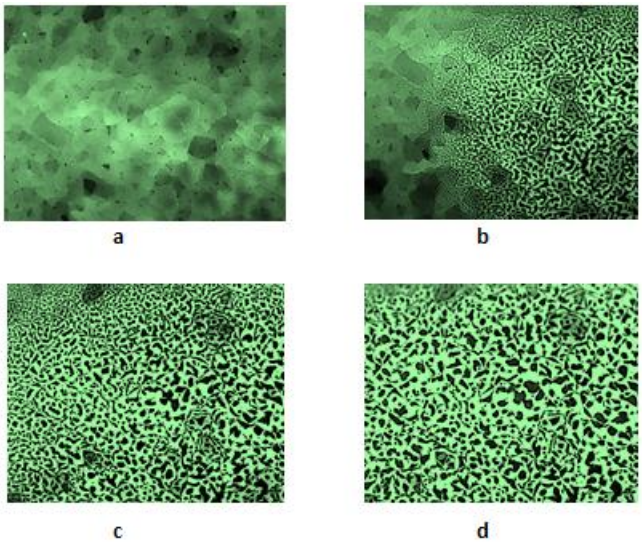


Fig 4.6: Fluorescence microscopy images of supported multilayers of POPC–16:1 PC (3:1) containing 20 mol% 27HCH at 100% RH; temperature was varied from 30^{o}C to 20^{o}C going from (a) to (d). The domains appeared at a temperature slightly below 24^{o}C (image size: 0.5 mm x 0.45 mm). Note that the dark patches in (a) are due to the non-uniform thickness of the lipid films.

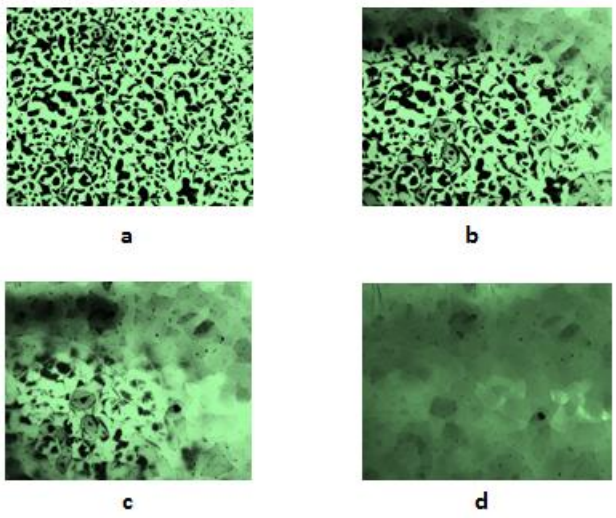
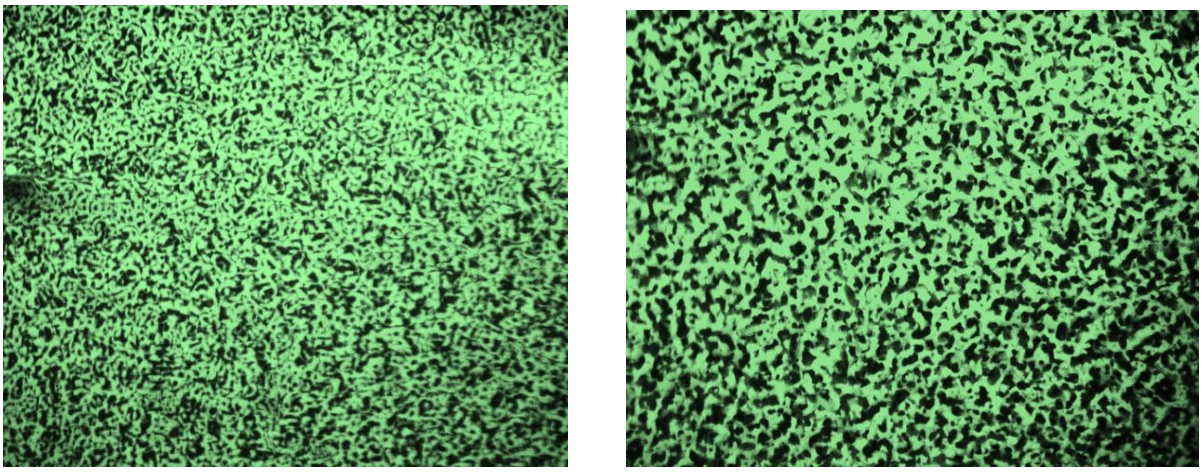


Fig 4.7: Fluorescence microscopy images of supported multilayers of POPC – 16:1 PC (3:1) containing 20 mol% 27HCH at 100% RH: The sample temperature was varied from 20^{o}C to 30^{o}C from (a) to (d); the domains disappeared at a temperature slightly above 24^{o}C (image size: 0.5 mm x 0.45 mm).

Coalescence of domains clearly indicates fluid nature of both the phases [Fig 4.8]. POPC- 16:1 PC (3:1) multilayers containing 10 mol% of 27HCH also shows fluid – fluid coexistence below 24^{o}C.

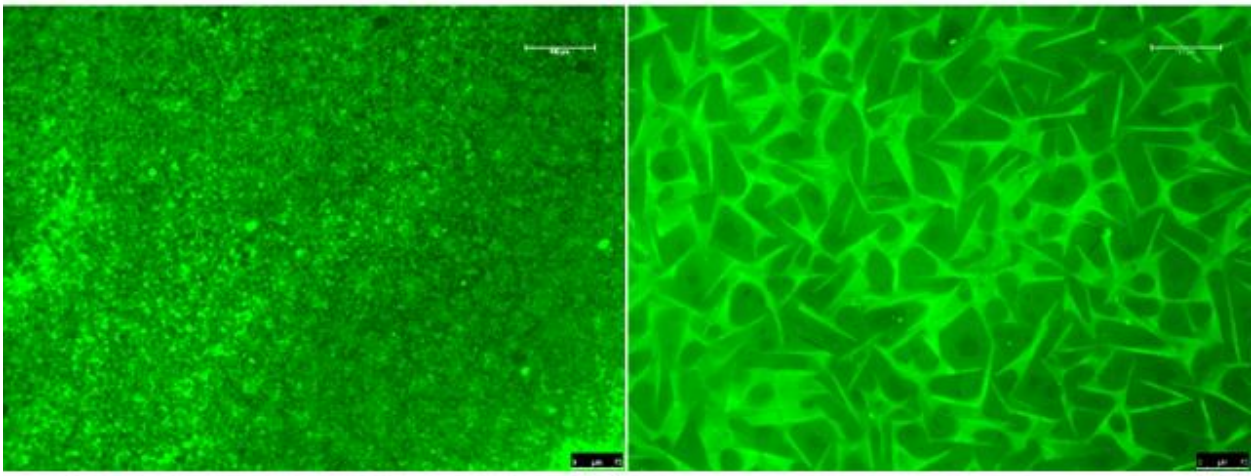


a b

Fig 4.8: Fluorescence microscopy images of supported multilayers of POPC – 16:1 PC (3:1) containing 20 mol% 27HCH at 100% RH: coalescence at a temperature slightly below 24^{o}C. (Image size: 0.5 mm x 0.45 mm). The time elapsed between the two images is of the order of a few minutes.

4.3.2 DLPE multilayers with 25HCH and 27HCH

To understand the dependence of the head group on fluid – fluid coexistence above T_m, supported multilayers of DLPE were prepared with 20 mol% of 25HCH and 27HCH. Observations were carried out under a fluorescence microscope from 30^{o}C to 60^{o}C (T_m of DLPE = 29^{o}C). DLPE membranes containing 25HCH do not show fluid – fluid coexistence above the chain melting temperature. Two phase coexistence is also not observed in DLPE membranes containing 27HCH. 27HCH is found to crystallize out of the membrane above the chain melting temperature of DLPE (29^{o}C) [Fig 4.9].

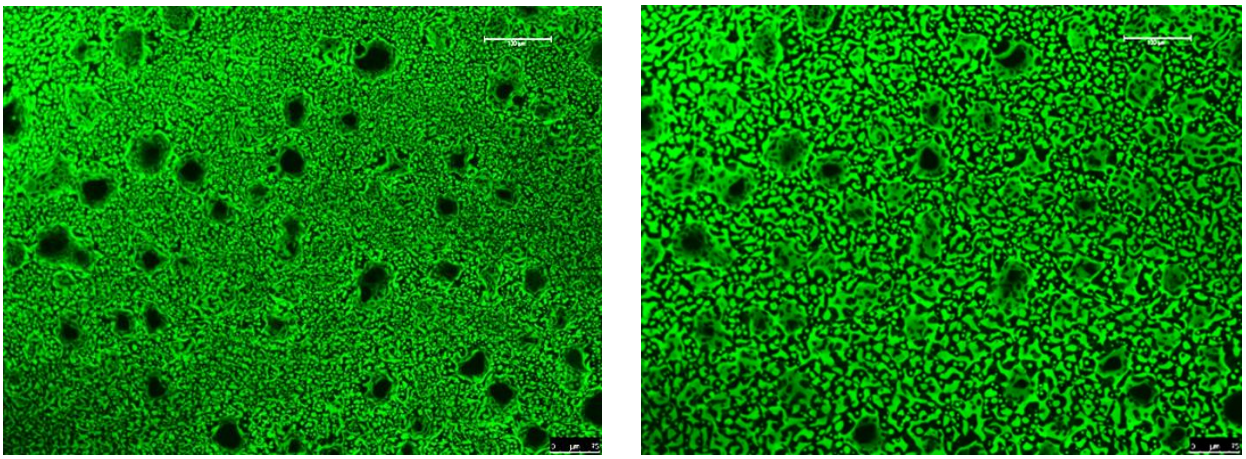


a b

Fig 4.9: Fluorescence microscopy images of supported multilayers of DLPE containing 20 mol% (a) 25HCH (b) 27HCH at 100 % relative humidity and 35^0C (scale bar 100μm).

4.3.3 14:0 diether PC multilayers with 25HCH and 27HCH

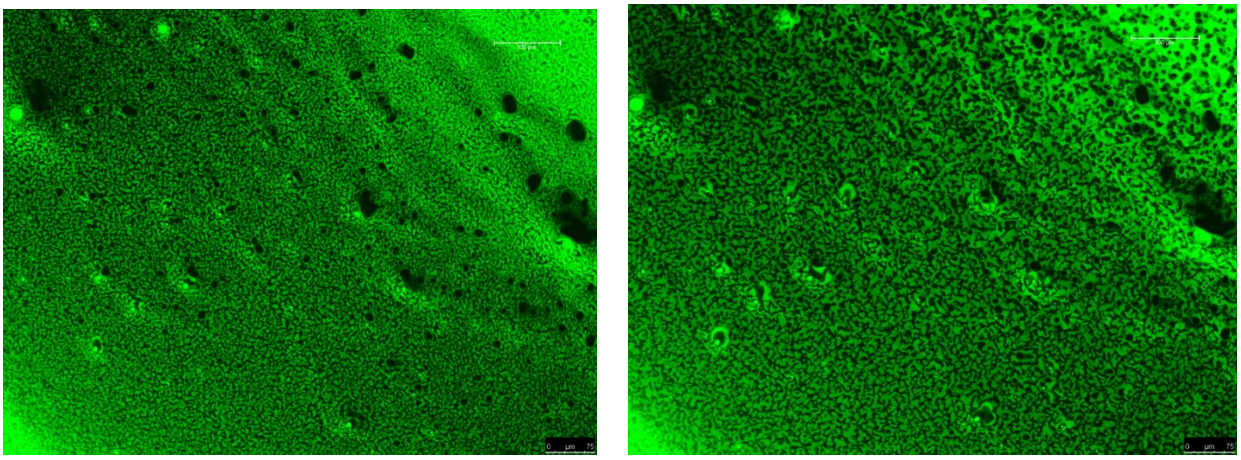
To study the role of the linkage between the glycerol backbone and the hydrocarbon chains on the fluid – fluid coexistence, supported multilayers of 14:0 diether PC with 25HCH and 27HCH were prepared. Fluorescence microscopy images of 14:0 diether PC membranes containing 20 mol% 25HCH show fluid – fluid coexistence above 31^oC. Coalescence of domains is observed, which confirms the fluid nature of the two phases [Fig 4.10].



a b

Fig 4.10 : Fluorescence microscopy images of supported multilayers of 14:0 diether PC containing 20 mol% of 25HCH at 100% RH at 31^0C (a) and at 38^0C (b). Coalescence of the domains shows the fluid nature of the two phases (scale bar 100 μm).

With 10 mol% of 27HCH, 14:0 diether PC membranes show domains above 30^oC and coalescence of domains confirms their fluid nature [Fig 4.11].



a b

Fig 4.11 : Fluorescence microscopy images of supported multilayers of 14:0 diether PC containing 10 mol% of 27HCH at 100% relative humidity and 31^0C (a) and 32^0C (scale bar 100 μm).

4.4 Discussion

Molecular dynamics simulations of 25HCH molecule in POPC bilayers show three preferred orientations in the membrane compared to a single orientation in the case of cholesterol. The three orientations are cholesterol-like, inverted and interfacial [11]. These different orientations of the oxysterol were explained based on the presence of a second -OH group at the tip of the oxysterol chain which is absent in cholesterol.

In the previous chapter, fluid – fluid coexistence in saturated PC- 25HCH/27HCH model membranes above T_m was discussed. From fluorescence microscopy observations and from SAXS studies [12] it is found that the acyl chain length of the saturated PCs plays an important role in the fluid – fluid phase separation behaviour. Molecular dynamics simulations clearly show that the observed phase separation is due to different orientations of the sterol molecules in the membrane which depend on the temperature [13].

Fluorescence microscopy observations of model membranes described in the previous chapter clearly show that DPPC, which has two saturated 16 carbon chains, does not show phase separation with 25HCH; but it shows with 27HCH. On the other hand, DLPC, which has two saturated 12 carbon chains, shows fluid-fluid coexistence with 25HCH, but not with 27HCH. As discussed in the previous chapter, these observations suggest that fluid-fluid coexistence in these symmetric saturated PCs occurs only for a narrow range of the bilayer thickness which depends on the specific oxysterol. The range for 27HCH extends from $16 \geq n \geq 13$, whereas that for 25HCH extends from $15 \geq n \geq 12$, where n is the number of C atoms in the chain. Since POPC has a saturated chain of 16 carbon atoms and an unsaturated 18 carbon chain, it is possible that its bilayer thickness falls only slightly above the range required for 27HCH, but much above the range for 25HCH. This conclusion is supported by the fact that GUVs prepared from POPC-27HCH membranes show fluid-fluid coexistence above a threshold value of the membrane tension, since membrane tension leads to the lowering of the bilayer thickness [3]. On the other hand, POPC-25HCH membranes do not exhibit this behaviour, suggesting that the bilayer thickness is not lowered sufficiently under tension in this case. It is therefore possible that the sporadic occurrence of fluid-fluid coexistence in POPC-27HCH membranes observed in the present study is a consequence of the fact that in some of the samples the membranes are under higher tension due to differences in the sample conditions, such as relative humidity. Our

observation of fluid-fluid coexistence in POPC-16:1 PC mixtures containing 27HCH is consistent with the above explanation, since the addition of shorter 16:1 PC to POPC will result in the lowering of the bilayer thickness. Thus these observations indicate that fine tuning of the bilayer thickness is required for the occurrence of fluid-fluid coexistence in lipid-sterol membranes. Moreover, this sensitivity to the bilayer thickness also suggests a possible role of hydrogen bonding between the -OH groups of the oxysterol molecules buried deep in the two leaflets of a membrane. Further studies are required to confirm this possibility. The above results also show that fluid-fluid coexistence can be induced in PCs with unsaturated chains. This behaviour could be of much biological relevance since all biomembranes are generally enriched in such lipids.

Phase diagrams of saturated PC membranes exhibit an intermediate ripple phase between the fluid and gel phases; whereas that of saturated PE membranes does not [5]. Saturated PEs exhibit an untilted gel phase, whereas saturated PCs exhibit a tilted gel phase. As a result phase behavior of DLPE- cholesterol bilayers is found to be different from that of saturated PC – cholesterol systems [5,14].Absence of a fluid – fluid coexistence region in DLPE -25HCH and DLPE -27HCH membranes may be due to the above mentioned inherent differences in the phase behaviors of PE and PC membranes. It may be noted here that POPC is also known to exhibit the ripple phase [15].

Different phase behaviors have been observed for ether lipid membranes and ester lipid membranes due to the higher hydrophilic character of the ester group [7-9]. The presence of the carbonyl group (-C=O) in ester lipids and its absence in ether lipids was found to affect the interaction between phosphate group and water molecules. Even then 14:0 diether PC membranes containing 25HCH and 27HCH exhibit fluid –fluid coexistence above T_m. So two phase coexistence above T_m is found to be independent of the type of the linkage between the glycerol backbone and the acyl chains.

4.5 Conclusion

Fluid – fluid immiscibility is observed in unsaturated PC model membranes containing 27HCH, and hence might be relevant in biological membranes. This behaviour is not observed in PE membranes. Occurrence of this phase behavior both in ester linked PC (dominant in Bacterial and Eukaryotic membranes) and ether linked PC (dominant in archaea membranes) is also biologically relevant.

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CHAPTER 5

Influence of cholestenoic acid and a few other cholesterol derivatives on the phase behaviour of saturated PC multilayers

5.1 Introduction

Influence of 25HCH and 27HCH on the phase behavior of saturated PC multilayers has been discussed in the previous chapters. In this chapter we discuss the influence of cholesterol, cholestenone, 27-hydroxy cholestenone and cholestenoic acid [Fig 5.1] on the phase behavior of saturated PC multilayers.

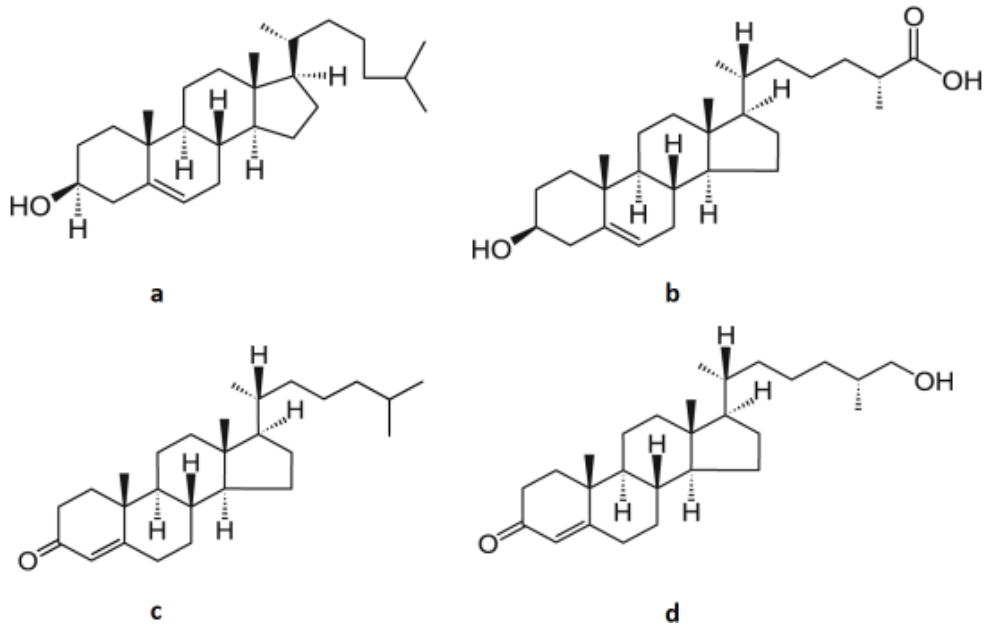

Fig 5.1: Structure of (a) Cholesterol, (b) Cholestenoic acid, (c) cholestenone and (d) 27-hydroxy cholestenone.

A partial phase diagram of DMPC – Cholesterol mixtures at 98% relative humidity has been determined using SAXS data [1]. A modulated phase was found in DMPC – Cholesterol membranes at intermediate cholesterol concentrations in addition to the ripple phase seen between the main and pre-transitions [Fig 5.2]. The modulated phase and the ripple phase were also observed in DPPC – Cholesterol membranes [2].

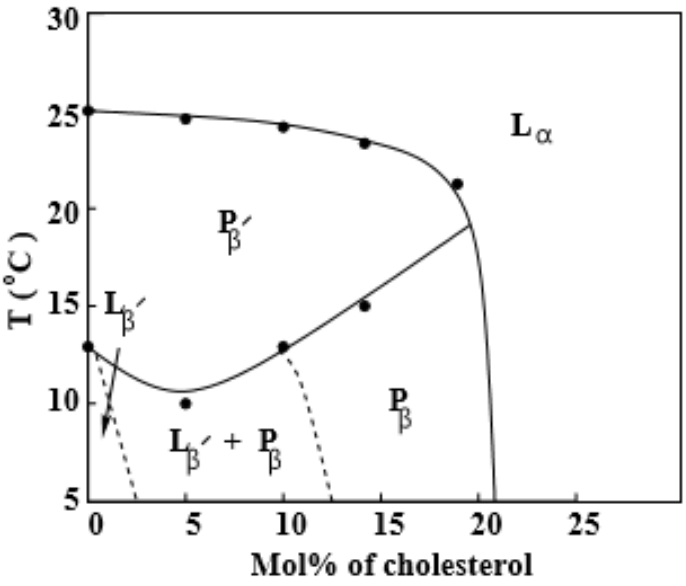

Fig 5.2: Partial phase diagram of DMPC – Cholesterol mixtures at RH 98% determined from SAXS data [1].

Oxysterols found in biological membranes mainly result from the enzymatic oxidation of cholesterol [3]. Addition or replacement of functional groups in cholesterol can change its interaction with other membrane molecules and can lead to changes in membrane properties. Oxidation of cholesterol can lead to the production of cholestenone by replacing the hydroxyl group of cholesterol by keto group [4]. Production of cholestenone from cholesterol is found to reduce membrane order or to increase membrane fluidity [5, 6]. Phase behavior of DPPC model membranes containing cholestenone has been studied using SAXS and the phase diagram [Fig 5.3] constructed is found to be very similar to that of DPPC – Cholesterol mixtures [7].

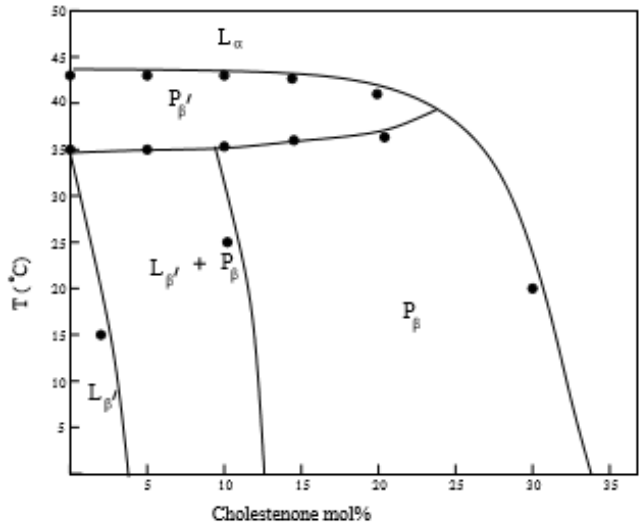

Fig 5.3: Partial phase diagram of DPPC – Cholestenone mixtures at RH 98% determined from SAXS data [7].

27-cholestenoic acid is found in the acidic cholesterol elimination pathway [12]. Elimination of cholesterol as cholestenoic acid from human lung has also been studied [13].

Influence of 27- cholestenoic acid on the phase behavior of DLPC, DMPC and DPPC model membranes is investigated in this chapter and the observed fluid-fluid coexistence is discussed. Effect of cholesterol, cholestenone and 27-hydroxycholestenone on the phase behavior of DMPC multilayers is also studied and no fluid-fluid coexistence above the chain melting temperature is observed in these systems.

5.2 Materials of Study

Saturated lipids DLPC, DMPC and DPPC were obtained from Avanti Polar Lipids in powder form. Cholesterol, Cholestenone, 27-hydroxy cholestenone and cholestenoic acid (3ß-hydroxy-5-cholestenoic acid) were also purchased from Avanti Polar Lipids. Stock solutions were made in chloroform (HPLC grade) obtained from Sigma-Aldrich. L_d phase marker Rhodamine-DHPE dye was obtained from Invitrogen bio services.

5.3 Experimental Results

5.3.1 DLPC multilayers with Cholestenoic acid

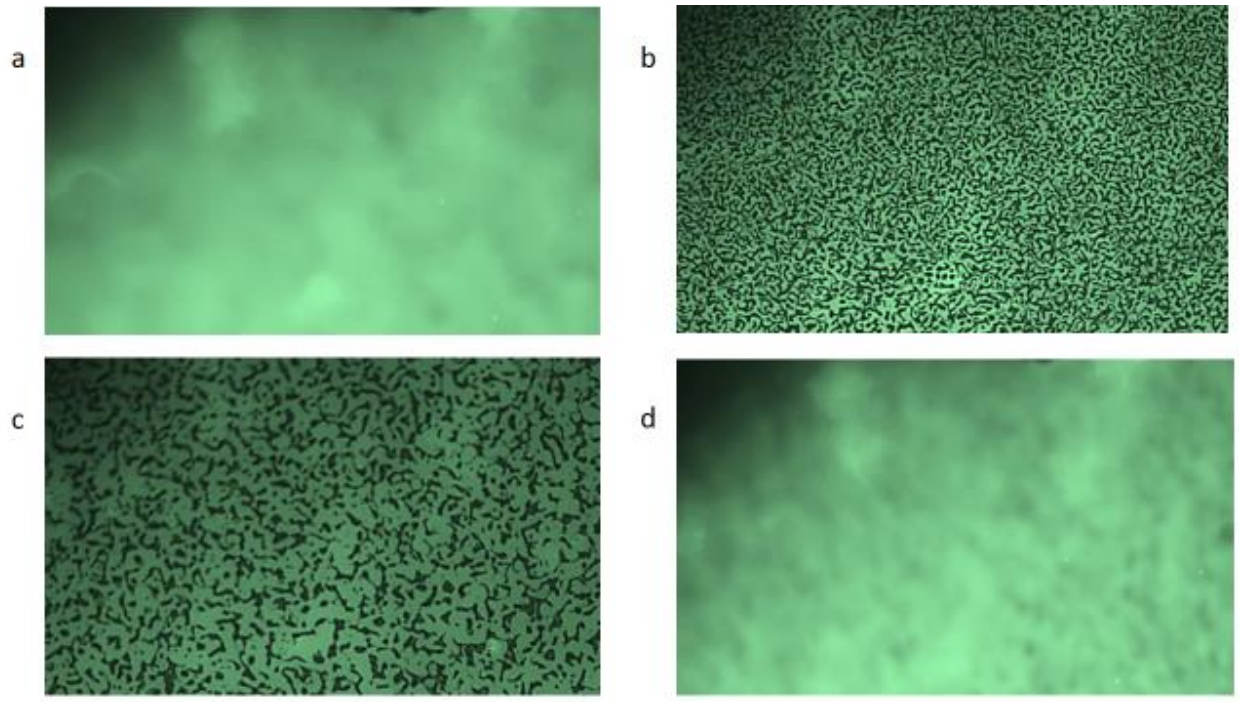

Fig 5.4: Fluorescence microscopy images of supported multilayers of DLPC containing 20 mol% cholestenoic acid at 100% relative humidity: fluid phase at 18^{o}C (a), fluid-fluid coexistence and coalescence at 22^{o}C (b, c) and disappearance of domains at 32^{o}C (d), (image size: 0.6 mm x 0.4 mm).

A systematic investigation on DLPC multilayers containing 20 mol% of cholestenoic acid was carried out using fluorescence microscopy. Sample temperature was varied from 15^{o}C to 35^{o}C. Fluid-fluid coexistence is observed between 18^{o}C and 32^{o}C [Fig 5.4]. Observed domains are found to coalesce, which confirms their fluid nature. Low temperature and high temperature boundaries of the two-phase region were determined both on heating and cooling.

5.3.2 DMPC multilayers with Cholestenoic acid

Supported multilayers of DMPC were prepared with 20 mol% of cholestenoic acid. Observations were carried out under fluorescence microscopy from 20^{o}C to 60^{o}C. Fluid – fluid immiscibility above chain melting temperature is observed in this case too. The low temperature

boundary of two phase coexistence was observed at a temperature of around 32^0C.Domains were also found to coalesce. The fluid – fluid coexistence was found to disappear above 60^0C [Fig 5.5].

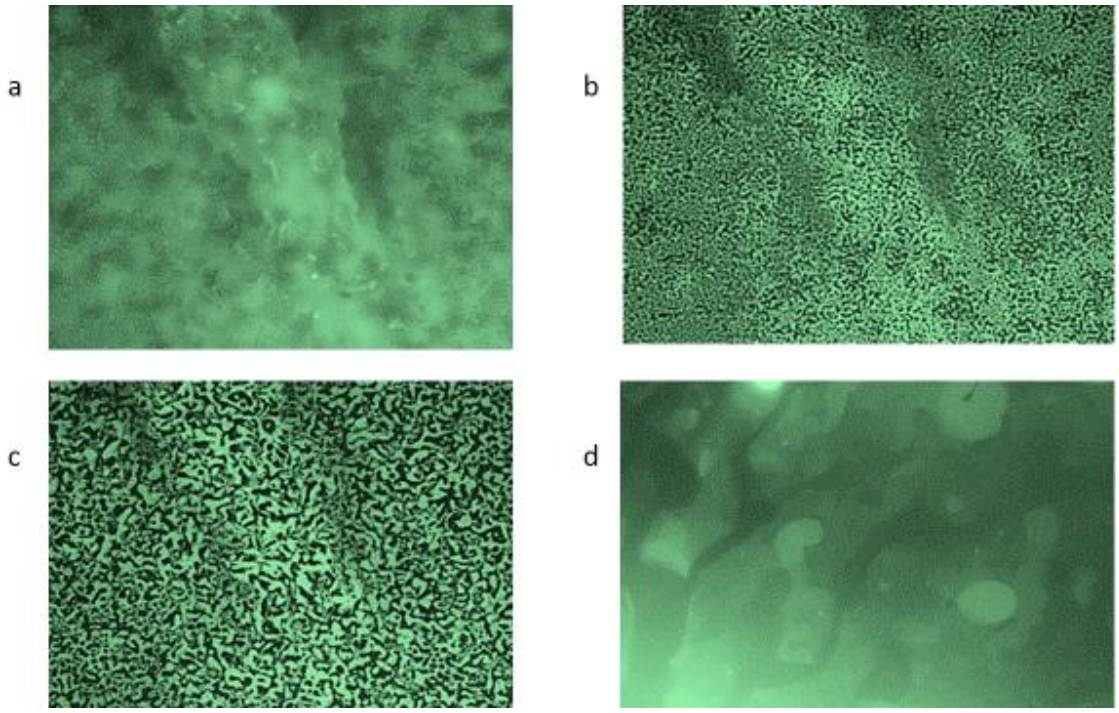

Fig 5.5: Fluorescence microscopy images of supported multilayers of DMPC containing 20 mol% cholestenoic acid at 100% relative humidity: fluid phase at 29^oC (a), fluid-fluid coexistence and coalescence at 32^oC (b, c) and fluid phase at 60^oC (d), (image size: 0.6 mm x 0.4 mm)

5.3.3 DPPC multilayers with Cholestenoic acid

Supported multilayers of DPPC containing 20 mol% of cholestenoic acid also show fluid – fluid coexistence from 40^0C to 48^0C. Coalescence of domains confirms their fluid nature [Fig 5.6].

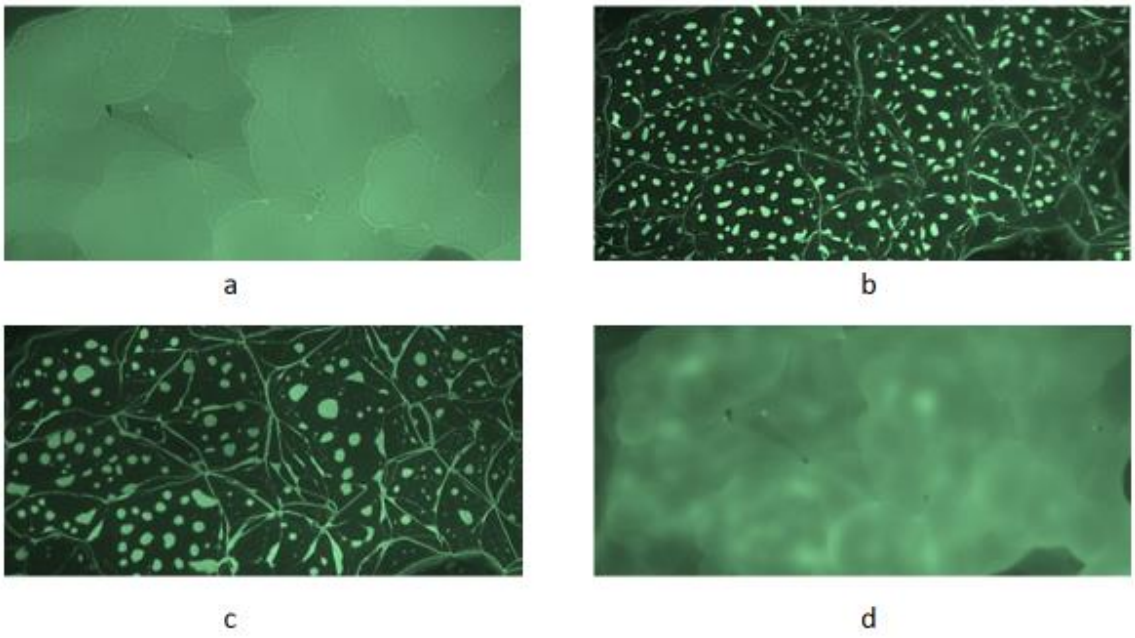

Fig 5.6: Fluorescence microscopy images of supported multilayers of DPPC containing 20 mol% cholestenoic acid at 100% relative humidity: fluid phase at 39^{o}C (a), fluid-fluid coexistence and coalescence at 45^{o}C (b, c) and disappearance of domains at 48^{o}C (d), (image size: 0.4 mm x 0.2 mm).

5.3.4 DMPC multilayers with Cholesterol

Phase behavior of DMPC multilayers with 20 mol% cholesterol was investigated and no fluid – fluid coexistence is observed above chain melting temperature. Sample temperature was varied from 25^{0}C to 45^{0}C [Fig 5.7].

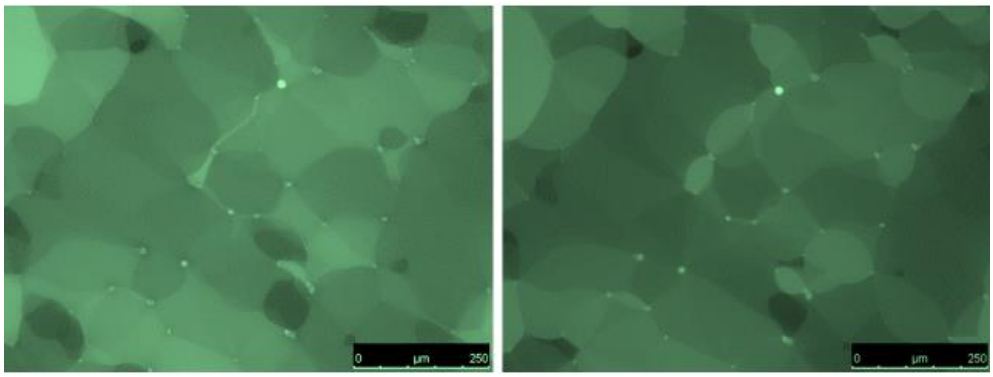

Fig 5.7: Fluorescence microscopy images of supported multilayers of DMPC containing 20 mol% Cholesterol at 100% relative humidity: fluid phase at 25^{o}C (a) and at 40^{o}C (b) Scale bar: 250 µm.

5.3.5 DMPC multilayers with Cholestenone

We studied the phase behavior of DMPC multilayers containing 20 mol% cholestenone. No fluid – fluid coexistence is observed above chain melting temperature. Observations were carried out from 25^{0}C to 45^{0}C [Fig 5.8].

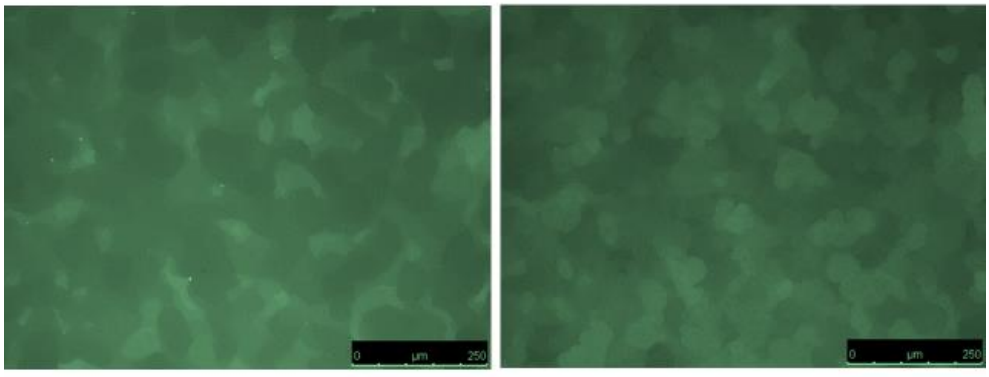

Fig 5.8: Fluorescence microscopy images of supported multilayers of DMPC containing 20 mol% Cholestenone at 100% relative humidity: fluid phase at 25^{o}C (a) and at 40^{o}C (b) Scale bar: 250 µm.

5.3.6 DMPC multilayers with 27-hydroxy cholestenone

Fluorescent microscopy observations on DMPC multilayers with 20 mol% 27-hydroxy cholestenone were carried out from 25^0C to 45^0C. No fluid – fluid coexistence is observed above chain melting temperature [Fig 5.9].

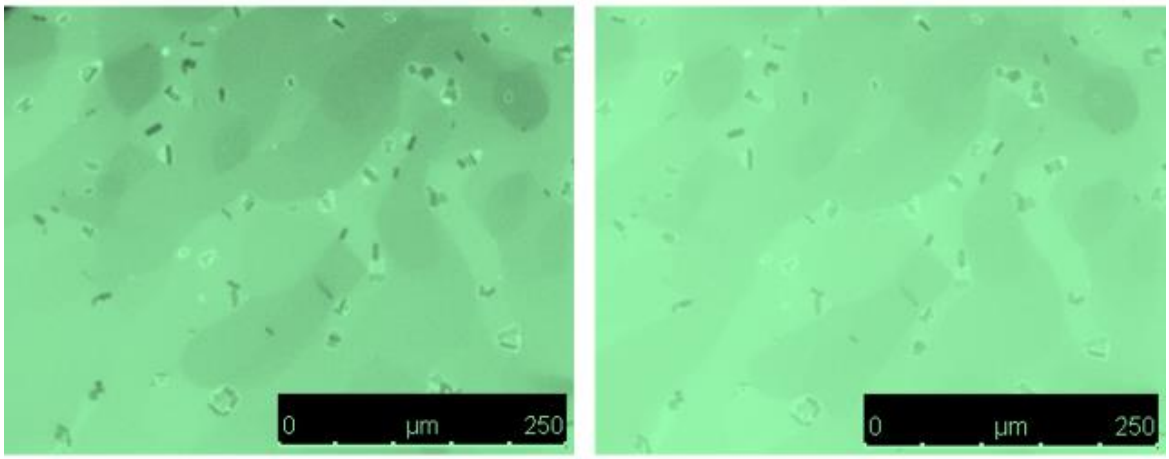

Fig 5.9 : Fluorescence microscopy images of supported multilayers of DMPC containing 20 mol% 27- hydroxy cholestenone at 100% relative humidity: fluid phase at 25^oC (a) and at 40^oC (b) Scale bar : 250 µm.

5.4 Discussion

Absence of fluid- fluid coexistence in DMPC- cholesterol membranes is consistent with previous studies [1]. It is also evident from computer simulations that cholesterol has only one orientation in saturated PC membranes [16]. It has also been shown that fluid- fluid immiscibility requires temperature dependent orientations of sterols in membranes. Phase behaviour of cholestenone in saturated PC membranes is similar to that of cholesterol [7] and no fluid- fluid coexistence is observed.

25HCH induces fluid – fluid coexistence in both DLPC and DMPC membranes whereas 27HCH induces fluid – fluid immiscibility in DMPC and DPPC bilayers. Fluid – fluid coexistence induced by cholestenoic acid is observed in DLPC, DMPC and DPPC membranes.

The stronger ability of cholestenoic acid to induce phase separation may be due to the presence of carboxyl group in the side chain of the sterol. Carboxylic acid moiety is considered as a more polar functional group than the hydroxyl group [14].

Side chain oxysterols are known to take different orientations in the membrane, such as cholesterol-like, inverted and interfacial orientations [9, 10]. Presence of keto group in 27-hydroxy cholestenone may prevent it from taking different orientations in the membrane. It has also been reported that the keto group cannot act as a hydrogen bonding donor [15], which might also have a bearing on the absence of fluid-fluid coexistence in this case.

Cholesterol and cholestenone do not contain any hydrogen bonding species in their side chain. Absence of phase separation in saturated PC membranes containing these sterols may be due to their inability to form hydrogen bonds, in addition to their inability to take different orientations in the membrane.

In this study fluid-fluid coexistence is observed in oxysterols having hydrogen bonding moieties present on both the steroid ring and at the tip of the side chain. It is, therefore, possible that these molecules can take different orientations in the membrane depending on the temperature, leading to phase separation. Further study is needed to confirm this hypothesis.

5.5 Conclusion

Fluid – fluid coexistence observed in model membranes containing side chain oxysterols which are found in the cellular cholesterol elimination pathways is biologically important. It is likely that formation of dimers of sterol molecules through hydrogen bonding plays a crucial role in the phase separation behaviour.

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CHAPTER 6

Summary and Future Directions

6.1 Summary

In this thesis we have presented first examples of fluid-fluid coexistence in binary lipid-sterol membranes. Earlier spectroscopic studies and supporting computer simulations had suggested the occurrence of fluid-fluid phase separation in binary lipid-cholesterol systems. However subsequent studies did not provide any evidence for such behaviour. Interestingly we have observed that various binary PC membrane systems containing 25HCH, 27HCH or cholestenoic acid exhibit two fluid phase coexistence above the chain melting temperature. The common structural feature of all these sterol molecules is that they contain hydrophilic groups at both ends.

Fluid-fluid coexistence in ternary systems containing a saturated PC, an unsaturated PC and cholesterol has been studied extensively. But this phase separation is observed only below the chain melting temperature of the saturated PC, where cholesterol converts fluid-gel phase separation into fluid-fluid phase separation.

We have presented fluorescence microscopy observations of fluid-fluid immiscibility both in supported multilayers and in GUVs of PC-oxysterol systems. Coexistence of two fluid phases in GUVs confirms that interactions within each bilayer are responsible for phase separation. X-ray diffraction studies on DLPC-25HCH, DMPC-25HCH, DMPC-27HCH and DPPC-27HCH provide clear evidence for this phase coexistence. Computer simulations on DMPC-27HCH membranes suggest that the observed fluid-fluid phase separation is associated with different orientations of oxysterol molecules in the lipid bilayer as a function of temperature.

We have also observed that the two fluid phase coexistence is sensitive to the acyl chain length of the saturated PCs. It implies either that the ability of the sterol to take different orientations depends on the membrane thickness, or that they are capable of forming dimers through hydrogen bonding. We have also found that the observed phase behaviour depends on the position of the hydroxyl moiety on the isooctyl side chain of the oxysterol.

Tension induced fluid-fluid phase separation has been observed in GUVs made from POPC-27HCH membranes. It clearly shows that fluid-fluid coexistence can be induced in bilayers of monounsaturated lipids by lowering membrane thickness. We have reduced the bilayer thickness by adding 16:1 PC to POPC and membranes formed from these mixtures containing 27HCH are found to exhibit fluid-fluid immiscibility. This result shows that fluid-fluid coexistence can be induced in membranes of unsaturated PCs. This has much biological relevance as biomembranes normally contain large fraction of unsaturated lipids.

Fluid-fluid coexistence is observed in membranes formed by ether-linked lipids as well as by ester-linked lipids. . This shows that the phase separation is independent of the linkage between the glycerol backbone and the acyl chains. This result is biologically relevant since ester linked PCs are dominant in bacterial and eukaryotic membranes, whereas ether linked PCs are dominant in archaea membranes.

We have observed fluid-fluid coexistence in PC membranes containing 25HCH, 27HCH or cholestenoic acid, but no phase separation with 27-hudroxy cholestenone. The above sterols showing this phase behaviour are biologically relevant as they are found in cholesterol elimination pathways. We speculate that sterols that have hydrogen bonding moieties on both the sterol ring and at the tip of the side chain show two fluid phase separation.

6.2 Future Work

1. Further studies are needed to understand the detailed mechanism of phase separation induced by temperature dependent conformations of the sterol molecule.

2. More studies are essential to understand the role of bilayer thickness in fluid-fluid immiscibility. Further studies are needed to confirm the role of hydrogen bonding in the phase separation behaviour.

3. Further studies are required to understand the dependence of fluid-fluid coexistence on the position of the hydroxyl moiety on the isooctyl side chain of the oxysterol.

4. Whether doubly unsaturated PCs with appropriate chain length show fluid-fluid coexistence is an open question.