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Translational molecular diffusion in phospholipid monolayers: substrate coupling and phase transitions

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Abstract. — The results of an investigation of the phases of a monolayer of the phospholipid dipalmitoylphosphatidylcholine, deposited by Langmuir-Blodgett techniques onto hydrocarbon-derivatized silicon substrates, are described. Measurements of the long-range translational diffusion of fluorescent lipid analogs in such monolayers reveal the presence of a phase transition not previously seen in phospholipids. It is marked by a large increase of lipid mobility at a temperature characteristic of the employed combination of lipid and substrate-attached hydrocarbon residue. A second transition into a high temperature phase, characterized by a rapid, homogeneous diffusivity, is observed at a temperature slightly above the temperature of the so-called chain melting transition in hydrated multibilayers of the lipid. In the novel intermediate state between the two transitions, long-range translational diffusion in the uniformly fluorescent monolayers is best described in a manner analogous to transport in composite materials. Lipid analogs containing a fluorophore in the polar headgroup portion of the molecule sample coexisting populations of host lipid whose diffusivities differ by as much as an order of magnitude in this temperature range. A chain labelled lipid analog is found to exhibit completely different behaviour, remaining largely immobile on the scale of the experimental observation times at temperatures below 43 °C. In samples cycled through the high temperature phase, this label exhibits increased mobilities at lower temperatures for several hours before returning to its original state. The measured diffusivities are compared with corresponding values obtained for monolayers spread at the air-water interface for which the average density is fixed, and also with available data for hydrated multibilayers. In natural bilayer membranes the two monolayer leaflets are generally not identical. They thus exhibit the same type of asymmetry as the one inherent in the system described here.

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1. Introduction.

Phospholipids are amphiphiles and exhibit a variety of lyotropic liquid crystalline phases [1], in which a bilayer forms the fundamental unit of a smectic-like layer structure. These molecules have received a great deal of attention because they form the major structural component of cellular membranes. In the course of the past ten to fifteen years there has been much effort to characterize the physical and chemical nature of phospholipid membranes, in most cases in the form of either bulk suspensions of vesicles or thick planar multilayers [2].

Phospholipid monolayers, deposited on substrates carrying a chemisorbed monolayer of long-chain hydrocarbon residues, have recently been introduced in an effort to gain insight into molecular events that play a role in the cellular immune response in vertebrates. Such monolayers and related thin supported planar layers have already been demonstrated to hold unique promise for the characterization of the molecular mechanisms underlying certain recognition and triggering events which initiate the response [3, 4]. Experiments carried out to date have shown that an understanding of the physical properties of the target membrane is imperative to explore the molecular basis for the events leading to an immune response. For example, the conditions ensuring the lateral mobility of monoclonal antibodies which are specifically bound to haptenated lipids incorporated in deposited monolayers, have only recently been established and reveal the critical role of the interactions between the lipid monolayer and the substrate [5].

Thin planar systems of liquid crystals provide an experimental testing ground for theoretical predictions concerning the effects of low dimensionality on atomic or molecular ordering and on phase transitions [6, 7]. Furthermore, they represent a class of models which permit the study of certain aspects of more complex systems such as polymers [8]. A growing number of applications is being considered for layered thin films of amphiphilic molecules [9] deposited on solid substrates in the way pioneered by K. Blodgett [10].

In the broader context of exploring the physical nature of phospholipid layers at different interfaces we have used a variety of techniques to address such problems as the interaction of an interfacial layer with the adjacent bulk phases [11] or with adjacent layers in thin multilayers [12, 13] as well as the intralayer ordering in substrate-deposited monolayers [14].

Here we present the results of a study of long-range molecular diffusion in such monolayers. Aside from magnetic resonance [15], the most wide-spread techniques for the measurement of translational and also of rotational diffusion of phospholipids in various model membranes as well as on cell surfaces [16] are those based on photochemical reactions, such as excimer formation [17] or bleaching of the fluorescence of a number of fluorophores. Among the methods relying on fluorescence bleaching, a variety of techniques have been developed, including «spot photobleaching» [18], «microphotolysis» [19], «time-resolved spatial photometry» [20], «fringe pattern photobleaching» [21], and «fluorescence recovery after periodic pattern photobleaching» [22]. The latter version referred to as FRAPP in what follows, is the method employed here.

On the basis of this technique heterogeneous diffusion with widely differing diffusion coefficients for various fluorescent lipids has previously been found in DMPC multibilayers in the $L_\alpha$ (1), and $P_\alpha$ («ripple») phases [23]. Data reported by Fahey and Webb [24] for hydrated DPPC multibilayers containing C12-NBD-PC and by Wu et al. [25] for hydrated DMPC multibilayers containing an egg phosphatidylethanolamine based, headgroup labelled fluorescent lipid (NBD-PE), do not reveal any significant features to indicate the presence of the ripple phase. The diffusivity of NBD-PC in a single DPPC bilayer, deposited on a silicon/silicon oxide substrate, remained below $10^{-10}$ cm$^2$/s up to the melting transition [26].

The objective of the present study was the characterization of phospholipid monolayers deposited on alkylated silicon wafers, using measurements of diffusive mass transport. The substrate-supported monolayers, submerged in an aqueous phase, closely resemble a phospholipid bilayer in a biologically relevant environment. The FRAPP measurements show that the nature of the substrate has a major influence on the state of the deposited monolayers. Specifically, they reveal the existence of substrate-specific interlayer coupling as well as the existence of a novel intermediate state of the lipid monolayers. A domain model is proposed as a description of this state, separated by first order phase transitions from a low temperature solid and a high temperature liquid phase.

An abstract reporting preliminary data was presented previously [27].

2. Materials and methods

2.1 Materials. — Phospholipids dipalmitoylphosphatidylcholine (DPPC), dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylethanolamine (DPPE), and dimyristoylphosphatidylethanolamine (DMPE) were obtained from Sigma (St. Louis, Missouri) and from Calbiochem (San Diego, California). An ultrapure preparation of DPPC was made available to us by Mr. Reichardt (Basel, Switzerland).

Derivatized phospholipids were purchased from Avanti Polar Lipids (Birmingham, Alabama). The

(1) The notation used to refer to the phases of lipid-water systems is that introduced by Tardieu et al. [1].
fluorescent lipid analogs used in this study were 1-palmitoyl-2-[n-(7-nitro-2,1,3-benzoxadiazol-4-y)aminocaproyl] phosphatidylcholine (C6-NBD-PC) and dipalmitoyl N-(7-nitro-2,1,3-benzoxadiazol-4-y)phosphatidylethanolamine (NBD-DPPE). Their structures are shown below:

\[
\begin{align*}
\text{C6-NBD-PC} & \quad (m = 5 \quad n = 14) \\
\text{NBD-DPPE} & \quad (n = 14)
\end{align*}
\]

P- and N-type single crystal silicon wafers of various resistivities and orientations and with «prime polished» surface were purchased from The Aurel Company (Santa Clara, California). Approximately 8,000 Å of SiO\textsubscript{2} were thermally grown on these wafers to prevent the quenching of lipid fluorescence due to the interaction of the dipole radiation field with the semiconductor. The oxidation was carried out at the Stanford Integrated Circuit Laboratory according to standard procedures.

The wafers were cleaned according to a procedure adapted from a «pre-diffusion clean» used in semiconductor processing. Briefly, this includes sonication in hot chloroform and subsequent exposure of the wafers to 5:1:1 solutions of H\textsubscript{2}O:N\textsubscript{2}H\textsubscript{4}OH:H\textsubscript{2}O\textsubscript{2} and of H\textsubscript{2}O:HCl:H\textsubscript{2}O\textsubscript{2}; this is followed by a short etch in 1:50 diluted hydrofluoric acid. Between cleaning steps, the wafers are rinsed extensively (10-20 min) in deionized water. After drying at approximately 150 °C for 90 min, the wafers were subjected to an alkylation reaction similar to that described by Sagiv and collaborators [28]. The substrate surface is thereby rendered hydrophobic.

Typically, contact angles of water on well alkylated surfaces were found to be in the range of 112°-116°, with hysteresis between advancing and receding angles [29] of 5°-8°. Such surfaces were not wetted by chloroform. Details on substrate cleaning, alkylation and characterization are given elsewhere [12].

All silane reagents applied to silicon oxide surfaces were purchased from Petrarch Systems, Inc. (Bristol Pennsylvania). Compounds with long-chain hydrocarbon residues used in this study were decyltrichlorosilane (DTS; C\textsubscript{10}), hexadecyltrichlorosilane (HTS; C\textsubscript{16}), and octadecyltrichlorosilane (OTS; C\textsubscript{18}).

2.2 Experimental methods.

2.2.1 Sample preparation. — Monolayers of phospholipid were spread in 5 μl aliquots to a low surface pressure (< 5 dynes/cm) at an air-water interface as 1.5 mM chloroform or 9:1 hexane/ethanol solutions. The aqueous subphase consisted of freshly purified water from a Milli-Q purification system (Millipore Corp. Bedford, Massachusetts). The pH was not adjusted and thus attains a value of approximately 5.5 in equilibrium with a standard atmosphere. All experiments were carried out at room temperature (22 °C-25 °C). The solutions typically contained one or two mole percent of a fluorescent lipid analog. Layers were monitored by epifluorescence microscopy and were uniformly fluorescent after spreading. They were compressed at typical rates of 1 Å\textsuperscript{2} per molecule per minute to a desired surface density. The Langmuir trough used in the present study, upgraded to include a low-light level (silicon-intensified target) video camera (Model 5150, Cohu Inc., San Diego, California) connected to a video monitor (Model VM-
Layer deposition was accomplished under feedback control of the surface pressure on a downward pass of an alkylated substrate through the layer (Fig. 1), at typical rates of 1-2 mm/min. Layers were deposited onto substrates varying in area between 5 cm² and 20 cm². Typically, two substrates derivatized with identical or different hydrocarbon residues were coated simultaneously, placing the substrates «back-to-back». After deposition the samples were covered with a thin (< 10 μm) mica plate, cemented, with room temperature vulcanizing rubber (RTV, RT 102 Silicone Rubber, General Electric Company, Silicone Products Division, Waterford, New York), to a Kapton spacer (Fig. 2) which varied in thickness between 25 μm and 125 μm. All sample mounting manipulations were carried out with the entire assembly kept submerged in water. Frequently, the sample was sealed by placing RTV around the edges of the mica plate. It was thus ensured that the monolayer was always covered with water. Occasional measurements were also performed without the cover, using a Zeiss 40X water immersion objective.

For heating scans, the sample assembly was placed on a small heating stage which could be mounted onto the stage of a Zeiss Photomicroscope III, equipped for epifluorescence. The stage (Fig. 2) consisted of a Peltier heat pump module (CP 1-4-127-10L; Melcore, Trenton, New Jersey), sandwiched between a heat sink (EG & G, Wakefield Engineering, Wakefield, Massachusetts) and a copper platform. Good thermal contact was ensured by applying silicone heat sink compound (Dow Corning 340, Dow Corning Corp., Midland, Michigan) to all areas of contact. A platinum resistance thermometer (118 MF, Rosemount Inc., Minneapolis, Minnesota), attached to the bottom of the copper platform, served as the temperature sensor. The temperature was controlled by a Lake Shore DRC-84C controller (Lake Shore Cryotronics, Westerville, Ohio) and was stable to within 50 mK. Absolute temperatures were calibrated against a surface sensor, placed on top of the copper sample platform, and are accurate to within 1 °C.

In preliminary FRAPP measurements at the air-water interface we employed a miniature trough, consisting of a cylindrical piece of Delrin with a bore of 18 mm to which a thin piece of mica was cemented with RTV. This fixed area trough was placed into a thermostated bath to control the temperature. To overcome the problem of convection [33] the subphase was kept shallow (< 6 mm); moreover, the monolayer was covered with a thin mica piece, leaving a narrow air-space between layer and cover.

Fig. 1. — Schematic illustration of the steps involved in Langmuir-Blodgett style transfer of a phospholipid monolayer from an air-water interface onto hydrocarbon-derivatized substrate. Deposition is carried out under feedback-control of the surface pressure. The deposited layer is kept submerged.

Fig. 2. — Sample mounting and geometry employed in measurements of fluorescence recovery after periodic pattern photobleaching.
Fig. 3. — Experimental set-up to measure fluorescence recovery after periodic pattern photobleaching. Side View: BLS.: Bleach shutter; HV: High Voltage; LPF: Long-Pass Filter; Ob.S.: Observation Shutter; PMT: Photomultiplier tube; PS: Pinhole Stop; Pr.S.: Protect Shutter. Top View: BLS.: Bleach Shutter; BS1, BS2: Beam Splitters; MI, M2, M3: Mirrors; ND: Neutral Density Filter; Ob.S.: Observation Shutter; RR: Ronchi Ruling.

The average density of molecules at the interface was fixed and determined by the amount spread. Typically, 1 mM 9:1 hexane/ethanol solutions of the lipid DMPC were spread with a 5 μl microsyringe (#701, Hamilton Corp., Reno, Nevada). The accuracy of the density values is estimated to have been on the order of 20%.

2.2.2 Translational diffusion. — The interfacial translational diffusion of fluorescent lipid analogs, contained in all monolayers at a concentration of one or two mole percent, was measured by monitoring the fluorescence recovery after periodic pattern photobleaching (FRAPP) [22]. The principle of the technique is closely related to that of forced Rayleigh scattering which has been employed to extract transport coefficients from planar systems of liquid crystals [34]. It relies on a periodic stripe pattern, generated by imaging a Ronchi ruling onto the sample [22]. The period of the Ronchi rulings used here varied between 80 μm (corresponding to 350 lines per inch) and 500 μm (corresponding to 50 lines per inch). Imaged onto the sample via a 40X microscope objective, these periods become 2 μm and 12.5 μm, respectively, in the plane of the sample. An epifluorescence micrograph of a Ronchi ruling, bleached onto a uniformly fluorescent monolayer, is shown in the inset of figure 4. A circular area of sample of approximately 150 μm diameter is illuminated. The fluorescence from a 100 μm diameter spot, defined by an aperture in the back focal plane of the objective, is collected. In contrast to other techniques such as inelastic neutron scattering which are sensitive to local motion, FRAPP measurements monitor translational diffusion on the length-scale of several microns, set by the stripe spacing.

Measurements were performed on the central portion of the sample to avoid edge effects. During temperature scans, several recovery curves were measured at each temperature. It is important to note that for each individual exposure a new area of sample was moved into the field of view of the microscope. Where appropriate, mobile fractions (see below) and diffusion coefficients were averaged.

The set-up is shown schematically in figure 3. Bleach and observation beams were delivered by a 5 W argon ion laser (Model 164-05, Spectra Physics, Mountain View, California), operating at 488 nm in the TEM00 mode. The intensity of the observation...
beam, set to approximately 0.1 % of the intensity of the bleach beam, could be further attenuated by a series of neutral density filters. Photon detection and counting were achieved using a cooled photomultiplier tube (RCA C31034-05, Lancaster, Pennsylvania), followed by an amplifier/discriminator (Model 511, Mec-Tronics, Addison, Illinois) and a photon counter (PRA 1770, Photochemical Research Associates, Oak Ridge, Tennessee). Timing of bleach and observation pulse sequences as well as data collection and on-line analysis were under control of a microcomputer (The Archives Inc., Davenport, Iowa; the interface was designed by Dr. R. M. Weis). Recovery curves were fitted to single or double exponentials, employing a Marquardt algorithm [35].

The time constant, τ, of the fluorescence recovery is related to the desired diffusion constant D by the expression

$$D = \frac{a^2}{\tau},$$

where $a = 2 \pi / P$, and $P$ is the period of the Ronchi-ruling, measured in the plane of the sample [22]. Introducing the symbols $I_\infty$, $I_0$, and $I(t)$ for the fluorescence intensities (with Ronchi-ruling in place) prior to, immediately following, and at time $t$ after the delivery of the bleach pulse, respectively (see Fig. 4), we may define a quantity

$$\phi = \frac{I(t_{obs}) - I_0}{I_\infty - I_0},$$

which we refer to in what follows as the mobile fraction (see also: [18]). It is a measure of the percentage of the original fluorescence $I_\infty$ which is recovered, due to translational diffusive motion of fluorescent probes in the sample, over a time period of the order of the experimental observation time $t_{obs}$. In the fitting routines $\tau$ is evaluated by extrapolating the fit to the recovery curve to « infinite » time. Under the experimental conditions pertinent here, the maximum possible value for $\phi$ is 1/2, or 50 %.

Values of $\phi$ lower than 50 % are obtained if the recovery time for diffusion exceeds the experimental observation time (see lower curve in Fig. 4). In that case the experiment only monitors a certain fraction of the entire recovery process. Hence, a computed value of $\phi$ less than 50 % implies that the intrinsic diffusion constant(s) is (are) too small to be conveniently accessible with our setup. This lower limit of reliably measurable values of D was set by maximal recovery by a quantity which varies on a scale of 0 % to 100 %. Such a quantity, $\phi_m$, was introduced in [5] and is related to $\phi$ by the simple expression $\phi = \phi_m / \phi_m$, where $\phi_m = \phi (t \to \infty)$.

3. Results

The bulk of the data to be presented in this section was obtained from monolayers of the phospholipids DPPC and DMPE, transferred at room temperature (~22 °C) and at high surface pressure (> 25 dynes/cm) from an air-water interface onto OTS-derivatized silicon/silicon oxide substrates. We provide a thorough characterization of the phase behaviour, as probed by FRAPP, of these systems. As will be seen, our data contain certain features which may be compared to the behaviour exhibited by the much studied multilayers of phospholipids. In addition, there are other, novel aspects, for which there is no known analog in the bilayer systems.

Before beginning with the detailed description of the diffusion measurements, we note that other combinations of a variety of lipid monolayers and substrate-bonded hydrocarbon residues have been investigated. Transfer experiments involving different lipids and different substrate-attached layers were carried out to address the question whether a general correspondence exists between phases of a given monolayer at a variety of different interfaces. Phosphatidylcholines and phosphatidylethanolamines of varying chain lengths were transferred, at given temperature and over a range of surface pressures, to silicon/silicon oxide substrates derivatized with hydrocarbon residues containing 10 (DTS), 16 (HTS) and 18 (OTS) carbons, respectively. Both the domain morphology, made visible by fluorescence staining techniques [33, 36, 37], and the translational diffusion of the monolayers were monitored. We find that the irreversible redistribution of fluorescent probe into domains, which are visible at the air-water interface and associated with a probe-excluding « ordered » (2) phase [38, 39], occurs after transfer to a substrate at a temperature which is characteristic of the particular combination of lipid and substrate chains.

We have reported such observations on DPPC/OTS and DPPE/OTS as well as on physisorbed bilayers of DPPC. For DPPC/OTS, probe redistribution was found to occur at a temperature $T_{sub}^*$ of approximately 35 °C [11]. The dynamic features accessible to FRAPP experiments which are associated with this phenomenon will be presented below. Our subsequent studies [12] have revealed that $T_{sub}^*$ increases monotonically, for given substrate chain length, with the chain length of a given type of lipid. For example, in the relevant case of an OTS-substrate, we find this to be true for both phosphatidylcholines and phosphatidylethanolamines:

<table>
<thead>
<tr>
<th>Lipid</th>
<th>DMPC</th>
<th>DMPE</th>
<th>DPPC</th>
<th>DPPE</th>
<th>DSPC</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_{sub}^*$</td>
<td>$&lt;25$ °C</td>
<td>$31$ °C</td>
<td>$35$ °C</td>
<td>$&gt;35$ °C</td>
<td>$\sim42$ °C</td>
</tr>
<tr>
<td>$T^*$</td>
<td>$23.9$ °C</td>
<td>$48$ °C</td>
<td>$41.4$ °C</td>
<td>$64.0$ °C</td>
<td>$54.9$ °C</td>
</tr>
</tbody>
</table>

$T_{sub}^*$ denotes the bulk chain melting transition temperatures obtained by differential scanning calorimetry (\(^{(2)}\) The phase referred to as « ordered » does not appear to exhibit long-range positional order when probed by electron diffraction after substrate deposition, but may be related to a bond-orientationally ordered phase [39, 40, 41].
metry for phosphatidylcholines [42] and phosphatidyl-
ethanolamines [43]. Conversely, for given lipid, \( T_{\text{sub}}^* \) is found to be higher on OTS than on HTS and DTS substrates. Preliminary experiments in which \(^{14}\)C-
labelled DPPC was transferred to HTS and OTS-
substrates indicate that the transferred monolayer
exhibits different final densities on these different
substrates [5].

In contrast to the final state attained by layers on
OTS-substrates, the state of layers transferred to
DTS-substrates is indistinguishable in its diffusive
behaviour from the \( L_\alpha \)-like state which the same mono-
layer on OTS only reaches at temperatures above
43 °C.

The morphology of DPPC and DMPE mono-
layers observed in fluorescence staining at the air-
water interface at surface pressures greater than
25 dynes/cm is substantially preserved when such
layers are deposited by Langmuir-Blodgett techniques
onto OTS-substrates. (For DPPC, see [11].) At room
temperature, the probe NBD-DPPE partitions into
both solid and fluid domains in DPPC monolayers
at air-water interface as well as subsequent to sub-
strate deposition, as already pointed out by McConnell
et al. [37]. In contrast, the same label partitions only
weakly into solid domains of DMPE at the air-water
interface. The apparent morphology, as characterized
by domain shapes and sizes closely resembles that
reported for dimitristoylphosphatidic acid (DMPA)
at pH 8 [44]. As in the case of DMPA, regions exhi-
biting superstructures with long-range order were
observed in which domains occupy distinct lattice
sites. Individual domains were found to exhibit diam-
eters of between 60 \( \mu \)m and 80 \( \mu \)m, thus exceeding
the average domain size outlined by C-12-NBD-PC
(up to surface pressures of approximately 12 dynes/cm)
and by C6-NBD-PC (up to surface pressures of
30 dynes/cm) in DPPC by a factor of five [12].

As is evident from the examination of the domain
morphology in the monolayers of DPPC and DMPE,
there are distinct variations in the partitioning of the
fluorescent probes employed here. Thus, as we noted,
at room temperature the "ordered" domains of
DPPC are stained by NBD-DPPE, but not by C12-
NBD-PC or C6-NBD-PC. In contrast, domains in
DMPE monolayers remain largely unstained by
NBD-DPPE.

When the recovery of fluorescence after photo-
bleaching in deposited monolayers is assumed to
exhibit a simple exponential kinetic behaviour, and
is consequently described by a single diffusivity \( D \),
the temperature-dependence of \( D \) exhibits the beha-
viour depicted in figures 5.1, 5.3, and 5.5. The
temperature dependence of the corresponding mobile
fractions is shown in the insets to those figures.
We reiterate that all measurements were carried out
on samples which were submerged in an aqueous
phase. When a DPPC layer, containing NBE-PE, was
removed from water and transferred into an atmo-
sphere of water-saturated helium, the diffusivity
remained below measurable limits up to a temperature
of at least 55 °C. The salient features are now described
for each of the figures.

Figure 5.1 shows the diffusivity as a function of
increasing temperature for a monolayer of DPPC,
containing 1 mole percent of a headgroup-labelled
probe, after deposition at approximately 25 °C onto
OTS at a surface pressure of 30 dynes/cm. The inset
shows the temperature dependence of the mobile
fraction. The most obvious feature in the data is the
large, precipitous increase in \( \phi \), at a temperature \( T_\phi \),
from values less than 5 % (on a scale of 0 % to a
maximum of 50 %, see Methods section), to values
between 40 % and 50 % at temperatures above \( T_\phi \).
This temperature coincides with the temperature
\( T_{\text{sub}}^* \) of reference [11], which marks the completion
of the redistribution of the chain-labelled fluorescent
lipid into domains, from which the chain labelled
probe C6-NBD-PC had been excluded at the air-
water interface. For all temperatures above \( T_\phi \), \( \phi \) is
seen to be essentially constant.
Fig. 5.2 and inset. — Part of the data of figure 5.1 replotted, after analysing recovery curves assuming double exponential recovery kinetics. It is seen that the intermediate region \( T < T_p < T \) is characterized by two distinct diffusivities of \( 2 \times 10^{-9} \text{ cm}^2/\text{s} \) and \( 1 \times 10^{-8} \text{ cm}^2/\text{s} \), respectively.

Individual pairs of diffusivities, corresponding to a single intermediate diffusivity in figure 5.1, are indicated, for example for the temperatures 37.30 °C, 38.70 °C and 39.70 °C, by pairs of open and filled semicircles. Open circles refer to data points which were not reanalysed. Filled circles mark such data points which were reanalysed, but found to be best represented by single exponential recovery. Typically, double exponential recovery was found to yield better fits, with diffusivities as indicated, whenever the diffusivity corresponding to single exponential recovery was intermediate between the two limiting values. The amplitudes of the two exponentials varied between ratios of 2:1 and 1:2, without obvious dependence on temperature. Lines are drawn to guide the eye.

The large increase in \( \phi \) reflects an increase in diffusivity to values which make the corresponding relaxation time \( T \) short compared to the observation time. The temperature \( T_p \) is a characteristic of the specific lipid-substrate combination (see below). The possible nature of the transition will be discussed below.

The temperature dependence of \( D \) displays several important features. The values of \( D \) obtained at temperatures below \( T_p \) reflect the mobility on the scale of our experimental observation time of only a small (< 10%) fraction of all fluorescent probes in the field of view. Fits to the data in this region are only marginally reliable and diffusivities cannot be taken literally. The implication of this behaviour, for temperatures below \( T_p \), is that only a small percentage of the NBD-DPPE molecules diffuse at a measurable rate while the diffusivity of the majority of probes is lower than the lowest detectable diffusivity of approximately \( 10^{-11} \text{ cm}^2/\text{s} \). As will be discussed below, we associate the small mobile fraction in this temperature region with defect diffusion.

Near \( T_p \), diffusivities are found to range between approximately \( 10^{-10} \text{ cm}^2/\text{s} \) and \( 10^{-9} \text{ cm}^2/\text{s} \) in different parts of the sample, indicating phase coexistence. In the temperature region between \( T_p \) and approximately 41.5 °C, individual measurements yield large fluctuations of \( D \) in the range \( 10^{-9} \text{ cm}^2/\text{s} < D < 10^{-8} \text{ cm}^2/\text{s} \), although \( D \) only rarely reaches this upper
Fig. 5.4. — (A) Diffusivities of chain labelled C6-NBD-PC in a monolayer of DPPC on OTS as a function of temperature between 42° and 46 °C, describing single exponential recovery in a « stored » sample (see text). The temperature dependence of the corresponding mobile fraction is marked by « o » (individual data points) in the inset of figure 5.3. In the present figure, open circles refer to individual data points, filled circles denote averages. (B) Part of the data of 5.4A reanalysed, assuming double exponential recovery. The meaning of the symbols is as follows. Open circles represent data of figure 5.4B which were not reanalysed, while solid circles mark data points which were reanalysed but found to correspond best to single exponential recovery. Solid semicircles represent pairs of diffusivities describing double exponential recovery. Any pair (:) of such points replaces a single data point in figure 5.4A. Coexistence of two populations is observed, characterized by diffusivities of 2 x 10^-9 cm²/s and approximately 2 x 10^-8 cm²/s. The coexistence region is approximately one degree wide, and is centered at 43 °C. Lines in (A) and (B) are drawn to guide the eye.

value. A straight line, corresponding to a linear dependence of ln (D) on T (and, in this temperature region, 1/T) and hence Arrhenius type activation of diffusion, provides a poor description of this behaviour. Rather, an apparent temperature-independent population with \( D = 3 \times 10^{-9} \) cm²/s is consistently observed, as is evident from figure 5.1. An increase in D up to \( 5 \times 10^{-8} \) cm²/s, accompanied by the disappearance of the fluctuations finally occurs at approximately 41.5 °C. Both the detected jump in \( \phi \) and the underlying increase in D from very low (<10^{-11} cm²/s) to intermediate values (10^{-9} cm²/s) show hysteresis. Significant hysteresis associated with the high temperature transition was also observed (data not shown).

Analysis of the data of figure 5.1 in terms of two exponential recovery fractions, was found to yield the best description for the recovery in the intermediate temperature range \( T_o < T < 41.5 \) °C. The results of this analysis are shown in figure 5.2. It implies the coexistence, in the intermediate temperature regime, of (at least) two widely different diffusivities of NBD-DPPE. The large fluctuations of D are readily understood as arising from a random sampling of regions of intermediate and fast diffusion. A more detailed model will be discussed below. The mobile fraction of figure 5.1 (inset), replotted in the inset of figure 5.2 after reanalysing the data, shows no major modifications compared to the original plot. As before, the transition near 41.5 °C is not marked by any change in the temperature dependence of the mobile fraction.

The diffusivity D of the fluorescent lipid C6-NBD-PC, incorporated into DPPC monolayers deposited onto OTS-substrates under conditions matching those in the experiments described above, exhibits a temperature dependence strikingly different from that just described for the headgroup-labelled NBD-DPPE. The relevant data are presented in figure 5.3. Let us again begin by examining the behaviour of \( \phi \) as a function of temperature. Given in the inset of figure 5.3 are data from two different samples recorded under the following conditions.

After substrate-deposition, one sample was heated to facilitate the redistribution of fluorescent label mentioned above and described in reference [11], and subsequently stored at room temperature for two days. During this period, the monolayer remained homogeneously fluorescent. The data points marked by « o » result from a heating run on this homogeneously fluorescent sample after storage. The mobile fraction is seen to remain low (< 5%) up to temperatures well above \( T_o \); the large jump in \( \phi \) detected with the headgroup-labelled fluorescent lipid is not observed here. Occasional large mobile fractions begin to appear at temperatures near 39 °C. Observations on DPPC-monolayers containing C12-NBD-PC transferred to OTS-substrate at lower surface pressures of 11 dynes/cm and 18 dynes/cm indicate similar behaviour, despite partial label redistribution into « ordered » domains immediately following transfer (data now shown). Full mobility across the sample is only achieved at approximately 43 °C as indicated by the cluster of points « D ». These data points were taken on the same sample while investigating the apparent coexistence of two diffusivities, indicated in the plot of D as a function of T near 43 °C (Fig. 5.3). This latter plot was obtained during a heating run
which followed a cycle of first heating a new sample briefly (20 min) to a temperature of 45 °C and subsequently cooling it to room temperature. The mobile fractions corresponding to this plot of D as a function of T are marked by « O » and « • » symbols in the inset of figure 5.3. In contrast to the heating run on a stored sample, φ now exhibits finite values at all temperatures, fluctuating between 10 % and 35 %, but remaining well below 50 %. Again, there is no significant change in φ at T_m in this heat-treated sample. The mobile fraction in this sample again « freezes in » at values below 5 % in the course of two days (data not shown), we return to this time-lag effect in the discussion.

The temperature dependence of D was measured in the course of a few hours following the heat treatment and is indicated by circles (« O ») and « • ») in figure 5.3. These data are in good agreement with those obtained in 1977 by Fahey and Webb on hydrated C12-NBD-PC ([24], Fig. 4; see also comments in Schneider et al. [45]). Their data are represented by « Δ » and « ▲ ». A broad transition is observed in both systems, centred at approximately 39 °C for the monolayer.

The diffusivities measured during a fine-scaled temperature scan after several cycles of heating to 45 °C and cooling to temperatures below 40 °C (Fig. 5.4A) were analysed assuming two recovering fractions. The results are contained in figure 5.4B and exhibit a coexistence region of approximately one degree width, with a transition centred at 43 °C. Hence, there are two types of transition present in the heat-treated monolayer sample: one type of « phase » melts at 39 °C while the second type, which is the only one present in stored samples, melts near 43 °C. At temperatures above approximately 44 °C, the diffusivities for C6-NBD-PC and NBD-DPPE behave identically.

Representative data describing the temperature dependence of the diffusivity D and the mobile fraction φ as probed by NBD-DPPE in a monolayer of DMPE, deposited onto OTS at room temperature and at a surface-pressure of 25 dynes/cm, are displayed in figure 5.5. The mobile fraction φ, for two samples, is seen to follow a temperature dependence analogous to that of the same probe in DPPC, with the important difference that the large jump in φ now occurs at a temperature of 31 °C, well below T_m for DPPC on OTS. (In monolayers of DMPC, which contain 5 mole percent of NBD-DPPE, the label only starts to diffuse rapidly (D > 5 x 10^{-8} cm²/s) at a temperature of 35 °C even though the matrix is liquid-like at temperatures above 24 °C [5, 42]).

Figure 5.5 contains a plot of the diffusivity D of the system DMPE/OTS as a function of T for temperatures T > T_m. While fluctuations in D are again observed, two important features distinguish the present data from those of figure 5.1 for NBD-DPPE in DPPC. First: the clearly predominating contribution to the measured values for D in the intermediate region comes from fast diffusion. Thus, D frequently exceeds 10^{-8} cm²/s, and rarely falls below 5 x 10^{-9} cm²/s. This is, in some sense, the opposite behaviour of that seen for NBD-DPPE in the intermediate regime in DPPC. Second: there is no significant increase in D in the vicinity of the chain melting transition of hydrated multibilayers of DMPE (48 °C [43]). At best, an increase of D from 5 x 10^{-8} cm²/s to 10^{-7} cm²/s in the neighbourhood of 50 °C might be

![Graph](image-url)
interpreted as evidence for a second transition. We note that data have not been analysed for the presence of a second component.

Measurements of the translational diffusion of chain-labelled fluorescent lipid, contained in a monolayer of DMPC at the air-water interface were also carried out. Temperature scans were performed under conditions of constant interfacial area and approximately constant average density. Some loss of lipid into vesicles sometimes occurs, as judged by examination of the layers under epifluorescence. In our preliminary set-up, we can furthermore not exclude the possibility of lipid creep onto the walls of the enclosure which for the small area of interface may cause significant deviations in density. Homogeneously fluorescent monolayers were obtained by spreading lipid at 24°C (above the bulk chain melting transition of DMPC) and subsequent cooling, or by spreading at 10°C. When heating such layers, D exhibits a temperature dependence of the type shown in figure 5.6. While these latter measurements can at present only

![Diagram](image.png)

Fig. 5.6. Diffusivity of the chain label C12-NBD-PC in a monolayer of DMPC at the air-water interface, as a function of temperature. Symbols «O» and «•» refer to a layer which was spread at 24°C from a 9:1 hexane/ethanol solution and subsequently cooled to 10°C. Triangles represent data obtained when heating a film spread at 11°C from a 9:1 hexane/ethanol solution. Open symbols denote individual measurements, while closed symbols represent averages. The average surface density was fixed by spreading appropriate amounts; it is estimated to be accurate to within 20%. The data were obtained at an average area per molecule of approximately 50 Å²/mol. A broad transition, centred near 17°C is observed. This is accompanied by local expansion of the layer, as judged by visual observation of specific «necklace» patterns shown in the inset. Individual points in the center of the transition have a somewhat greater uncertainty than usual, indicated by .

be taken as qualitative characterizations, they permit the following conclusions. Diffusivities, even at the highest (nominal) densities and well below the chain melting transition are relatively rapid and do not fall below 5 × 10^{-10} cm²/s (see also [33]) as compared to values of 5 × 10^{-11} cm²/s and below for C6-NBD-PC in substrate-deposited DPPC. The mobile fraction is always maximal (ϕ = 50%). The broad transition is centred at approximately 17°C, i.e. below the bulk transition temperature. At the temperature marking the first increase in D the monolayer appeared to enter a coexistence region, as judged by a characteristic change in sample morphology, i.e. the appearance of unstained regions in the previously homogeneously fluorescent layer. Particularly striking were patterns of unstained strands (see inset to Fig. 5.6). Similar to the «necklace patterns» observed when certain lipid monolayers are reexpanded subsequent to compression [12, 37]. While values of D measured in homogeneous portions of the sample as well as in those containing unstained domains of finite size were high (10^{-7} cm²/s), those obtained in regions with necklace patterns were an order of magnitude lower, indicating that the unstained strands represent a barrier for long-range probe diffusion. We interpret the necklace patterns as evidence for the local expansion of the «confined» monolayer, implying that the increase in diffusivity around 17°C is due to this change in surface density. That D is a sensitive function of the density in the range corresponding to 50 Å²/molecule to 60 Å²/molecule has been demonstrated by Peters and Beck [33].

We note that an attempt was made to monitor global expansions of deposited monolayers by visually inspecting the «edge» of a transferred monolayer. To accomplish this, monolayers were deposited only in the central portion of a large, hydrocarbon-derivatized substrate, defined by an approximately sized Kapton mask which was removed after layer deposition. While this edge does become «rough» in the course of temperature scans, and weak fluorescence can be detected a small distance outward from the original position, no significant global expansion was observed in this way. Necklace patterns such as those seen at the air-water interface were never observed in substrate-deposited monolayers.

We end this section by summarizing the salient experimental findings which must be accounted for.

1. There is a temperature T_0, characteristic of the particular combination of the deposited lipid monolayer and the substrate-attached hydrocarbon layer, which marks a sharp increase of the mobile fraction of the headgroup label NBD-DPPE, from below 5% to maximal values. This temperature is 31°C for DMPE/OTS and 35°C for DPPC/OTS. A large increase in ϕ at 35°C is also observed when large (≈ 5 mole percent), presumably phase-separated quantities of NBD-DPPE are present in a liquid DMPC matrix. For given substrate, e.g. OTS, T_0 increases...
with increasing lipid chain length of a given lipid type, reflecting the trend of the corresponding bulk chain melting temperatures \( T^* \). Comparing phosphatidylecholines with phosphatidylethanolamines, \( T_d/T^* \) is found to be lower for the latter class of lipids. We refer to the phenomenon occurring at \( T_d \) as a substrate-induced transition of the monolayer. While no significant effect is observed in FRAPP experiments on DPPC/OTS containing the chain-label C6-NBD-PC, \( T_d \) coincides with the temperature \( T_d \) of [11], which marks the completion of the redistribution of that label into originally «ordered» domains after transfer from the air-water interface.

2. A transition, characterized by an increase in diffusivity \( D \) to values of \( 5 \times 10^{-8} \text{ cm}^2/\text{s} \) into a phase exhibiting homogeneous diffusion, is observed in DPPC at 41.5 °C with NBD-DPPE and at 43 °C with C6-NBD-PC, temperatures slightly larger than the chain melting transition temperature in bulk multilayer systems of this lipid. Only a weak effect on \( D \) is seen with NBD-DPPE in DMPE near \( T^* \) of 48 °C. We refer to the temperature which marks the transition into the high temperature phase of the monolayer as \( T_D \). Heat treated DPPC monolayers containing C6-NBD-PC show, in addition to the transition at 43 °C a broad transition near 39 °C, also seen in multilayers. The high temperature phase referred to above is indistinguishable, by FRAPP, from the \( L_p \) phase of multilayers of the corresponding lipid and we refer to it as such in what follows.

3. In the intermediate temperature range between \( T_d \) and \( T_D \) the label NBD-DPPE samples widely differing diffusivities. In DPPC, two apparent individual diffusivities of \( 10^{-8} \text{ cm}^2/\text{s} \) and \( 2 \times 10^{-9} \text{ cm}^2/\text{s} \), respectively, are measured. In DMPE, diffusivities at temperatures above \( T^* \) are predominantly in the vicinity of \( 10^{-8} \text{ cm}^2/\text{s} \), with only occasional values below \( 5 \times 10^{-9} \text{ cm}^2/\text{s} \).

The diffusivity of the probe C6-NBD-PC remains low (< \( 10^{-10} \text{ cm}^2/\text{s} \)) over the entire range of intermediate temperatures. The corresponding mobile fraction is negligible in stored samples, but is found to fall into the range of 20 % to 35 % following brief sample heating. The temperature dependence of the corresponding diffusivity closely resembles that of hydrated multilayers of DPPC and shows a broad transition near \( T^* \), the chain melting transition temperature (as probed by FRAPP). In the course of subsequent storage the mobile fraction returns to values below 5 %.

4. Monolayers of DMPC containing 1 mole percent of C6-NBD-PC, spread at the air-water interface, always exhibit a maximal mobile fraction. Diffusivities never fall below \( 10^{-10} \text{ cm}^2/\text{s} \) in the range of temperatures and surface densities investigated. On heating runs, the onset of rapid diffusion appears to coincide with the local expansion of the layer, even under conditions of fixed surface density.

4. Discussion

4.1 Interfacial equilibrium of transferred monolayers. — To provide the appropriate starting point for the discussion of the dynamic phase behaviour of lipid monolayers transferred to hydrocarbon-derivatized substrates, it is important to reiterate that the state of local equilibrium of an interfacial monolayer is dependent on the bulk phases which form the interface [11, 46]. For the phospholipids, this is clearly demonstrated by the metastability of an unstained solid phase, exhibited by certain monolayers at the air-water interface. In the course of substrate deposition, the original phase must undergo a structural modification which is sufficient to permit probe partitioning.

The comparison of final states resulting after deposition of identical monolayers from the air-water interface onto DTS- and OTS-substrates (the latter after probe redistribution), lends strong support to the notion that the state of local equilibrium which an interfacial layer seeks to attain after transfer depends critically on the nature of the layer formed by the substrate-attached chains [11, 12]. A transfer ratio of unity [47] during Langmuir-Blodgett style layer deposition may be interpreted as an indication of the presence of a density constraint [11] during transfer. As a result, the deposited layer can be trapped in a metastable state. Major structural reorganization can occur in such instances [48].

We assume that monolayers after transfer to OTS are in a state of local equilibrium following the redistribution of fluorescent probe in the course of a first heating run to a temperature \( T_d \). This assumption is based on the fact that the subsequent dynamic behaviour of monolayers does not change significantly, barring occasional chemical degradation, after repeated heating (to the \( L_p \)-like phase) and cooling (to room temperature) and prolonged storage.

4.2 Substrate-induced transition at \( T_d \). — The most unusual features in the FRAPP data of the OTS-deposited monolayers are those revealing heterogeneous diffusivity in the temperature interval between the substrate-induced transition at \( T_d \) and the transition into an \( L_p \)-like phase at \( T_D \).

Let us first examine the transition at \( T_d \). As mentioned above, \( T_d \) increases for given substrate chain type, e.g. OTS, in proportion to the bulk chain melting transition temperature \( T^* \) of the deposited lipid. This indicates that the transition involves work against the attractive van der Waals interactions between lipid chains. Depinning of isolated, individual lipid molecules is ruled out as a potential mechanism for the increase in diffusivity at \( T_d \) on the grounds that NBD-DPPE shows liquid-like behaviour in (liquid) DMPC at room temperature when present in low concentrations (< 1 mole percent) whereas the characteristic increase in \( \phi \) at 35 °C is observed when larger concentrations (> 5 mole percent) of label are
used. It is possible that at such concentrations NBD-DPPE phase separates from DMPC [49].

The temperature $T_\phi$ for DPPC-OTS coincides with that reported for the appearance at the « ripple » (P$_r$ [50] or P$_s$ [51]) phase of multibilayers of DPPC [50, 52]. However, behaviour analogous to that of DPPC/OTS, but marked by a $T_\phi$ of 31 °C, is observed for DMPE/OTS (Fig. 5.5). Neither differential scanning calorimetry [43] nor saturation transfer electron spin resonance [53] have detected evidence for a transition into a « ripple » phase in hydrated multibilayers of DMPE. In view of this fact, the occurrence of a substrate-driven transition in DMPE/OTS is strong evidence that the intermediate phase observed in substrate-supported monolayers is not the ripple phase. The only possible alternative is the assumption that it is the intrinsic asymmetry of the DMPE/OTS system which leads to the formation of a ripple or analogous phase, not observed in symmetric DMPE multibilayers. While this would be highly interesting in regard to existing models for the formation of the superstructure [54], only direct structural examination of the intermediate phase in the monolayers can ultimately resolve this question.

That $T_\phi$ does not just mark a transition into an isotropic L$_a$ phase is ruled out by the complex behaviour of the diffusivity at temperatures above $T_\phi$, and by the second transition detected at $T_D$, close to $T_\phi$. The analysis of the FRAPP data presented in figure 5.2 provides direct evidence for the considerable heterogeneity of the molecular diffusivity in the uniformly fluorescent monolayers in the intermediate temperature interval between $T_\phi$ and $T_D$. Furthermore, the behaviour of chain labelled and headgroup labelled fluorescent analogs in the L$_a$ phase is identical, in contrast to the significant differences observed for the two labels in the intermediate state.

The microscopic nature of, and the molecular ordering in, the lipid phase in the interior of the domains can only be identified by suitable structural probes such as infrared spectroscopy [8, 55, 56], Raman scattering [55] or surface X-ray scattering techniques [13, 14, 57] of sufficient sensitivity. However, these techniques are only beginning to be extended to liquid-vapour interfaces and have not been applied to monolayers adsorbed at the interface between a substrate and a bulk fluid. The FRAPP data represent the only information presently available on the phase behaviour of such monolayers. With regard to the molecular ordering, we restrict ourselves to the observation that cooperative chain melting, possibly into a state retaining partial chain order, which may or may not involve a change in the average tilt angle for sections of the lipid chains [58], would be consistent with the observed increase in $D$ and would account for the dependence of $T_\phi$ on the lipid chain length.

To provide a qualitative explanation for the sudden increase in mobility at $T_\phi$, two limiting situations may be envisioned.

The first involves a weakening of the interaction locking the monolayer to the substrate. The monolayer is thereby released into an intrinsic state of interfacial equilibrium. It has been suggested for smectic phases of thermotropics that potentials mediating interlayer coupling may depend on hydrocarbon chain order [59]. A theory of the chain melting transition in symmetric lipid bilayers has recently been discussed which takes into account the interaction between the two halves of the bilayer [60]. The coupling term, which is shown to have a considerable effect on the critical temperature of the system, depends explicitly on the degree of hydrocarbon chain order in apposed monolayers.

In the second scenario, the intermediate state of the monolayer is the result of a competition between lipid-lipid interactions and those coupling the lipid monolayer to the substrate-attached layer of OTS. For example, the substrate layer may constrain the lipid layer to a preferred average density. This situation would be akin to that of the commensurate phases encountered for monolayers of simple atoms physically adsorbed to crystal surfaces [61]. Alternatively, the substrate-layer may induce a preferred average alignment of lipid chains, in analogy to wall alignment phenomena in nematics [62]. In such a case the intermediate state of the lipid monolayer would not be expected to exist in « symmetric » systems. It may be added that only a gradual increase of chain disorder with temperature, but no cooperative « melting » has been detected for substrate-attached monolayers of OTS [63]. The cooperativity of the transition at $T_\phi$, evidenced by the jump in the mobile fraction, is therefore attributed to the lipid monolayer.

### 4.3 Domain model for intermediate state ($T_\phi < T < T_D$) : novel intermediate phase or two-phase coexistence.

A model of the intermediate state which is consistent with all our observations is the one sketched in figure 6. The salient feature of this model is the presence of a domain structure in the deposited lipid monolayers with average domain sizes in DPPC. Two thermodynamically distinct but otherwise closely related arrangements are consistent with the proposed domain model.

In the first of these, the heterogeneous molecular diffusivity is characteristic of a novel intermediate phase for which we favour a generic description as an « ordered fluid ». As discussed above, this ordered fluid may appear as a result of a reduction in monolayer-substrate coupling at $T_\phi$. More likely it may result from a competition between lipid-lipid and lipid-OTS interactions. The order parameter characterizing this novel phase is unknown, but is speculated to be related to the surface density or the chain tilt. In this picture, rapid ($D > 10^{-8} \text{ cm}^2/\text{s}$) diffusion occurs in the interior of domains formed by this ordered fluid.
Fig. 6. — Schematic illustration of domain structure proposed for the intermediate temperature regime (\( T < T_D \)).

The upper right half of each circular field refers to DMPE on OTS, while the lower left half depicts the situation envisioned from DPPC on OTS. For clarity, the grid bars of the Ronchi ruling were made unequal in width from the width of the exposed regions. In actual fact, the two widths were equal, producing a square profile of fluorescence intensity immediately following the bleach pulse. Domains of average diameter \( L \) are surrounded by a « boundary region » which represents a barrier to rapid diffusion. Diffusion is assumed to be isotropic both in the interior of domains \( (D_d) \) and in the boundary region \( (D_b) \), with diffusivities differing by a large factor. For DMPE: \( L > P \); for DPPC: \( L < 1/2 P \).

Boundary regions, separating individual domains, are thought to be « defect-rich », i.e. characterized by a high degree of disorder and may be related to a « glass ». We assign the diffusivity of approximately \( 5 \times 10^{-11} \) cm\(^2\)/s observed for the chain-labelled lipids at temperatures below \( T_D \) (Fig. 5.3) to such boundary regions which therefore represent a barrier to the rapid diffusion of both types of fluorescent lipid probes.

We note that the existence of an anisotropic fluid, separating a tilted « crystalline » and an isotropic fluid phase has been postulated for DPPC monolayers at the air-water interface on the basis of film balance studies [31].

Local chain ordering in lipid multibilayers has been investigated extensively and it has been pointed out that the concepts of « chain disorder » and membrane « fluidity » are not identical in content [65, 66]. Seelig and collaborators have compared chain order parameter profiles for fatty acids and DPPC [67] and have also extracted values for the corresponding translational diffusion coefficients, in both cases at the respective bulk melting transitions. They find that translational diffusion is more rapid by two orders of magnitude in the fatty acid bilayers which retain a higher degree of chain order [66, 67].

In keeping with these findings, we suggest that the diffusivity reflects local chain order in such a way that diffusion in the interior of domains can be rapid \((D > 10^{-8} \) cm\(^2\)/s) if the residual chain order is not disturbed by the fluorescent probe. Rapid diffusion of the head-group label in the interior of domains in the intermediate monolayer phase would therefore reflect the fact that the label is matched to the state of chain orientational order of the lipid matrix. In contrast, the chain-labelled fluorescent lipid is expected to interfere with the chain ordering of the matrix and would consequently diffuse slowly.

An alternative implementation of a domain topology of the type introduced above may be considered. It invokes the coexistence, over the range of temperatures defined by \( T_D \) and \( T_F \), of a uniformly fluorescent solid phase, and the high temperature \( L \)-like phase. This model can account for the observed transport behaviour if domains of fluid lipid (\( L \)-phase) are embedded in a stained « solid » phase such as that observed below \( T_D \). In this case, rapid diffusion is expected for both chain and headgroup labelled lipids in the interior of domains, now consisting of a fluid lipid phase.

That solid-liquid coexistence exhibiting the topology considered here does indeed exist in lipid monolayers was confirmed by direct visual observation in DMPC monolayers at the air-water interface, as described in connection with figure 5.6 in the section on results. As described there, bands of unstained solid, delineating regions of fluid monolayer, lead to the reduction of effective diffusivities for long-range transport by more than an order of magnitude.

The present version of the domain model also readily explains the absence of any significant effect on the diffusivity of DMPE at temperatures near \( T^* \) (= 48 °C), as large domains of the monolayer would already be in the \( L \)-phase at much lower temperatures. We note that an estimate of 53 kJ/mole for the activation energy is obtained from a straight line fit, between 35 °C and 55 °C, to the data of figure 5.5. This is within a factor of two of activation energies quoted for diffusion in the \( L \)-phase of lipid bilayers [22, 23, 26].

In the context of this model, the widely differing diffusivities of chain-label and headgroup-label between \( T_F \) and \( T_D \) are again due to the suggested connection between higher chain order and more rapid diffusion. The argument given above must now be applied to the solid phase, in which the headgroup-label \((D = 2 \times 10^{-9} \) cm\(^2\)/s) is assumed to be more mobile than the chain label \((D < 10^{-11} \) cm\(^2\)/s).

While thermodynamically quite distinct, the two realizations of the proposed domain model invoke the same set of assumptions to account for the experimental results. The remainder of the discussion thus applies to both. However, it is to be kept in mind that in the case of two-phase coexistence the connected regions form a distinct solid phase, while in the presence of one thermodynamic phase domains of ordered fluid are separated by defect-rich (« grain ») boundaries.
Schneider et al. suggest that the data of Fahey and Webb ([24]; see also Fig. 5.3) obtained with the chain-labelled probe C12-NBD-PC were dominated by defect diffusion [45]. The value they attribute to the diffusivity of their fluorescent label in « ordered » regions of the sample is several orders of magnitude below our detection limit and would therefore not contribute to fluorescence recovery on the scale of our observation time. The behaviour of the chain-labelled probe in DPPC monolayers on OTS, particularly the transient increase in $\phi$ following heat treatment, is entirely consistent with that suggestion. The temperature dependence of the diffusivity associated with the transient mobile fraction closely matches the data of Fahey and Webb [24] and, like the hydrated multilayers, exhibits a broad melting transition near 39°C.

Rapid cooling after heating the sample into its L$_s$-like phase is expected to result in a high concentration of defects. Following Schneider et al. [45], we attribute the diffusive transport associated with the transient intermediate mobile fractions at temperature up to $T_D$ (Fig. 5.3) to « defect diffusion », characterized by a slow ($D \sim 5 \times 10^{-11}$ cm$^2$/s) diffusivity, which is usually only exhibited by a small fraction of the lipids. We propose that prolonged storage leads to annealing of defects as a result of which ordered domains are restored. The low mobile fraction of C6-NBD-PC in stored samples for $T < T_D$ indicates that the diffusivity of the chain-label remains below our detection limit, as would be expected from the findings of Schneider et al. [45]. In stored samples, rapid diffusion is observed only at $T_D$ (see Fig. 5.4), while the broad transition associated with the defects is absent. We note that phase separation of the label into « defect-rich » regions of sample, or into microclusters whose life-time is long compared to local diffusional jump times of matrix molecules, cannot be ruled out. This may explain the remarkable fact that the cooperative effect leading to the transition at $T_*$ is not sensed by the chain-labelled probe.

Both versions of the domain model imply that the transport characteristics of the intermediate monolayer phase are those exhibited by disordered composite materials [68]. On this basis we now examine briefly the diffusivities measured with the headgroup label in the intermediate phase.

Consider the schematic shown in figure 6. Since domains (of ordered fluid or of L$_s$-phase) are not connected, but separated by regions representing a barrier to rapid long-range transport, pathways for rapid diffusion ($D > 10^{-8}$ cm$^2$/s) are limited to a length of the order of $\bar{L}$, the average diameter of the domains. To contribute to the recovery of fluorescence in a FRAPP experiment, a fluorescent probe must diffuse, in the direction perpendicular to the stripes of the Ronchhi-ruling, over a distance of the order of $P$ (see Fig. 6). Hence, rapid intradomain diffusion dominates the recovery if the average domain size $\bar{L}$ is larger than $P(\bar{L} > P)$. In our domain model, this is the case for DMPE and accounts for the predominance of rapid diffusion at temperatures above $T_D$ (Fig. 5.5). In the case of DPPC, the average domain size $\bar{L}$ is comparable to or smaller than $P(\bar{L} < 1/2 P)$. Under these conditions, diffusion over a distance $P$ generally involves the traversal of at least one boundary region or strip of solid phase. The diffusivity $D$ describing the recovery will reflect the two individual diffusivities for intradomain and interdomain diffusion and the fractions of the total area sampled by a diffusing particle in either region.

Assuming isotropic intradomain ($D_a$) and boundary ($D_b$) diffusivities, a simple expression for $D$ can be derived by an argument given by Owicky and McConnell [69]. If $S$ is the total area sampled by a particular diffusing fluorescent lipid during the observation time, and $s_a$ and $s_b$ are the fractions of $S$ represented by the interior of domains and by regions between domains, respectively, $D$ is given by

$$1/D = s_a/D_a + s_b/D_b$$

where $s_a + s_b = 1$. To obtain a quantitative description of the actual fluorescence recovery, suitable distribution functions are needed to account for variations in domain shapes and sizes, and widths of domain boundaries which enter a calculation of a properly weighted composite diffusivity. Treatments such as those given for stochastic transport in disordered media may be useful in dealing with this type of problem [70]. The analysis of the data of figure 5.1 in terms of double-exponential recovery kinetics, in fact represents a test of a description in terms of a composite diffusivity, defined in the expression above. The result of this test, shown in figure 5.2, argues in favour of the presence of two exponentially recovering components. An internal consistency check can be performed in form of an order of magnitude calculation. Assuming $D_a = 10^{-8}$ cm$^2$/s and $D_b = 10^{-9}$ cm$^2$/s, as suggested by the results given in figure 5.2, we obtain a value for a composite $D$ of $5 \times 10^{-9}$ cm$^2$/s (see Fig. 5.1) if $s_a/s_b = 0.05$. This ratio compares favorably with that derived from photographs of the domain structure characterizing the unstained solid phase (see e.g. Fig. 1 in reference [11], or [37]).

We conclude that a model based on a domain structure with large ($\bar{L} \gg P$) domains for DMPE/OTS and smaller ($\bar{L} < 1/2 P$) domains in DPPC/OTS can account for the diffusivities observed with NBD-DPPE in the intermediate temperature regime ($T_* < T < T_D$) in the two monolayer systems.

We suspect that the measured composite diffusivity in general contains both contributions from heterogeneity and local diffusion anisotropy, which might be realized, for example, in the presence of molecular tilt. Closer inspection of the relevant azimuthal averaging shows that such in-plane anisotropy would also lead to intermediate composite diffusivities, but non-
exponential recovery kinetics. However, the simpler assumption of locally isotropic diffusion, in connection with the existence of domains, suffices to account for our data.

We suggest that a domain structure is preserved up to a temperature \( T_D \) which marks the appearance of a homogeneous phase, most probably the \( L_a^- \)-phase. The extent of the temperature interval \( [T_4, T_D] \) may be a measure for the degree of asymmetry between the substrate-attached hydrocarbon and the deposited lipid monolayer. For substrate-deposited DPPC bilayers, \( T_D^b \) does indeed appear to coincide with the chain melting transition, as measured by FRAPP [11].

Based on a comparison of diffusivities for substrate-deposited monolayers and those measured in monolayers at the air-water interface in comparable phases (Fig. 5.6, see also [33]), it appears that lower values are obtained in the former case. We interpret this to be an indication of «frictional» effects for substrate-deposited layers. Such residual interactions between monolayers and substrate would also explain the absence of convective flow in these layers.

As recently demonstrated, the lateral mobility of monoclonal antibodies, bound in low concentrations and with high specificity to target molecules present in a substrate-deposited lipid monolayer of the type described here, is controlled by the characteristic interactions between the lipid monolayer and the substrate-attached hydrocarbon chains [5]. Natural bilayer membranes exhibit an asymmetry between intracellular and extracellular sheets of the bilayer which is analogous to the inherent asymmetry of our substrate-deposited lipid monolayer/substrate-layer system. Our results raise the question as to whether the mobility of membrane components associated with one half of the cellular membrane can be regulated by events on the opposite side [71].

5. Summary.

In this article we have presented an extensive set of measurements of long-range translational diffusion in lipid monolayers at the interface between a hydrocarbon-derivatized substrate and a bulk aqueous phase. No other characterization of the phase behaviour of, and transport in such systems is presently available. The data support the conclusion that lipid monolayers, deposited on certain hydrocarbon-derivatized substrates, exhibit a novel intermediate state, not observed in multibilayers of the lipid. The heterogeneous character of this state, as evidenced by measurements of long-range molecular diffusion, is consistent with a domain topology. Domains are arranged in such a way that regions of rapid diffusivity are embedded either in a «glass» or other solid phase. The interior of the domains is suggested to be formed by an \( L_a^- \)-like phase, or, alternatively, to consist of an «ordered fluid». In the latter case, the observation of rapid diffusion in the intermediate state for a fluorescent lipid probe with intact hydrocarbon chains, but not for a probe with modified chains, suggests that rapid intraplanar diffusion in liquid crystalline phases can be aided by a high degree of chain order. The domain picture accounts for the essential features contained in our data, summarized in points (1) through (4) at the end of the section on «Results».

Two first order transitions are observed which separate the intermediate regime from a low temperature \( L_a^- \)-type phase and a high temperature \( L_a^- \)-like phase. The lower transition is dominated by characteristic interactions with, and insightful comments by Mr. S. Subramaniam who also participated in the measurements of long-range molecular diffusion, is consistent with a domain topology. Domains are arranged in such a way that regions of rapid diffusivity are embedded either in a «glass» or other solid phase. The interior of the domains is suggested to be formed by an \( L_a^- \)-like phase, or, alternatively, to consist of an «ordered fluid». In the latter case, the observation of rapid diffusion in the intermediate state for a fluorescent lipid probe with intact hydrocarbon chains, but not for a probe with modified chains, suggests that rapid intraplanar diffusion in liquid crystalline phases can be aided by a high degree of chain order. The domain picture accounts for the essential features contained in our data, summarized in points (1) through (4) at the end of the section on «Results».

Two first order transitions are observed which separate the intermediate regime from a low temperature \( L_a^- \)-type phase and a high temperature \( L_a^- \)-like phase. The lower transition is dominated by characteristic monolayer-substrate interactions.

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References


