Reorganization and nanoscale domain formation in phospholipid Langmuir-monolayer

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Reorganization of DPPC at air-water interface is studied by Langmuir-Blodgett technique. Reorganization process is found to be surface-pressure and temperature dependent. Surface pressure dependent study shows that the domains formation rates are different at different pressure region. Two-dimensional reorganization process is the main reason for domain formation. Surface morphology of transferred layer is studied by FE-SEM. At higher pressure, supported film shows a framework of nanometer sized condensed domains of DPPC. Domain sizes are in the range of 20 – 30 nm. Holes in the range of few nm to 100 nm are also present.

Introduction

Supported lipid membranes/films on solids have attracted great and considerable interest because of their scientific importance and practical applications in bio-functionalization [1]. They have also played a significant role in developing protocols for drug delivery, immunosensing system as well as biosensors [2, 3]. Phospholipids, the most essential composition of cell membrane has attracted enormous attention because of its ultra first self-aggregation. Though the hydrophobic effect is qualitatively found responsible for self-aggregation [4], actual process is more complicated.

Langmuir Blodgett (LB) method is one of the most versatile and convenient techniques for designing ultrathin artificial films with biological functions [5,6] as well as to produce active surfaces for interaction with nano metals [7]. LB monolayers formed from phospholipids, such as 1, 2-dihexadecanoyl-sn-glycero-3-phosphocoline (DPPC), are prototypical systems for investigating the structural properties of thin films. After prediction of fluid mosaic model of cell membrane [8], many studies have been done on phospholipids aggregated domain structure. Aggregated domain structures have been studied by atomic force microscopy (AFM) [9], fluorescence microscopy [10, 11], Brewster angle microscopy (BAM) [12], near-field scanning optical microscopy (NSOM) [13, 14] as well as by simulation [15]. The most obvious features of the structure of LB films are their similarity to biological membranes [16, 17]. In this respect, most of the studies of this nature are confined to the examination of monolayers on the water surface [18]. Few cases of successful transfer of single layer of phospholipids onto solid substrate have been reported [19, 20]. Hence, LB technique may be a useful tool to study phospholipid monolayer at air-water interface and subsequent transfer of film on the appropriate solid substrate.

Previously many studies had done on monolayer of single phospholipid component or on phospholipid mixtures. However, most of the studies involved in monolayers created at low surface pressures correspond to gaseous/liquid-expanded or liquid expanded/liquid condensed phase coexistence region on LB isotherm [21]. Moreover, mimicking cell membrane generally requires a higher monolayer surface pressure [22] and a few studies have done at surface pressure greater than liquid expanded and liquid condensed region. Out of these studies, the studies of surface morphology by high-resolution field emission scanning electron microscope (FE-SEM) are rare.

In the present work, the investigation was done on the formation of the LB monolayer of DPPC at different controlled surface pressures. After transferring the monolayer on hydrophilic glass by LB technique, surface morphology was observed by means of FE-SEM.

Experimental

DPPC, purchased from Sigma (>99% pure), was used as received without further purification. Teflon-bar-barrier type LB trough (Model 2000C, Apex Instruments Co., India) was used for monolayer film preparation and characterization. Spectral grade Chloroform (SRL India) was used as the spreading solvent. The subphase was triple-distilled water, deionized with a Milli-Q water purification system from Millipore (USA). The pH and resistivity of the deionized water were 6.8 and 18.2 MΩ cm respectively. All the studies are done at room temperature (26°C) unless otherwise mentioned.

For the study of surface pressure-area (π-A) isotherm, 1 mM chloroform solution of DPPC was spread on the water subphase. After a delay of ~20 minutes (to allow the solvent to evaporate), the film at the air/water interface was compressed at the rate of $1 Å^2/$ (molecule min).

The area-relaxation experiment at constant pressure was done by compressing the film at first to a predetermined surface pressure, followed by an automatic adjustment of the surface area to keep the surface pressure fixed. A relaxation curve is obtained by recording the surface area during the relaxation period.

After waiting about two hours, the monolayer was transferred onto a hydrophilic glass substrate. The glass substrates were cleaned in a liquid soap ultrasonic bath followed by repeated rinsing with Millipore water. They were then immersed in acetone in an ultrasonic bath. Finally, they were cleaned with Millipore water in the ultrasonic bath. Uniform layer of water indicates the hydrophilicity of the slide. For monolayer transfer, glass slide kept initially under water was lifted with a speed of 1mm/min. Transfer ratio was found to be unity for first layer. The surface morphology of the films was studied by high-resolution field emission scanning electron microscope (FE-SEM, Model No; JEOL JSM-6700F).

Results and Discussion

1. π-A isotherms of DPPC

Figure 1 shows π-A isotherms of DPPC measured at different temperatures. The inset shows the molecular structure of DPPC. These π-A isotherms are found to be highly temperature sensitive. Though similar in shape, a plateau like region appears in the isotherms and the pressure at which the plateau appears, increases with increasing temperature. This type of behavior gives a strong impression that the plateau originates from temperature dependent phase co-existence, associating with the first order phase transition between the liquid-expanded (LE) and liquid-condensed (LC) states [10, 23]. This is a process of reorganization of molecules. Plateau indicates the region of reorganization. The onset of reorganization is temperature dependent. With increasing temperature, reorganization starts at higher surface pressure. In the high-pressure condensed region, area/molecule is found to be temperature independent and all the isotherms tend to overlap. Only the process of reorganization is temperature sensitive. Another interesting feature is that collapse pressure is found to decrease with increase of temperature.
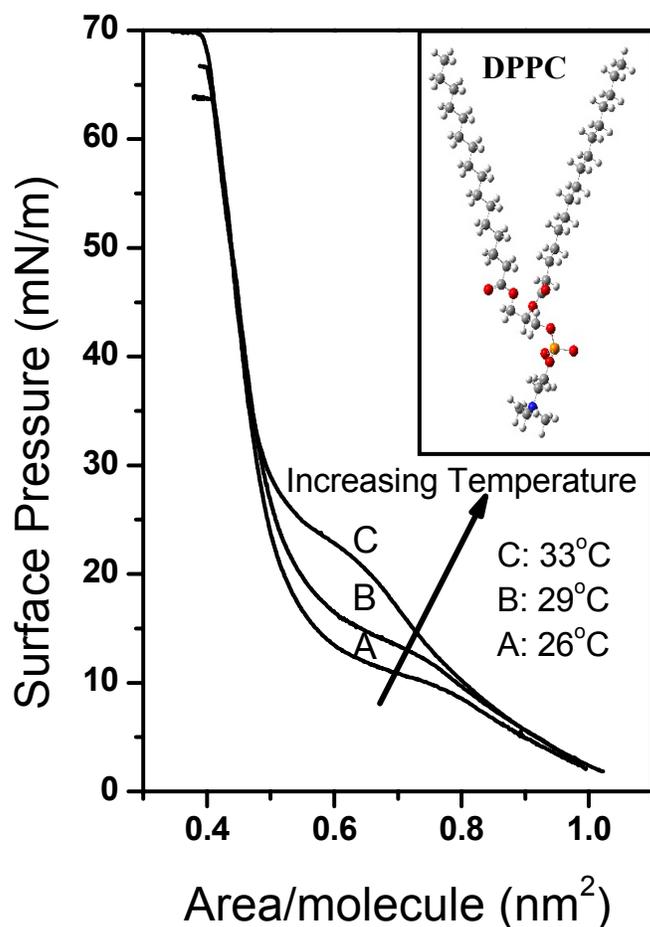

Figure-1. π-A isotherms of DPPC at different temperatures. The inset shows structure of DPPC molecules.

2. Area relaxation behavior of the monolayer.

Figure 2 shows the results of the area relaxation behavior of the monolayer at different control pressures. At constant surface pressure, the area of relaxation of insoluble monolayer was theoretically described by transformation of monolayer material into an overgrown 3D phase by Vollhardt et al [24, 25]. This model is based on the homogeneous nucleation and succeeding growth of the centers of monolayer. Simultaneous nucleation rate, growth rate and overlapping of growth centers are also considered.
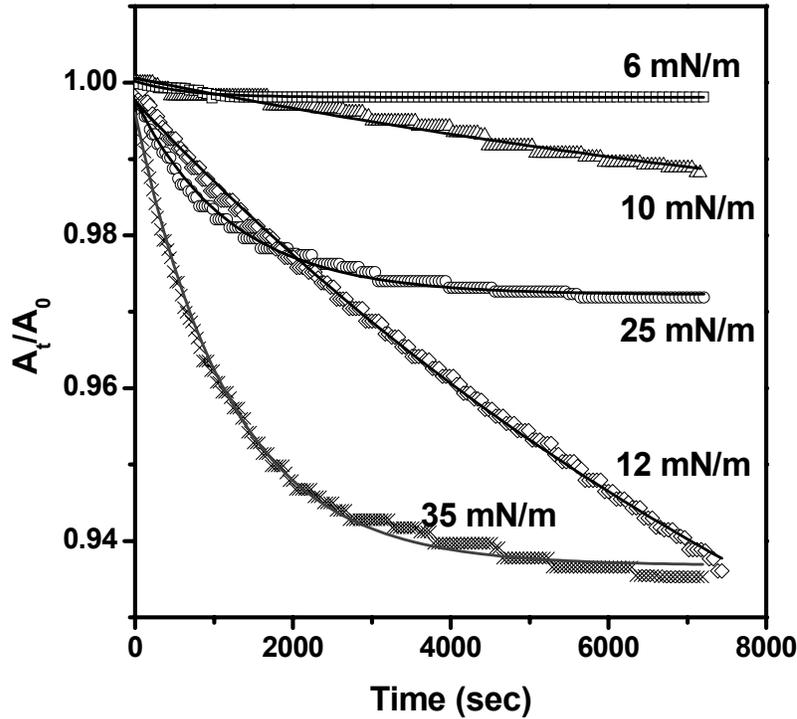

Figure-2. Relaxation curves for DPPC at different control pressures at 26°C.
Symbols are experimental data and the curved lines are the fitting with eq.2.

A general expression for different mechanisms of nucleation and growth was expressed by the formula as [25]

$$(A_0 - A_t)/(A_0 - A_\infty) = 1 - \exp(-K_x t^x) \dots\dots (1)$$

Where A_0 is the initial monolayer area, A_t is the monolayer area at time t and A_∞ is the area of the monolayer for t =∞. The exponent x is the characteristic quantity related to a particular nucleation and growth mechanism specified by the overall transformation constant K_x. The value of A_∞ can be obtained by plotting A_t/A_0 values versus 1/t. The intersection obtained by extrapolating the curve to 1/t = 0 gives a good approximation of the A_∞/A_0 value. By non-linear least square fitting to the experimental data of the fig. 2 with eq.1, the K_x and the exponent x were evaluated for each curve. The estimated A_∞/A_0 values and best fit values of x and K_x as well as the residual square correlation coefficient (R^2) for DPPC at various control pressures are listed in table 1. R^2 values in the table indicate that the eq.1 fitted well specially in higher-pressure region. A_∞/A_0 values indicate that at very low pressure (<6 mN/m) there is no appreciable change in area. Reduction of area increases with increasing pressure in the plateau region (10-12 mN/m). This implies that the phase change from LE to LC dominates at plateau region showing maximum loss in area. In the condensed region (35-65 mN/m), area reduces differently compared to plateau region with increasing pressure. According to Vollhardt's model, the value of the exponent x should be more than 1.5 depending on the nature of nucleation. However, in the higher-pressure region our results show values consistently less than unity (Table-1). Lee et al [26] also observed the similar contradictory findings in octadecylamine.

Similar relaxation behavior for lecithin was observed by Smith et al [27] and was explained this phenomenon as molecular reorganization. We believe that in our case two-dimensional structural rearrangement is the main reason for differing from Vollhardt's model. If we closely look at the eq.1 we find that, it simply represents single exponential decay if x is nearer to unity. Then the representative equation will be

$$A_t/A_0 = a \exp(-t/\tau) + c \ldots\ldots\ldots (2)$$

where τ is the lifetime of the reorganization process, a and c are constants. All our curves except for 6 mN/m, are found to be fitted well in single exponential decay with eq.2. In fact, the change in area for the curve at surface pressure 6 mN/m is too small to be fitted meaningfully. All the fitted data are tabulated in Table 2.

Table 1. Model parameters of relaxation at different control pressures for DPPC at air/water interface using equation 1. Details of parameters are mentioned in the text.

Pressure (mN/m)	A_∞/A_0	x	K_x (10^{-3})	R^2
35	0.935	0.832±0.006	2.85±0.11	0.99
25	0.971	0.794±0.006	3.71±0.163	0.99
12	0.305	0.789±0.002	0.08±0.00173	0.99
10	0.931	1.139±0.056	0.007±0.00005	0.98
6	0.998	1.620±0.108	0.02±0.02	0.98

Table 2. Model parameters of relaxation at different control pressures for DPPC at air/water interface using equation 2. Details of parameters are mentioned in the text.

Pressure (mN/m)	a	τ (sec)	c	R^2
35	0.059±0.0002	1194±56	0.93±0.001	0.99
25	0.025±0.0001	1214±59	0.97±0.001	0.99
12	0.125±0.0005	11473±452	0.8721±0.005	0.99
10	0.030±0.0006	14689±803	0.970±0.008	0.97
6	0.002±0.0009	642±90	0.998±0.001	0.93

In table 2, at pressure 6 mN/m the value of c indicates that the area loss is negligible. Plot of A_t/A_0 vs. t in fig. 2 is almost straight line parallel to time axis. R^2 value indicates poor fitting. In situ Brewster angle microscopy study [28] shows the DPPC domain formation at air water interface even at very low surface pressure. Our result at low pressure shows no appreciable change in area with time. This observation implies that at very low surface pressure, the domains/molecules (species) are well separated to reorganize themselves efficiently with time, indicates that at very low surface pressure, the reorganization process in this system is not dominated by long-range type of interaction. Since the separation between species on the average decreases with the increase of surface pressure, the reorganization rate increases as shown in our result. The most interesting phenomenon is that the lifetime of reorganization in plateau region is higher than the condensed region. Here (10 and 12 mN/m), the relaxation times are one order of magnitude higher that of at 25 and 35 mN/m. In addition, through out the condensed region lifetime is found to be almost same. In plateau region, the species come closer and domain formation facilitate. This is a mid range type of interaction and the lifetime of reorganization is relatively large. In condensed region, reorganization takes place within the domains those are already formed. This is a short-range interaction. Hence, the lifetime of reorganization for this interaction is less.

Recently, [29, 30] reorganization process at constant pressure are analyzed by fitting curves in one, two or even multi exponential decay indicating different steps of reorganization. Our results indicate that two-dimensional rearrangement of DPPC molecules at constant pressure at air water interface is one-step

process because it can be fitted well to eq.2 as discussed above. This reorganization may be due to adjustment of charges and chain orientation, symmetry and mean distances among DPPC molecules.

3. Surface morphology of LB films

The surface morphology of the single layered LB films transferred to hydrophilic glass slide was studied by FE-SEM. Figure 3 shows the FE-SEM images in the micrometer scale resolution at low pressure (6mN/m) and high-pressure (35mN/m) region. The results indicate that in the micrometer scale, void spaces are present at low surface pressure. With increasing surface pressure, void spaces are reduced. In addition, the texture of the image indicates that at high pressure film is more condensed. Pressure dependent in situ domain formation for DPPC was studied by Brewster angle microscopy by many authors [10, 31]. Micro domain of LC phase separated by LE phase was observed. With increasing pressure, domains became more compact. Domain shape and size are also found to be time and ambient dependent. Here the striking feature is that when the monolayer is transferred on hydrophilic glass slide no such micrometer range domain was observed on transferred film. This may indicate some interaction with the substrate during transfer process. LC domains are aggregated on the glass slide squeezing out the LE region showing uniform structure in micrometer range. To elucidate the compactness of the film we have studied the surface morphology in the nanometer scale resolution. Figure 4 shows the FE-SEM images (in nanometer scale resolution) of DPPC LB film lifted at different control pressures. FE-SEM image of bare glass slide in the same resolution is also included.
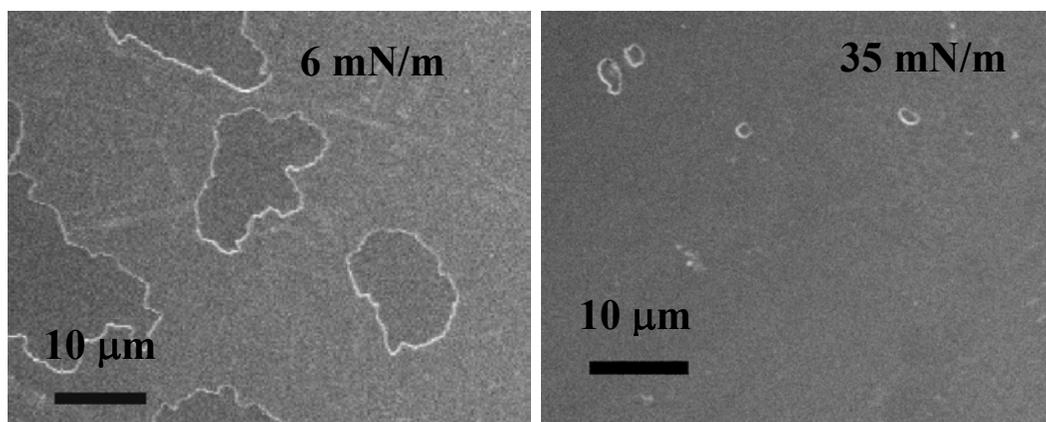

Figure 3. Micrometer scale resolution FE- SEM images of DPPC LB films lifted at low (6 mN/m) and high (35 mN/m) control pressures. The accelerating voltage used is 5KV.

At liquid expanded region (6 mN/m), film is not compact and domain formation is just beginning to start. A strip like structure of phospholipids domain is observed. Similar observation was found by others [30].

In the plateau region (12 mN/m), film is becoming more compact and domains are not fully formed. In the condensed state (35 mN/m), the film is compact with distinct domain formation. Figure 4(A) and Figure 4(B) show the representative relative surface contrast profile of line A and line B. From Figure 4(A) diameters of these domains are found in the range of 20-30 nm. It is interesting to observe that nano holes in the range of few nm to 100 nm are also formed. With further increase of pressure, no appreciable change is observed. Near collapse (~ 65 mN/m), one can clearly see the folding or buckling of the film.

These nano domains at higher pressure are aggregates of phospholipids. Nano domains are found in the range of 20 to 30 nm. Holes are in the range of 30 to 100 nm. Our results indicate that after spreading the lipid on air water interface, reorganization of lipid takes place with increasing applied pressure forming nano scale domains with some nano scale holes. Recently, Marsh [22] suggested that mimicking cell membrane generally requires a higher monolayer surface pressure. Our results validate Marsh's suggestion.

Conclusions

We have studied reorganization behavior of DPPC at air water interface. Reorganization process is found to be temperature dependent. Surface pressure dependent study shows that domains are formed with different formation rate at different pressure region. Moreover, not only the reorganization kinetics but also the critical pressure for reorganization or phase transition is temperature dependent.

FE-SEM studies indicate that the film consisting of nano domains of DPPC with some nano holes greatly dependent on surface pressure. Domain size is in the range of 20 – 30 nm and holes are in the range

of few nm to 100 nm. This supported film will be helpful for future study of protein adsorption in lipid membrane.
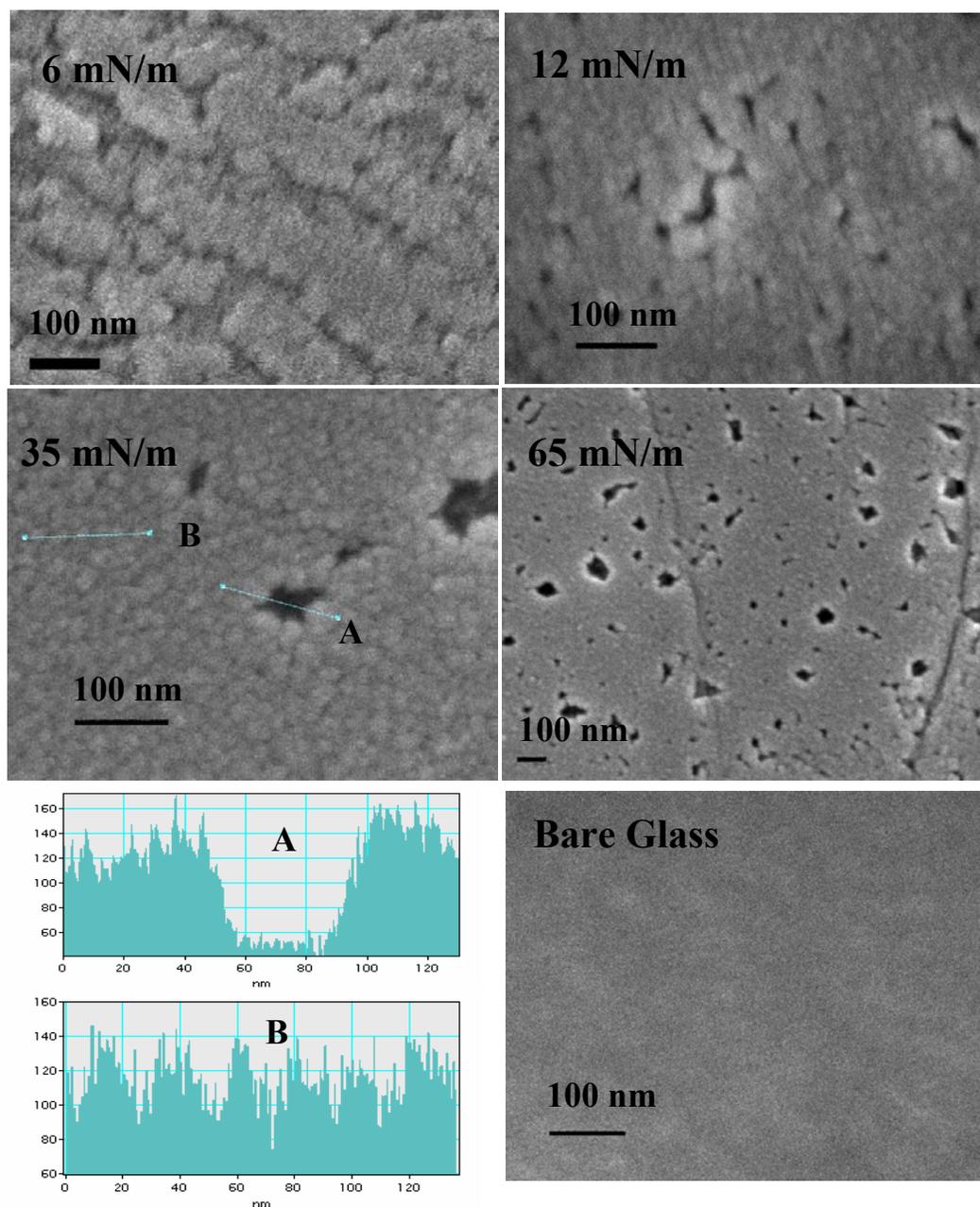

Figure 4. Nanometer scale resolution FE- SEM images of DPPC LB films lifted at different control pressures. FE-SEM image of bare glass slide is also shown. The accelerating voltage used is 5KV. A and B shows relative surface contrast profile of line A and line B. Here in the ordinate scale 0 represents black and 255 represents white.

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