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Merocyanine 540 as a probe to monitor the molecular packing of phosphatidylcholine: a monolayer epifluorescence microscopy and spectroscopy study

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The characteristics of the fluorescent dye, merocyanine 540 (MC-540), incorporated in monolayers of 1,2-dipalmitoyl-phosphatidylcholine (DPPC), and 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) were studied in different states of molecular packing. Conditions for phase separation in these monolayers were defined by their pressure/area (π–A) isotherms. Within the liquid expanded (LE) and the liquid condensed (LC) coexisting phases of DPPC monolayers, low light level epifluorescence microscopy revealed 'dark' discoid domains embedded in a 'bright' matrix. Under the same conditions, and at temperatures as low as 12°C, the π–A isotherms of POPC demonstrate the existence of a single phase, and no fluorescent domains were observed. Fluorescence spectra of MC-540 labelled monolayers, recorded in different structural states, reveal three distinct emission peaks: a 572 nm peak, present for monolayer packing conditions at low surface pressures; a 585 nm peak, similar to that obtained from dye molecules in fluid phase lipid bilayers, and observed here within the respective area/molecule ranges of 54–62 Å^2 and 62–69 Å^2 for monolayers of DPPC and POPC with diminishing intensity at increasing surface pressure; and finally, a peak at 560 nm, which predominates in densely packed POPC monolayers. Our results are interpreted on the basis of dye partitioning between monolayer and subphase, and different orientations of the dye with respect to the monolayer in various structural states. The usefulness of MC-540 to differentiate lipid packing in cell membranes is discussed.

Introduction

MC-540 is widely used as a fluorescent probe in membrane studies [1,2], in clinical applications to differentiate normal and malignant cells and in photodynamic therapy [3–6]. The partitioning between different states of packing of phospholipids and the environmentally sensitive emission characteristics of this dye have been utilized as an indicator of the 'tightness' of lipid packing in the bilayer membrane [7,8]. However, the fluorescence spectral shift observed as a function of the state of the host lipid has never been quantitatively established, nor is the term 'tightness of packing' defined in molecular terms.

The MC-540 dye molecule consists of two butyl groups and a quaternary ammonium and sulfonate group. The chromophore ring in the MC-540 molecule is uncharged. The only charge present on the MC-540 molecule is located on its functional group [9–11]. Charge delocalization through lipid–dye interactions can affect the fluorescence emission spectrum. When partitioned in fluid phase phospholipids, which are disordered or 'loosely packed', MC-540 emits a strong 585 nm fluorescence. This emission peak is absent when MC-540 is mixed with gel phase phospholipids, which are more ordered or 'tightly packed' [7,8]. These characteristics supposedly make MC-540 a sensitive probe of the 'tightness' of lipid packing.

Lipid monolayers at the air/water interface permit explicit control of the molecular packing of membrane constituents. Fluorescence spectroscopy of monolayers may therefore provide more detailed information concerning the relation between MC-540 fluorescence and the molecular packing of the host lipid; this complements spectroscopic studies of MC-540 labelled bilayers in their gel and fluid states [8]. Furthermore, the results derived from experiments on lipid monolayers may be applicable to the study of the interactions occurring in biological membranes [12].

The phases of a phospholipid monolayer below the critical temperature are generally defined as liquid expanded (LE or L_1), coexistence of liquid expanded/

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liquid condensed (LC or L_2) and solid condensed (SC or S) [13]. The LE phase is a two-dimensional anisotropic fluid state of high compressibility. Upon compression, the LE state undergoes a transition to an LE/LC state, possibly of first order, involving order/disorder chain transitions [14,15]. During the transition, the monolayer is heterogeneous, as observed by fluorescence microscopy [16–18]. In the high surface pressure region, the monolayer assumes a so-called solid condensed (SC) state, resembling the gel state of the bilayer.

To investigate the effects of monolayer phase transitions, domain formation, and molecular packing on the fluorescence characteristics of MC-540, we have incorporated the dye in DPPC and POPC monolayers. At 23°C, the former has a distinct LE/LC phase transition, while the latter remains in a single phase at temperatures as low as 12°C. This is indicated by their respective isotherm: a 'kink', marking the beginning of LE/LC phases, appears in all DPPC monolayer isotherms below 40°C, but does not exist in POPC monolayer isotherms at these same temperatures down to 12°C. We constructed an accessory to our fluorometer to collect and analyze spectral signals from these monolayers, in addition to low light level fluorescence microscopy imaging. The information so obtained enables us to establish the usefulness and the accuracy of MC-540 as a fluorescent probe of the lipid packing in corresponding bilayers.

Materials and Methods

DPPC and POPC were purchased from Avanti Polar Lipids Inc. (Birmingham, AL). Merocyanine 540 was purchased from Molecular Probes Inc. (Eugene, OR). Phospholipids and the fluorescent dye were mixed in chloroform in pre-determined ratios prior to spreading on the air/water interface of a Langmuir trough. Doubly distilled water was used as the sub-phase. Solvent was evaporated for at least 20 min before any measurement was taken. Isotherms of DPPC and POPC containing 0–50 mol% of the dye were recorded. The lipid/dye ratio of 1 mol% was chosen for domain imaging. The isotherms of monolayers with this lipid/dye ratio were similar to the isotherms of pure lipids. An initial dye/lipid ratio of 15 mol% was used in monolayer spectroscopy, due to the poor signal/noise ratio from monolayers of lower dye content.

The relationship between surface pressure and surface area per molecule of monomolecular films (π–A isotherms) was measured by a π–A recording system. The custom-built, four compartment, environmentally controlled Langmuir trough and barriers were made of teflon. The size of the final trough where measurements were made was 15×22 cm^2. The movement of the barrier (1–10 mm^2/s) was connected to a potentiometer, which converted the position of the barriers to a voltage signal. The output of the potentiometer was connected to the X-input of an LY-1600 X-Y recorder (Linseis Inc., Princeton Junction, NJ). Surface pressure measurements were made with a Wilhelmy plate. The change of surface tension was measured by a Cahn RG electric balance. The surface pressure is defined as:

$$\pi = F_0 - F$$

where F_0 is the surface tension of the pure substrate, such as water, and F is the surface tension of the film covering the substrate. The amplified signal was connected to the Y-input of the X-Y recorder which plotted the π–A isotherms.

Adsorption isotherms were recorded by maintaining either a DPPC or a POPC monolayer at given constant surface pressures, using a servo-controlled bar placement mechanism. The change in total surface area of the monolayer over a period of 3 h was recorded, with increasing concentration of the dye injected and mixed into the subphase through the other side of the bar. The surface pressure of water with and without the MC-540 dye was also recorded.

Epifluorescence microscopy images were recorded by an American Optical (Buffalo, NY) fluorescence microscope AO-2071 with a $40\times$, (NA = 0.85) objective lens. A retaining ring with a small opening was attached to the objective lens, and immersed through the air-water interface to minimize monolayer drift. A Dage-MTI (Michigan City, IN) silicon intensified target (SIT) video camera MCP-SIT66 was attached to the microscope. A Magnavox-80 monitor was used to observe the image, which was recorded by a Panasonic Industrial Co. (Secaucus, NJ) AG6050 video recorder. The processing of images from the video tape cassette was carried out in a Datacube (Peabody, MA) Maxvision AT-1 imaging system.

For spectroscopic recording, a set of Schott (Duryea, PA) flexible fiber optic cables were used as excitation and emission light guides. A 50 W mercury lamp provides the excitation light through a 500 nm interference filter. The fluorescence emission from the monolayer was collected at the entrance of the emission light guide. The other end of the light guide was connected to the input port of the emission monochromator of an SLM (Urbana, IL) 8000 fluorospectrometer.

The spectra of MC-540 labelled monolayers were extremely noisy due to the small number of fluorophores present in the sampling area. In a typical experimental setup, about 5% of the fluorescence emission photons were collectable by the end plate of the fiber optics, from an area of about 1 cm^2. If 2 mol% of the dye remained in the monolayer at say, 10 dyne/cm pressure, only an equivalent of 10^{12} molecules, or

about 0.2 fmole of dye was available for spectral study at a time. In order to collect more photons to increase the signal to noise ratio, spectra were scanned at 0.1 nm/s. Scanning 840 points (84 nm) took about 13 min Because the monolayer was constantly flowing through the illuminated area, as evidenced by video recording of images, the photobleaching problem was not a serious one. Spectra were usually reproducible upon rescanning. Typical photon counts were at a 1000 cps level. This accounted for the low signal/noise ratio of the spectra.

Because the emission collector gathered light from the surface as well as the subphase, which also contained the dye, a spectrum was recorded without monolayer and subtracted from each spectrum recorded in the presence of a monolayer. The difference spectra were extremely noisy, as expected when subtracting two large and nearly equal quantities. A slight error in placing the base line after the subtraction would induce a significant error in measuring the half peak width. These difference spectra were smoothed through a low pass filter which cut off fluctuations below 10 nm. This criterion was chosen to be less than half of the full width at half peak maximum (FWHM, 20–25 nm) of any known emission peak of the MC-540 dye [8]. Two of the resolvable emission peaks were found to be centered at either 572 or 585 nm. These two peaks coincided with those observed from the dye in either an aqueous or in a non-polar medium such as hydrocarbons. The shapes of these two peaks were used to simulate the overlapping spectra observed from the monolayers. The contribution of these individual peaks, extracted from the observed spectra, were plotted as peak intensity versus surface area.

Results

π–A isotherms

The π–A isotherms of DPPC containing 0–23 mol% dye were measured at 23°C. Up to 15 mol% of the dye, the total area per DPPC molecule increased by only 2–3%, manifesting itself in Fig. 1a as a slight shift of the π–A curve to a larger area per molecule. All of the π–A isotherms exhibited a readily detectable onset liquid expanded/liquid condensed (LE/LC) transition at about 7–8 dyne/cm. Following the onset of this transition, a plateau region could be recognized. This is a typical characteristic of monolayers below the critical temperature. The curves in Fig. 1a were obtained at an ambient experimental temperature of 23°C, which was well below the DPPC monolayer critical temperature of 42°C [19]. The lower the experimental temperature, the wider the plateau region (data not shown). Further compression resulted in the formation of a solid condensed (SC) phase which was highly incompressible.

Fig. 1b shows the π–A isotherms of POPC monolayers labelled with MC-540 at increased dye/lipid ratios at 12°C. The addition of the dye, even up to 60 mol%, did not alter the area per POPC molecule by more than 2%. It was particularly interesting to note that none of the π–A isotherms showed an LE/LC transition. This was expected because our experimental temperature of 12°C was above the monolayer critical temperature (T_c) of the unsaturated lipid POPC monolayers. Were it possible to perform experiments at temperatures below the monolayer critical temperature, the LE/LC phase transition would have been observed.

In order to understand the partitioning of MC-540

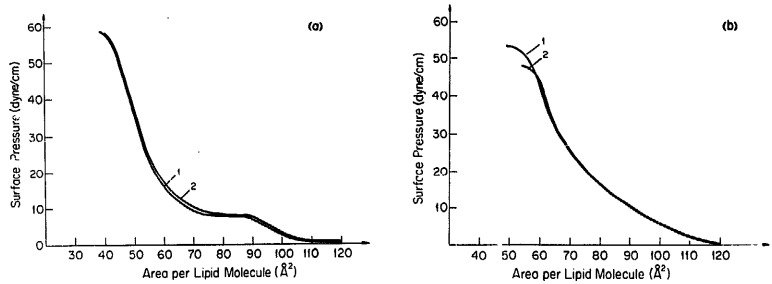

Fig. 1 (a). The $\pi - A$ isotherms of DPPC labeled with MC-540 dye at 23°C. Curves 1 and 2 represent a dye molar concentration of 0 and 15 mol%, respectively. The onset of LE/LC transition is detectable in all curves at 7–8 dyne/cm. (b) The $\pi - A$ isotherms of POPC labeled with MC-540 dye Curve 1 and 2 represent POPC containing 0 and 15 mol% of MC-540 at 12°C. No abrupt phase transition occurs in any of the isotherms.

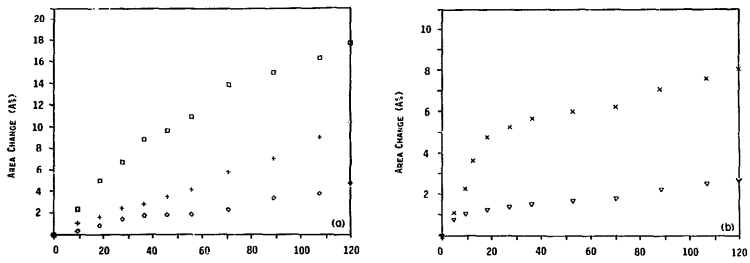

Fig. 2. Equilibrium adsorption isotherms of (a) DPPC and (b) POPC monolayers at varying concentrations of MC-540 injected and mixed in the subphase. The monolayers are maintained at fixed surface pressures of 4 (□), 9 (+), 27 dyne/cm (◇) for Fig. 2a and at 11 (×) and 33 dyne/cm (▽) for Fig. 2b.

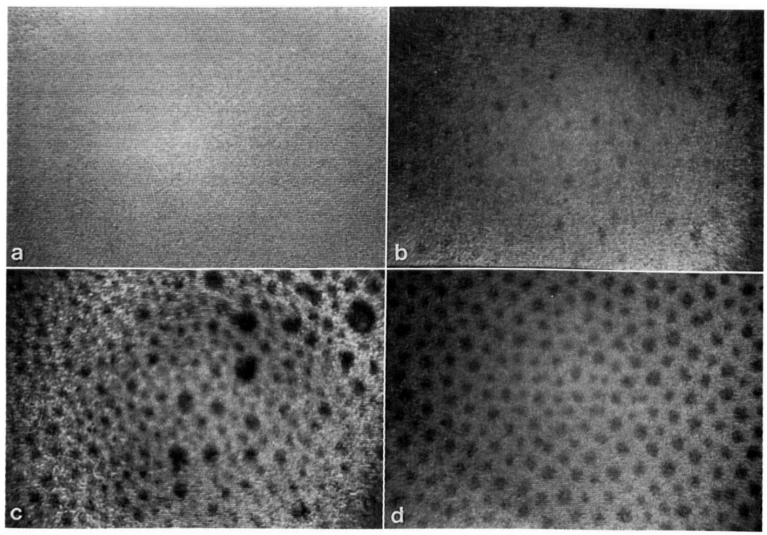

Fig. 3. Fluorescence micrographs (from a to h) of DPPC labeled with MC-540 dye (1 mol%) showing the lipid domain formation upon increasing surface pressures. Surface pressures (in dyne/cm) are (a) 1, (b) 8.2, (c) 13.6, (d) 16.5, (e) 19, (f) 23, (g) 27 and (h) 32. Lipid domains are seen at a pressure of 8 dyne/cm and above. Bar is 20 μm.

between the surface monolayer and the subphase, isobaric equilibrium adsorption experiments were performed for both DPPC and POPC monolayers. The results are shown in Figs. 2a and 2b, respectively. Without a lipid monolayer at the air/water interface, MC-540 dissolved in water did not influence the surface tension of water, indicating that the dye itself was not surface active. This was supported by the measurement of the concentrations of the dye in the subphase. The absorption at 500 nm by the subphase fluid withdrawn 3 h after 'spreading' the dye on the surface was identical to that of a bulk solution of the dye of an equivalent concentration, up to 100 nM (Table I). However, in the presence of a lipid monolayer, an increase of the surface area of the monolayer maintained on the dye-containing subphase was registered. The area increment was higher at lower surface pressures (Figs. 2a, b), and at higher dye concentration in the subphase. Apparently, some dye molecules in the subphase were being recruited to the surface by the lipid monolayer.

Because of the small amount of lipid and dye used in pressure-area studies, the loss of dye in the subphase due to adsorption is beyond our limit of spectroscopic detection. In one experiment we deliberately increased the dye concentration to an equivalent bulk concentration of 80 nM, and observed a detectable reduction of the dye in the subphase due to the presence of a DPPC monolayer (Table I).

TABLE I

Concentration of MC-540 in the subphase fluid

Dye added (nmol)	Lipid added (nmol)	Surface pressure (dyne/cm)	Subphase concentration * (nM)
13.6	0	0	14±1
13.6	32 DPPC	0	12±1
13.6	32 DPPC	9	13±1
13.6	32 DPPC	37	14±1
80	0	0	80±1
80	61 DPPC	1.5	45±1
80	61 DPPC	9	67±1
80	61 DPPC	36	78±1
80	60 POPC	0.8	79±1
80	66 POPC	9	84±1

* Measured by absorption at 500 nm.

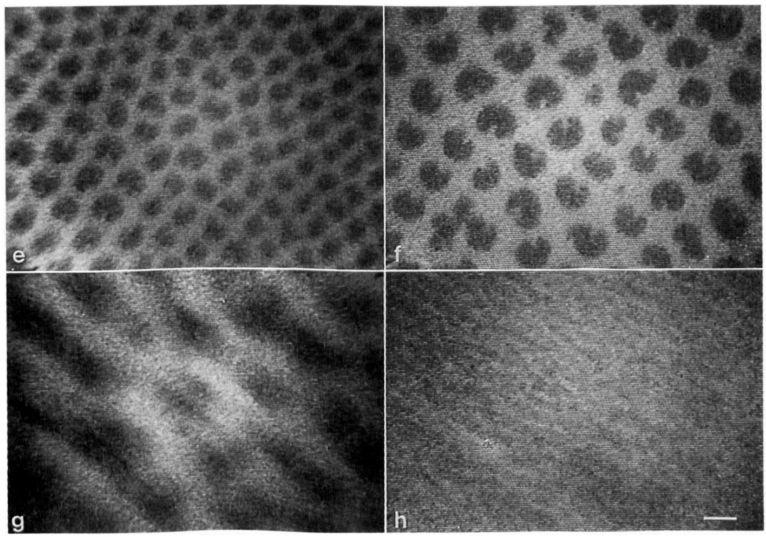

Fig. 3. (continued).

Epifluorescence microscopy

Fluorescence microscopy is a useful method to observe the heterogenous molecular packing of lipid monolayers. We have employed fluorescence microscopy to investigate the morphological characteristics of DPPC monolayers labelled with MC-540 at various surface pressures and dye concentrations. The images shown in Fig. 3 (a–h, 1 mol% dye concentration) documented the DPPC monolayer phase transition and domain formation in the experimental surface pressure range of 0–32 dyne/cm. Surface pressures of 1–7 dyne/cm corresponded to an LE phase in DPPC monolayers (Fig. 1a). In the LE phase (Fig. 3a), the lipid molecules exhibited homogenous packing. In addition, the uniform background of the LE phase monolayer had a higher fluorescence intensity than that in the solid condensed phase (shown in Fig. 3h).

Around the onset of the LE/LC transition of 7–8 dyne/cm, at 23°C, the monolayer started to form dark patches (Fig. 3b). The initially formed domains tended to aggregate during compression. The resulting patches looked circular in shape. With increasing surface pressure, the lipid monolayer domains gradually increased in size and resembled coffee beans in shape. These dark, coffee bean shaped domains of the DPPC monolayers represented lipid in a tightly packed solid-like state [17,20–22]. During the formation of solid domains the dye was excluded and the bright region of the image thus represents the fluid phase.

Figs. 3f–h show that the size of the solid domains increased with increasing lateral pressure. When different compressional rates ranging from 1 to 10 mm^2/s (corresponding to 3.0×10^{-3} to 3.0×10^{-2} $Å^2$ molecule^{-1} s^{-1}) were used, it was found that the solid lipid domains varied in their size due to the speed of compression (data not shown). The compressional speed we used to record video images was 5 mm^2/s (corresponding to 1.5×10^{-2} $Å^2$ molecule^{-1} s^{-1}). Generally, slower compression rates allowed more time for annealing to form larger domains. Upon increasing lateral pressure, the domain packing gradually assumed a hexagonal pattern, with domains remaining separated from each other (Fig. 3f), due to the repulsive dipolar force between domains. Upon further compression,

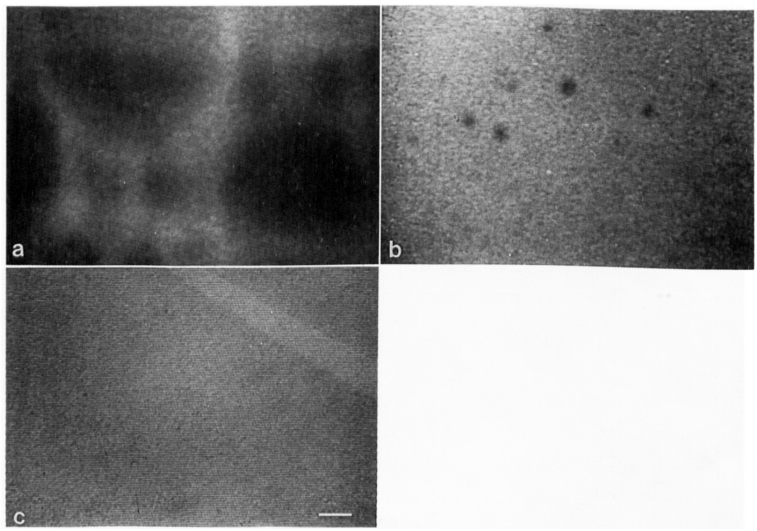

Fig. 4. Fluorescence micrographs (from a to c) of POPC labeled with MC-540 (1 mol%) show no phase separation in surface pressure range. There are some uneven patches at pressure below 1 dyne/cm. In the high surface pressure range, no domain formation is seen. Micrographs depicted are at surface pressures of (a) 0, (b) 1, and (c) 29 dyne/cm. Bar is 20 μm.

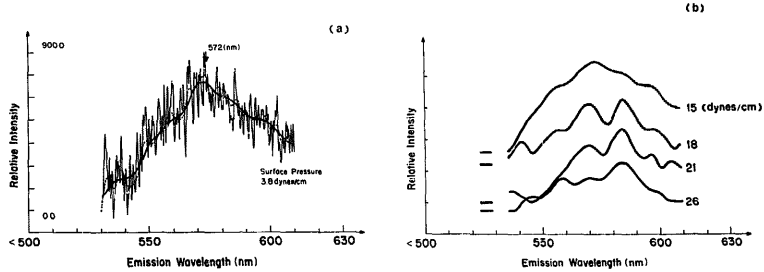

Fig. 5. The differential fluorescence spectra of DPPC monolayers at 23°C, labelled initially with 15 mol% of MC-540 and after the subphase spectrum is subtracted. (a) At 3.8 dyne/cm (corresponding the 96 Å^2/lipid molecule). The original differential spectra is represented by the fluctuating tracing. The dashed line and the solid line represent the results of smoothing by a different low pass filter. (b) Smoothed differential spectra at surface pressures of 15, 18, 21 and 26 dyne/cm corresponding to 64, 60, 57 and 54 Å^2/lipid molecule, respectively. The scale is the same as in (a). The baselines are shifted as indicated for each curve, for the sake of clarity in presentation.

dark domains of DPPC continued to expand, and bright areas emitting MC-540 fluorescence continued to diminish into inter-domain boundaries.

At 12°C, monolayers of POPC showed no lipid domains throughout the entire experimental pressure range. At a surface pressure of below 0.5 dyne/cm, epifluorescence microscopic imaging showed that some uneven holes in both POPC and DPPC monolayers were diminishing with increasing pressures (POPC case only in Fig. 4a, b). At higher surface pressure, POPC monolayers became uniform (Fig. 4c). Fluorescence microscopy recordings agreed with the π–A relations of DPPC and POPC, in which the DPPC monolayer at 23°C displayed a plateau region after the onset of the LE/LC transition, within which region both solid and fluid domains were visible. On the other hand, the POPC monolayer displayed no LC/LE phase transition within the experimental pressure range, and no domains were observed.

We also noted by fluorescence microscopy that increasing the dye concentration of MC-540 from 1 mol% to 15 mol% enhanced the emission intensity. However,

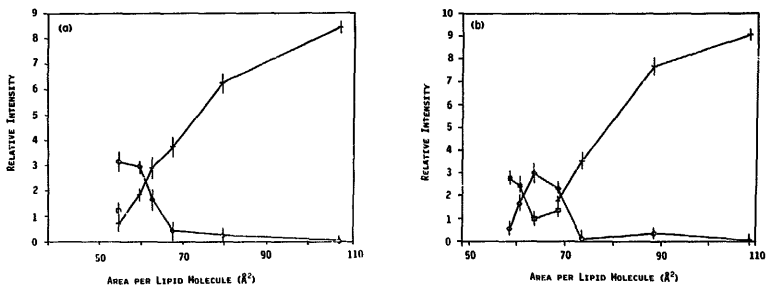

Fig. 6. The deduced fluorescence intensities I_{572} (+), I_{585} (◇) and I_{560} (□) for the respective emission peaks. (a) DPPC monolayers and (b) POPC monolayers.

the shape and size of the monolayer domains with 15 mol% dye were similar to those of lower dye content of 1 mol%.

Fluorescence spectroscopy

The fluorescence spectrum (not shown) of MC-540 dissolved in water gave a single broad peak centered at 572 nm, with or without the presence of DPPC vesicles below the phase transition temperature. When spreaded with 50 mol% of DPPC at near zero pressure, the spectrum was similar to that observed with the dye dissolved in water.

Figs. 5a, b show the fluorescence emission spectra of DPPC labelled with MC-540 dye at an initial concentration of 15 mol%. At low surface pressure, only the 572 nm emission peak was observed. Upon increasing pressure, this emission peak broadened but did not shift, and two peaks at wavelengths of 572 ± 5 nm and 585 ± 5 nm became resolvable when the surface pressure reached 18 dyne/cm. The peak intensities I_{585} and I_{572} were deduced as explained in Materials and Methods, and were plotted in Fig 6a. The single emission peak at 572 nm, observed at lower surface pressure, corresponded to an area per molecule greater than 97 Å^2. Upon compression from very low pressure, I_{572} decreased and I_{585} increased gradually until the area per molecule of DPPC reached 60 Å^2, then the change becames more pronounced. At 26 dyne/cm (corresponding to 54 Å^2), a third peak centered at 560 nm appeared. At this point, dark domains became predominant in fluorescence images (see Figs. 3f, g) At higher surface pressures, all emission peaks diminished below background level.

Fig. 7 shows the fluorescence emission spectra of POPC labelled with MC-540, at an initial dye content of 15 mol%, at 12°C. A single emission peak centered at 572 nm was observed at low surface pressure. As the surface pressure increased to 26 dyne/cm, three emission peaks at 560 nm, 572 nm and 585 nm were observable. The 560 nm and 585 nm peaks became more apparent and the 572 nm peak vanished, after increasing the surface pressure to 40 dyne/cm. The surface pressures of 26 and 40 dyne/cm corresponded to the area of 62–69 Å^2/lipid molecule. The deduced peak intensities I_{560}, I_{585} and I_{572} were plotted in Fig. 6b. Again an abrupt decrease of I_{572} and increase in I_{585} were detected at the area per lipid molecule range of 62–69 Å^2. At higher surface pressures, when the area was reduced to less than 60 Å^2/molecule, all emission peaks diminished below background level.

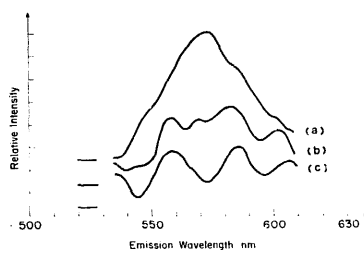

Fig. 7. Smoothed differential fluorescence spectra of POPC monolayers labelled initially with 15 mol% of MC-540 and recorded at 12°C, at pressures of (a) 0 (b) 26 and (c) 40 dyne/cm corresponding to (a) 120, (b) 69 and (c) 62 Å^2/lipid molecule, respectively.

Discussion

MC-540 has been used as a fluorescent probe to characterize the molecular packing in lipid bilayers and cell membranes [4–7]. The emission spectrum of MC-540 in the presence of fluid phase vesicles shows a pronounced 585 nm peak [8], whereas MC-540 with gel phase vesicles shows a broad, weak 572 nm peak which may represent the dye in water. Lipid-phase-dependent absorption curves (A_{564}/A_{500}) were also reported for MC-540 in DPPC [9]. However, a quantitative assessment of the spectral characteristics and spatial distribution of the dye by means of variable lipid packing in lipid monolayers has not been reported. Since DPPC and POPC monolayers respectively represent heterogenous and homogenous structures at 23°C, this system allows us to study the characteristics of the fluorescent dye under all conditions.

The amphiphilic dye MC-540 is soluble in both water and lipid. The partitioning of the dye between subphase and surface monolayer must first be understood to interpret our experimental data. If we assume that the area occupied by a dye molecule at the surface is of the same order of magnitude as that of a lipid molecule, we may regard the majority of the dye to be in the subphase for any monolayer at a surface pressure of greater than 5 dyne/cm. In a heterogeneous monolayer such as DPPC in the LE/LC phase, the dye further partitions between the solid and fluid domains of the monolayer. The partitioning between solid and fluid domains was not determined, but judging by the fact that at high surface pressure when the area percentage of solid domains increases, the area occupied by the dye molecules decreases, we may speculate that the solid domains are less favorable than fluid domains for dye partitioning between lipid monolayer and subphase.

Upon increasing surface pressure to 8 dyne/cm at 23°C, DPPC monolayers begin to exhibit two states which are recognizable by epifluorescence microscopy as dark domains on a bright background. Evidence that the dark domains correspond to a solid-crystalline

phase in monolayers was provided by electron diffraction [21,23] and X-ray diffraction of monolayer [20,22,24,25]. The solid domains keep a roughly circular boundary due to the balance between interphase line tension and preferred molecular arrangement, which varies with temperature and impurity [26–29]. The lipid packing in monolayers labelled by NBD-PC and MC-540 are visibly different (not shown). In NBD-PC labelled DPPC monolayers, the solid domains change from a circular shape to multi-boundary shapes upon compression. This could be a result of the NBD-PC label being concentrated along the domain boundary as the domains expand, leading to uneven, fractal growth [30]. In MC-540 labelled DPPC monolayers, dye molecules re-partitioning from the surface monolayer to the subphase as solid domains expand upon compression, up to the collapse pressure of 72 dyne/cm [19].

Since all spectra are recorded with a subphase background, the contribution of the subphase spectra must be subtracted to obtain the contribution from the surface monolayer alone. The spectral subtraction is justified if the background is taken from a subphase of identical dye concentration at equilbrium with the monolayer in question. After subtracting the counts from the background, which come from the subphase and the residual scattering of the trough bottom, there often is only a few hundred cps per channel. In spite of the noise level, the resolution of the spectra is sufficient to distinguish emission peaks at 560, 572 and 585 nm. MC-540 dissolved in water or in a gel-phase vesicle suspension also gives a broad emission peak centering at 572 nm. This peak coincides with the 572 nm peak which is observed from all monolayers adjusted to an area per molecule greater than 70 $Å^2$, regardless of molecular species, temperature or surface pressure. This peak is absent in unlabelled phospholipid monolayers. We interpret this peak to represent the fluorescence emission from MC-540 molecules orientating with their long axis parallel to the air/water interface, and thus being largely in contact with water [9,31]. The dye molecules may be interspersed with lipid molecules, which at this low surface density are also partially lying parallel to the air/water interface [31].

As the monolayer is compressed to a surface density of less than 70 $Å^2$ per POPC lipid molecule and less than 60 $Å^2$ per DPPC lipid molecule, the emission peak centered at 585 nm becomes dominant. This is the case regardless of surface pressure (> 15 dyne/cm for DPPC at 23°C or > 26 dyne/cm for POPC at 12°C) and phase state (two phase domains in DPPC and a homogeneous phase in POPC) of the monolayers. This peak is believed to represent the fluorescence emission from dye molecules re-orienting approximately perpendicular to the air/water interface, and among lipid molecules of similar orientation [31,32]. Because the dye molecule is now predominantly in contact with a hydrocarbon matrix, a 585 nm peak characteristic of dye molecules in such a non-polar environment is expected. The thresholds of 70 and 60 $Å^2$ per molecule of POPC and DPPC, respectively, are believed to represent the maximal expansion of the monolayer in which the dye molecule may retain an approximately upright orientation. This configuration is likely to correspond to that of the dye molecule in a fluid lipid bilayer [31,32]. In the case of phase separated monolayers such as DPPC at 23°C, the threshold is smaller due perhaps to the inclusion of solid phase domains in the averaging of area per molecule. Further compression leads to the diminishing of all fluorescence emission. We believe that the decline of fluorescence intensity is due to the expansion of the dark, solid phase domain area, which is much less favorable than fluid domains for adsorption of the dye at the interface. The possibility that dye molecules are concentrated at the solid domains to the extent of self-quenching is unlikely. With the amount of dye we used for imaging and spectroscopic studies, at high surface pressures, the dye molecules can occupy only up to 2% of the total surface area, while solid domains represent 80% of the same. However, the self-quenching of increasing concentrations of the dye in the diminishing fluid domain, in addition to repartitioning of the dye to the subphase, may also be responsible for the reduction of all fluorescence intensity at high pressures. The self-quenching mechanism is more imporfant in the case that the dye is not water-soluble [33].

Further compression of the monolayers to less than 54 $Å^2$/molecule and 62 $Å^2$/molecule for DPPC and for POPC, respectively, results in the appearance of a peak at 560 nm (Fig. 6). This peak is clearly resolved at 60 $Å^2$/molecule of POPC. The origin of this emission peak is uncertain, but is likely to represent the state of dye molecules associated with an incompressible monolayer at temperatures above the critical point. In the case of homogeneous monolayers such as POPC at 12°C, the compression results in a uniform reduction of area per molecule. Under increasing pressure, the lipid molecules in such a homogeneous monolayer may have less area per molecules than those in the fluid domains of a 2-phase monolayer in the highly compressible state. In DPPC monolayers, only those dye molecules associated with 'fluid state' lipids at the solid domain boundaries at the incompressible SC state may experience such a surface pressure. The 560 nm emission peak may be a characteristic of the dye in association with this compact, but non-solid phase, molecular packing.

Because of the difficulty of low signal level, fluorescence spectroscopic studies of monolayers at the air/water interface have been achieved only very recently. Initial attempts showed that by using high con-

centrations (23 mol%) of dye, a broad spectrum of fluorescence emission was detectable [34]. A subsequent study showed that the fluorescence of cyanine-dye-impregnated arachidic acid monolayer decreased monotonically with increasing surface pressure [35]. Our present study, the first one on phospholipid monolayers, and in conjunction with fluorescence microscopy, has revealed that the fluorescence behavior of a lipid packing indicator dye in a monolayer can be very complex, especially if the dye is soluble in both water and lipid. It requires microscopic observation to interpret local (in domains) and global changes in molecular packing derived from spectroscopic data in a heterogeneous monolayer.

From this study, we conclude that the fluorescence of MC-540 is related to the molecular packing of the lipid with which it associated. By drawing a parallel between the spectra of MC-540 labelled monolayers of DPPC and POPC, and those of the fluid state bilayer of these lipids, the molecular packing of the latter would correspond to a pressure of at least 27 dyne/cm and may be as high as 40 dyne/cm when I_{585} is dominant and I_{572} diminishes. These values agree with those proposed by Hui et al. [19] and Simon and McDonald [36], suggesting that bilayers have an equivalent surface pressure of greater than 40 dyne/cm, based on entirely different approaches.

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