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Epifluorescence microscopic observation of monolayers of dipalmitoylphosphatidylcholine: dependence of domain size on compression rates

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A fluorescence microscopic technique was used to observe phase transitions in monolayers of DPPC. The sizes of the domain structures observed were found to be dependent on the rate of compression of the monolayer. The distribution of domain sizes for different rates of compression were unimodal, but the scatter in the sizes was greater during slow compressions.

Dipalmitoylphosphatidylcholine (DPPC), a major component of pulmonary surfactant, when compressed in a monolayer film at the air/water interface, can sustain very high surface pressures and reduce the surface tension to low values. The properties of monolayers of DPPC have been extensively studied in vitro using surface balance techniques by which individual isotherms of surface pressure (or surface tension) versus molecular area have been characterized. DPPC undergoes a series of pressure induced phase transitions when it is compressed in a monolayer below its chain melting temperature [3].

Recently fluorescence microscopic surface balance techniques have been used to observe the compression of lipid monolayers [4–6]. The technique requires small amounts (0.5–2.0 mol%) of a fluorescent lipid analogue in the monolayer. The probe employed in this study is preferentially incorporated into the less densely packed (liquid-expanded) phase [4,5]. In the region of two-phase coexistence of the isotherms, tightly-packed phases (liquid-condensed or solid phases) can be observed in the form of dark patches, from which the probe is excluded, against a background of a continuum of fluorescence. The condensed areas have been suggested to be domains exhibiting certain liquid-crystalline properties [7]. The liquid-condensed domains have been characterized as having varying shapes and sizes depending on chirality of the molecules [8], temperature [9], subphase pH [5] and externally applied electrical field [9]. Some of the fluorescence results have been corroborated using charge decoration electron microscopy and electron diffraction techniques on monolayers transferred to solid substrate [7]. No quantitative studies on domain size distribution and their dependence on compression rates have been reported to date. Since the properties of monolayer films under dynamic conditions are particularly relevant to lung surfactant, and perhaps to membranes under some conditions, we have undertaken a quantitative study of domain distribution in DPPC monolayers.

We have studied the phenomena using a custom-designed surface balance consisting of a fluorescence microscopic attachment on a surface balance as discussed elsewhere [10]. Monolayers can be compressed with velocity ranging from 20 mm^2/s to 600 mm^2/s, by using a computer-controlled linear actuator motor. The instrument has an associated image analysis system which permits quantitative analysis of domain sizes. This is not only useful for equilibrium or near-equilibrium studies, but is essential for proper analysis of films under dynamic conditions.

Monolayers were formed at relatively low packing density and correspondingly large molecular areas (120 $Å^2$/molecule) by spreading DPPC containing 1 mol% of the fluorescent lipid analogue NBD-PC (1-palmi-

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toyl-2-{12-[(7-nitro-2-1,3-benzoxadiazole-4-yl)-amino]-dodecanoyl}phosphatidylcholine) in chloroform/methanol (3:1, v/v) onto a subphase of 0.15 M NaCl made with deionized, double-distilled water, the second distillation being from dilute potassium permanganate solution. Careful surface cleaning was done before spreading the monolayer on the subphase. Sufficient time (no less than 30 min) was allowed for solvent evaporation and monolayer spreading before any compression was performed.

The monolayers were compressed at two different velocities of 0.13 $Å^2$/molecule per s (slow) and 4 $Å^2$/molecule per s (fast) at an ambient temperature of $21 \pm 1°C$. The barrier was stopped at various surface pressures and visual recording performed by using a low light level video camera. For the fast compression rate, video recording was started immediately after stopping the barrier and continued for a minute. For the slow compressions a 10-min waiting time was introduced before recording at each selected pressure, thus the recordings were taken at times 10 to 11 min after barrier stoppage. After each video recording period compression was continued to the next selected pressure. The total times taken to compress the monolayers from an area/molecule of 113 $Å^2$ to 52 $Å^2$ were 127 min for the slow compression and 12 min for the fast compressions. The images were recorded on video tape, and randomly-selected recorded images were subsequently analyzed by a process discussed elsewhere [10]. Domain sizes and their number per frame were calculated using menu-driven image-analysis software. Frequency distributions of domains sizes were obtained at various surface pressures.

At both speeds of compression dark domains were observed to grow out of a fluorescent background at surface pressures of 4–5 mN/m at molecular areas of 74 ± 3 $Å^2$/molecule. There were more nucleation sites observed per frame during the fast compressions than during the slow ones. The dark domains seemed to grow with finger-like projections occurring at their boundaries during compression, and to revert to more regular shapes within seconds of stopping of the compressing barrier. At surface pressures near 9 mN/m in homogenous distributions of shapes were seen for fast rates of compression, whereas more homogenous kidney-shaped domains were observed for slow rates.

Fig. 1 shows typical images of the surface during fast (Fig. 1A) and slow (Fig. 1B) rates of compression, both images being obtained at a surface pressure of 9 mN/m. A greater diversity of sizes was seen during slow compression, whereas more irregularity of shape was seen in the domains formed during the rapid compression (Figs. 2 and 3). Slower compression rates, with the 10 min waiting periods introduced, allow for sufficient time for diffusion to occur so as to approximate static or quasi-equilibrium conditions. When a fast compression was followed by a waiting period of up to 45 min, domain sizes remained small in comparison to domains achieved at the same surface packing density during slow compression. This suggests that during and after fast compression there may be a complex route for domain formation in which there may be some kinetically-hindered step.

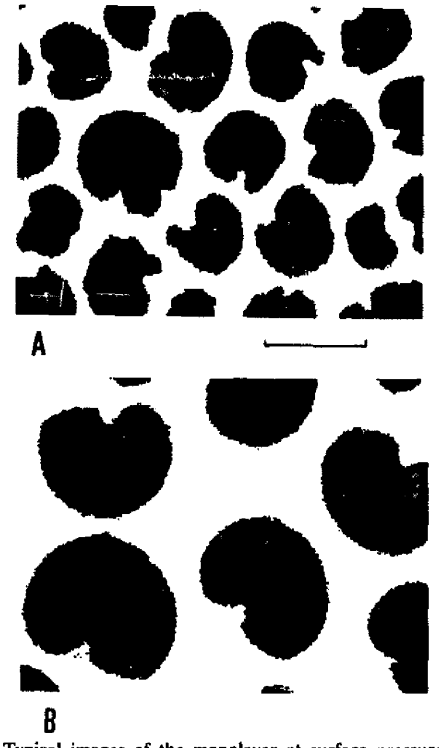

Fig. 1. Typical images of the monolayer at surface pressures of 9 mN/m achieved during fast compression (4 $Å^2$/molecule sec) (A), and at 30-fold slower compression rate (B). Scale bar is 25 microns. The reproductions were printed on a dot matrix printer from an image which was frame-grabbed from video tape.

Fig. 2 shows frequency distributions of the domains at a surface pressure of 9 mN/m for the fast (Fig. 2A) and for the slow rate (Fig. 2B) of compression. The frequency distributions of domain sizes in square microns for both rates are unimodal. As shown in Fig. 2A the peak of the distribution indicates an average size of about 250 square microns for domains formed during fast compression, whereas for the slow compression the average was near 1000 square microns. The greater range of domain sizes seen during slow as opposed to fast compression is evident in the frequency distributions.

Average size of the domains versus their molecular areas show two distinctly differing growth patterns for different compression velocities as shown in Fig. 3. During slow compressions the domains grew from an

average size from 150 square microns at molecular areas of 74 Å^2/molecule to 1500 square microns at molecular area of 54 Å^2/molecule (shown as open squares in Fig. 3). Comparatively, the average size of domains observed during fast compressions were smaller at the same molecular areas (filled triangles in Fig. 3). Fig. 3 also shows that the scatter in the sizes of

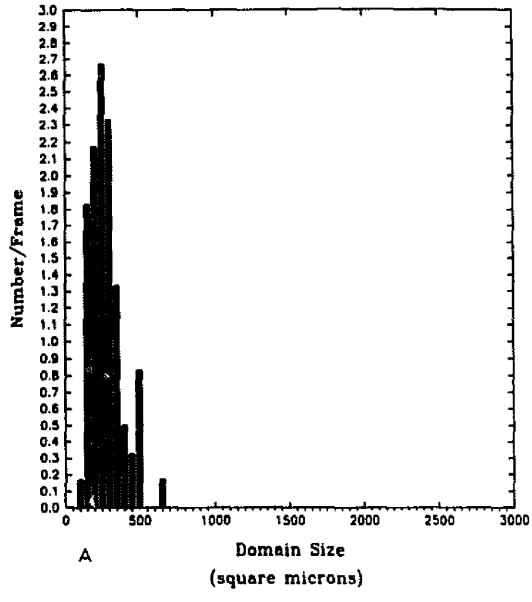

Fig. 2. Frequency distributions of domain sizes. The sizes of the domains were counted for 20 randomly-selected frames. Frequency distributions were calculated for images, recorded at surface pressure of 9 mN/m during fast (A), and 30-fold slower compression (B).

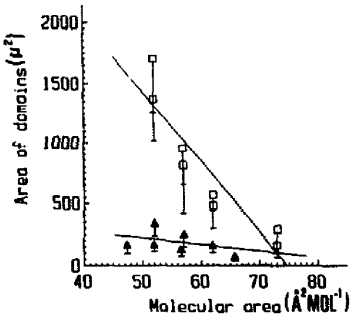

Fig. 3. Average size of the domains vs area per molecule at which the images were obtained in the monolayer. The blank squares represent results of two experiments performed at 21°C at slow compression rates. The solid triangles represent results of two experiment performed at the same temperature but with 30-fold faster rates of compression. The total time taken between 73 and 52 Å^2/molecule was 5 min for fast compressions and 53 min for slow compressions. The error bars represent standard deviations of the sizes of domains.

the condensed domains was greater during the slow compressions. The surface pressure-area isotherms for a number of these experiments were well-matched, with values of area/molecule varying by no more than $\pm$ 2Å^2/molecule at any given surface pressure over all isotherms studied.

The percentage of the total area occupied by the condensed domains was essentially the same for slow and fast compressions. At 62 Å^2/molecule, for example, the dark domains occupied 30 $\pm$ 3% of the fields for both types of compression. Florsheimer and Mohwald [9] also found that at 62 Å^2/molecule, 30% of the total area was covered by condensed domains when DPPC monolayers were compressed at 10 Å^2/molecule per h. Fig. 2 shows that there was a greater frequency of smaller domains during fast compressions, and a smaller number of large domains during slow compressions.

The difference in appearance between monolayers compressed at fast or slow rates persisted at surface pressures above 30 mN m^{-1}. At high pressures up to 70 mN m^{-1}, monolayers which were compressed slowly showed irregularly shaped bands of condensed phase, whereas rapidly compressed ones showed large numbers of small domains in contact with one another. These shapes have not yet been quantitatively analyzed.

During expansion of compressed monolayers the condensed domains became smaller and disappeared at pressures of about 5 mN m^{-1}, indicating that their formation was a reversible process. Continued expansion to very low pressures led to an appearance similar to that seen when the monolayers were spread at 110–120 Å^2/molecule.

DPPC domain architecture was studied by others in order to characterize molecular orientational order at

the air/water interface [9]. Still others have shown that these domain structures are susceptible to phospholipase A_2 hydrolysis [11], and shape instabilities occur at the domain boundary when trace amounts of other lipid species are present in the monolayer [12]. For these types of studies quasi-equilibrium conditions or slow rates of compression of 10 $Å^2$/molecule per h [9] were employed. Quantitative shape analysis was not done. Our intention in studying DPPC was to observe domain formation under fast dynamic compression and, eventually, expansion, so as to simulate conditions of cycling in monolayers during the respiratory cycle in vitro.

Our results indicate surface pressure-area isotherms did not show any significant differences when compression occurred at either of the two velocities, but there was a large difference in domain growth between the two rates of compression. The presence of smaller average domain sizes during fast compression leads to the suggestion that diffusion of the molecules in the surface is slow enough so as to be limiting for full-sized domain formation over the time periods studied. Peters and Beck [13] observed that the translational diffusion coefficient of a probe similar to NBD-PC in DPPC monolayers was about 30 $\mu m^2/s$ at a surface pressure of 5 mN m^{-1} in the liquid-expanded film, and that it decreased through the expanded-condensed region to be about 100-fold less at pressures of 20–30 mN m^{-1}. Thus, attaining the final size and shape will be a diffusion-limited process, and diffusion itself will decrease with increasing pressure. In addition, the small domains may persist because those lipid arrangements formed during fast compression are in some way different from those formed during slow compression, and the full growth of the dark domains is kinetically-hindered after fast compression. Measurement of variation of domain size with compression rate and time required to reach maximum domain size could provide useful information about surface diffusion and solid phase growth. Non-equilibrium conditions may be important in pulmonary surfactant dynamics.

Ultimately this instrument can be used to study lung surfactant dynamics at the air/water interface. The system may enable study of surface 'squeeze-out', surface readsorption and other parameters using the whole surfactant and its constituents. Rigorous quantitative molecular understanding of these processes have eluded workers in the last two decades [14].

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