Introduction

Oriented layer assemblies built up by the Langmuir-Blodgett (LB)-technique, have widespread applications in physico-chemical studies. Their electrical properties are gaining interest because of their possible future usefulness as electronics material. Recently, the importance of monolayers as test objects for high resolution electron microscopy has been shown. Furthermore, LB-layers may have aspects of ultrastructural and molecular organisation in common with certain domains existing in biological membranes. They can therefore serve as models for experiments that might lead to a better understanding of the possible local states in these very complex systems.

In a previous study, we reported on electron microscopic observations of ultrastructural changes that occur during the ageing of LB-layers of barium stearate and tripalmitin as well as on the occurrence of perturbations in layers of barium stearate and cadmium arachidate that became more and more severe as the number of transferred layers increased. In the case of tripalmitin, the ultrastructural indications of the transformation were confirmed by the results of an infrared spectroscopic investigation. With the aid of attenuated total reflection infrared spectroscopy (ATR-IR) we were able to show that a transition from a liquid crystalline state to a crystalline state takes place.

The aim of the work presented here was to investigate the time course of the rearrangement process in multimolecular LB-layers of tripalmitin.

Experiments

a. Attenuated total reflection infrared spectroscopy and specimen preparation

Attenuated total reflection spectroscopy was applied in order to get sufficient intensity of the infrared spectra. Germanium single pass reflection plates (50 × 20 × 1 mm) were used as a support for the lipid films.

Transfer of the monomolecular layer of tripalmitin from the air-water interface onto the germanium ATR plates was performed following the method of Blodgett. The plates were cleaned thoroughly with ethanol and twice distilled water in an ultrasonicator followed by a short plasmacleaning. Prior to the deposition of tripalmitin, the clean hydrophilic germanium plates were rendered hydrophobic by deposition of one monomolecular layer of barium stearate. The barium stearate layer was prepared by spreading of stearic acid on a subphase consisting...
of \(3 \times 10^{-5}\) M \(\text{BaCl}_2\) and \(4 \times 10^{-4}\) M \(\text{KHCO}_3\) pH 7.7), followed by transfer of one layer (film pressure: \(15 \pm 0.15\) dyn/cm; subphase temperature: \(16.5 \pm 0.5\) °C; ambient temperature: \(25\) °C). Four monomolecular layers (two inverted bilayers) of tripalmitin were then deposited on the hydrophobized germanium plate using the same subphase conditions as for the barium stearate. The transfer speed was \(2.2 - 2.7\) mm/min. The reproducibility of the quantity of the transferred tripalmitin was within 6%.

Immediately after the deposition of the lipid layers, the germanium plate was placed in a ATR sample holder which could be kept at a constant temperature (\(\pm 0.5\) °C). Spectra were scanned with a Perkin-Elmer 225 spectrophotometer equipped with two internal reflection attachments (Wilks Mod. 9 and Mod. 50). The angle of incidence of 30 degrees resulted in about 80 reflections. The mean spectral slit width was 1 cm\(^{-1}\). Spectra were scanned from 1800 cm\(^{-1}\) to 1100 cm\(^{-1}\) at arbitrarily chosen intervals over a period of up to one week. The ageing process of the specimens was observed at 20, 30, 40 and 50 °C. During the course of the entire experiments the specimens were maintained under constant conditions. The spectrometer was flushed continuously with dried air.

**b. Electron microscopy**

For electron microscopic investigation the layers were prepared the same way as for spectroscopy except that small pieces of germanium plates (smaller than 1 cm\(^2\)) were used as solid substrate. At different time intervals the ageing specimens were frozen in liquid Freon 22 (monochlorodifluoromethane, m.p. \(-146\) °C) and then transferred to liquid nitrogen (m.p. \(-196\) °C). To avoid contamination, the specimens were put in a brass specimen holder (still under liquid nitrogen) which could be closed by a tight fitting cover. The holder was quickly transferred into the vacuum chamber of a Balzers freeze-etch device BA 500 M where it was mounted on a precooled support (\(-170\) °C). The chamber was evacuated to a vacuum better than \(10^{-5}\) Torr and the object holder warmed up to \(-90\) °C until the outer surface of the still closed holder was free of ice. After the specimen holder had cooled again to \(-100\) °C and a vacuum \(2 \times 10^{-6}\) Torr had been reached, the holder was opened and the exposed sample shadow-casted by evaporation of 2 nm of platinum at an angle of 35 to 40 degrees by means of an electron beam gun. The platinum replica thus formed was reinforced by an additional deposition of 20 nm of carbon evaporated perpendicular to the object plane. The specimens where then brought to room temperature, the vacuum broken and the replicas floated onto a clean water surface. Sometimes the replicas failed to detach readily and the germanium plates had to be soaked for some hours in a detergent solution at \(40\) °C before the replicas could be lifted off. The replicas were then picked up with Formvar coated EM grids which before had been reinforced by evaporation of 10 nm of carbon. Cleaning of the replicas was found to be unnecessary.

Micrographs are photographically reversed so that the evaporated platinum appears white, whereas the shadows are black. The pictures are arranged in a way that the shadowcasting direction is from top left to bottom right.

**c. Chemicals**

The water used for the film balance was twice distilled in a quartz apparatus after it had been passed through an ion exchange column. Potassium hydrogen carbonate (pro analysis) was obtained from E. Merck, Darmstadt, Germany, and barium chloride, stearic acid and tripalmitin (all puriss.) from Fluka AG, Buchs (SG. Switzerland).

**Results and Analysis of Data**

**a. Ultrastructural aspects of the rearrangement process**

In a previous paper \(^8\) we showed that four monolayers of tripalmitin deposited on a hydrophobized glass slide spontaneously undergo ultrastructural and molecular transitions in the course of which the original Langmuir-Blodgett films brake down and the entire material is ultimately rearranged into randomly distributed microcrystals up to 1 \(\mu\)m in diameter and several tenths of a nanometer thick.

The electron micrographs presented here (Fig. 1 *) show that an analogous transformation occurs in LB-layers of tripalmitin deposited on an optically plane polished germanium plate. Fig. 1 a represents the situation found immediately (within 5 min) after deposition of 4 layers of tripalmitin on germanium. The germanium surface, which exhibits a great number of polishing traces, is covered by the lipid. But the covering is not as homogeneous as one would expect if the monolayer transfer from the air-water interface had been performed without any disturbance or redistribution of the material. Some areas of the deposited layers look quite smooth (top

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\* Fig. 1 see Table on page 712 b.
left) but large areas are covered by a kind of a network rather than by a homogeneous film.

If such layers are kept at room temperature for 24 h before freezing and replication, a large fraction of the lipid is found in the microcrystalline state shown in Fig. 1 b. In the course of this process tripalmitin molecules must move laterally over distances on the order of μm and vertically over several tenths of nanometers.

b. Spectroscopical aspects of the rearrangement process

The ATR-IR-spectra shown in Fig. 2 a and 2 b are examples out of a series of spectra taken from 4 layers of tripalmitin maintained at a constant temperature of 20 °C. Fig. 2 a shows the spectrum of a freshly deposited set of layers (start of the scan at 1800 cm⁻¹, 12 min 30 sec after the deposition of the lipid was completed; end of the scan at 1100 cm⁻¹, 29 min after deposition).

In Fig. 2 b a spectrum of the same specimen as in Fig. 2 a is shown after it had aged for about one week (start of the scan 164 h 30 min, end of the scan 164 h 47 min after the deposition of the lipid layers).

Only a short description of the spectra will be given here. For a more complete assignment of the absorption bands and analysis of the polarized spectra, the reader is referred to 8, 10.

1. The carbonyl stretching vibration (νC=O; 1743, 1736, 1728 cm⁻¹)

The νC=O stretching absorption band of the LB-layers at 20 °C is broad and not structured (Fig. 2 a). Its shape corresponds well with that exhibited by the equivalent band of molten tripalmitin 10. During ageing, the short wavelength part decreases (1743 cm⁻¹) whereas two peaks at 1736 and 1728 cm⁻¹ become more and more prominent (Fig. 2 b). These two peaks are attributed to the three carbonyl groups of the glycerol triester, whereby the larger one at 1736 cm⁻¹ is assigned to the νC=O vibrations of the symmetrically arranged two outer ester groups and the smaller one (1730 cm⁻¹) to the central ester group 10.

2. The –CH₂OCOCH₂-group vibrations

(1182 cm⁻¹, 1169 cm⁻¹)

Closely related to the crystallization of the lipid layers, an absorption band with rather large half width at 1169 cm⁻¹ is replaced by a sharper band at 1182 cm⁻¹ (Fig. 2 a and 2 b). The broad band corresponding to the LB-state of the layers indicates the existence of a variety of conformations in this more liquid-like phase. It was reported earlier 10 that in crystalline tripalmitin a very large band appears at 1155 cm⁻¹ at temperatures above 50 °C. This band was assumed to correspond to the 1180 cm⁻¹ band of the low temperature spectra of the same specimen. The analogy to the molten state is once more demonstrated by this behaviour.

3. The methylene bending vibration [δ(CH₂); 1473 cm⁻¹, 1469 cm⁻¹]

Fringeli et al. 10 reported a continuous broadening of the low wavenumber part of the CH₂-bending mode with increasing temperature. We found 8 that in the case of eight tripalmitin layers at short ageing times (1 h 30 min) the band consists of two components of which the one at higher wavenumber is assigned to the band observed in crystalline tripalmitin (cf.
Fig. 2 a and 2 b). The doublet 1473/1469 cm⁻¹ proved to be a suitable probe for the analysis of the time-dependent behaviour of the tripalmitin layers, as will be shown below.

4. The sequence of wagging vibrations \( \gamma_w(CH_2); 1310 - 1190 \text{ cm}^{-1} \)

The wagging sequence is known to exist only if the saturated hydrocarbon chains are in the all-trans conformation. In crystalline tripalmitin, the wagging progression is not affected up to 59 — 60 °C but between 60 and 61 °C an abrupt breakdown of the sequence occurs.

In the LB-state of the tripalmitin layers, this sequence is faintly visible (Fig. 2 a) but concomitant with the rearrangement becomes more and more prominent (Fig. 2 b). The formation of all-trans configuration is therefore involved in the rearrangement.

In order to get more details on the time course of the rearrangement process occurring in the tripalmitin layers, we investigated the time-dependent changes in the CH₂-bending region at different temperatures. In a few cases a similar analysis of the \(-CH_2OOCOCH_2-\) group vibrations was carried out too. It was found that the bending doublet can be well approximated by super-imposing two Lorentz functions, one corresponding to the LB-state the other to the crystalline (CR) state. This enabled us to estimate the amounts of the substance in the two states as a function of time.

To perform the lineshape analysis, the coordinates of 20 to 40 points of the absorption band were digitally read out from each spectrum investigated. Then, the least squares fit of the data points were computed for the following function

\[
a(\tilde{v}) = \frac{1}{\pi} \left[ \frac{A_{LB} \cdot A_{LB}^{'}}{(\tilde{v}_{LB} - \tilde{v})^2 - (A_{LB}^{'})^2} + \frac{A_{CR} \cdot A_{CR}^{'}}{(\tilde{v}_{CR} - \tilde{v})^2 - (A_{CR}^{'})^2} \right]
\]

where \(a(\tilde{v})\) is the absorption coefficient, \(A_{LB}\) an \(A_{CR}\) are the areas of the LB and CR absorption bands, \(\tilde{v}_{LB}\) and \(\tilde{v}_{CR}\) are the positions of the LB and CR absorption bands, \(A_{LB}^{'}\) and \(A_{CR}^{'}\) are the half of the half widths of the LB and CR absorption bands and \(\tilde{v}\) equals the wavenumber.

The mean values found for positions and half widths of the CH₂ and the \(-CH_2OOCOCH_2-\) bands are given in the following Table.

<table>
<thead>
<tr>
<th>Group</th>
<th>State</th>
<th>Wavenumber [cm⁻¹]</th>
<th>Half width [cm⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH₂-bending</td>
<td>LB</td>
<td>1469</td>
<td>8.4</td>
</tr>
<tr>
<td></td>
<td>CR</td>
<td>1473</td>
<td>4.0</td>
</tr>
<tr>
<td>(-CH_2OOCOCH_2-)</td>
<td>LB</td>
<td>1169</td>
<td>(32)</td>
</tr>
<tr>
<td></td>
<td>CR</td>
<td>1182</td>
<td>9.0</td>
</tr>
</tbody>
</table>

Fig. 3 shows typical examples of the Lorentz line shape approximation in the case of the CH₂- bending mode at different times and temperatures. The solid line connecting the measured points (line with kinks) can hardly be distinguished from the Lorentz function (1) (continuous solid line S). When comparing the curves obtained from the same specimen at different times (Fig. 3 a to c) it can be seen that the LB-state is continuously transformed into the CR state. Fig. 3 a shows a typical \(\delta(CH_2)\) absorption band of the LB layers about 20 min after deposition to the germanium plate. The relatively broad shape compared with the one of the crystalline form (Fig. 3 d) reflects the larger population of conformational states expected in this more liquid-like state.

The appearance of a small shoulder at 1473 cm⁻¹ is due to the crystallization which already has started at the moment of deposition. After the layers had been stored for one day at 20 °C (Fig. 3 b) the peak at 1473 cm⁻¹ has become more prominent than the original one at 1469 cm⁻¹ but as can be seen from the area/time curve in Fig. 4a the crystalline fraction comprises only one third of the tripalmitin present on the plate (level indicated by triangular points).

The velocity of the rearrangement process slows appreciably from its initially high value (Fig. 4 a) reaching a constant level after about one day. After one week at 20 °C about half of the tripalmitin has been converted from the LB-state to the crystalline one (Fig. 4 b). At 50 °C the crystallization proceeds much faster. More than half of the tripalmitin has already crystallized within one hour.

The line shape analysis by means of Eqn (1) involves the assumption the sum \(A_{LB} + A_{CR}\) to be constant over time. This was confirmed reasonably well by our results (triangular points in Fig. 4). However, in earlier stages a tendency to slightly smaller values was sometimes found (Fig. 4 a). It could not be determined whether these small de-
viations were due to a systematic error (introduced, for instance, by an incorrect positioning of the 100%-line leading to a larger relative deviation in the area of the broad LB-band which is more prominent in the earlier stages; note the increased fluctuation in the measured points of the LB-curve in the first ten hours compared with the accuracy of the corresponding points in the CR-curve).

The general course of the rearrangement in the tripalmitin layers is also confirmed by the similarity of the dotted lines E in Fig. 4 a and b, which represent the increase of crystallinity in the layers as reflected by the $\text{CH}_3\text{OCOCH}_2$ group vibrations of the crystalline tripalmitin. However, the accuracy of the line shape approximation of this band by (1) was found to be less than in the case of the $\delta(\text{CH}_2)$ absorptions. The broad band at 1169 cm$^{-1}$ assigned to the LB-form especially, cannot be approximated by a single Lorentz function. For this reason, only the time course of the formation of the crystalline form is given in Fig. 4.

**Discussion**

*a. Reproducibility of the time-dependent changes*

The original aim of our work was to get more detailed information about the kinetics and mechanism of the rearrangement process taking place in Langmuir-Blodgett layers. However, it turned out that the reproducibility of the decay velocity at

![Fig. 3. a–c. Three typical examples of the Lorentz lineshape approximation (Formula I) of the $\delta(\text{CH}_2)$ band at 20 °C resulting from four tripalmitin layers. The area enclosed by the measured adsorption band (kinked solid line) is approximated by the superposition S (continuous solid line) of the two areas enclosed by the two bands attributed to the crystalline (CR; broken line) part and the part of the substance which is in the Langmuir-Blodgett state (LB; dotted line). The $\delta(\text{CH}_2)$ band is shifted from 1469 to 1473 cm$^{-1}$. d. Shows an example similar to those in a–c. except that the temperature during the ageing process was 50 °C, leading to a greater quantity of crystalline material although the time was shorter than in e.*
Fig. 4. The time course of the rearrangement process in four layers of tripalmitin is shown at 20 °C (a) and at 50 °C (b). Each point and circled point corresponds to the area of a Lorentz curve of the CR band at 1473 cm⁻¹ and the LB band at 1469 cm⁻¹ respectively, computed as shown in Fig. 3. The sum of two corresponding values of the LB and CR state is given by a triangular point. The line E in a. and b. shows the time course of crystallization in the layers as demonstrated by the change in area of the 1182 cm⁻¹ band assigned to the —CH₂OCOCH₂— group vibrations.

constant conditions was very hard to achieve. We were also unable to observe significant differences in the rate of decay when the experiments were performed at 20, 30, and 40 °C. For these reasons we decided to present only two typical experiments performed at 20 °C and 50 °C, respectively. The rate of decay at these two temperatures was found to be distinctly different in all experiments. Possible reasons for the problems in reproducibility are:

1. Irregularities in the monolayers at the air-water interface which can act as crystallization nuclei in the deposited layers 11-13.

2. Crystallization occurring during the dipping cycle when the previously deposited layers are submerged. Such a behaviour was reported for barium stearate 8 and for electrolytically deposited lauric acid 14.

The model proposed by Honig 17 concerning overturning phenomena under water during the deposition could be an explanation for the formation of traces of X type (head-tail) domains in our layer assemblies which than could have acted as crystallization nuclei.

3. The possible occurrence of the “zippering” phenomenon (drainage of the water film enclosed between the substrate and the deposited layer 15, 16).

4. Contaminations of any kind (traces of impurities on the substrate, in the subphase or in the atmosphere) which could induce crystallization.

5. Humidity of the atmosphere. All our experiments were performed in an atmosphere with 1% rel. humidity (25 °C). We observed a significant reduction in the rate decay when the relative humidity was about 50%.

6. Influence of substrate. In order to render the germanium reflection plate hydrophobic, one monolayer of barium stearate was deposited. As was shown earlier, this first layer was found to be unstable on a carbon film 8 but stable on germanium. Based on this observation we assumed that this layer would not influence directly the crystallization of the upper tripalmitin layers.

b. Kinetic models of the rearrangement process

Because we were not able to control all the parameters influencing the decay rate of LB layers (cf. a) we did not attempt to arrive at a model to fit the experimentally determined kinetic data, although the accuracy of the measurements presented in Fig. 4 is good enough for kinetic analysis. While the results presented in Fig. 4 can be fitted by means of a simple kinetic model, it is obvious that
the actual mechanism of the rearrangement process is very complex. This is confirmed by the manifold ultrastructural states observed.

c. Remarks concerning earlier investigations of oriented layers of tripalmitin

In an ATR-IR investigation of oriented layers of tripalmitin10 which were produced by the LB technique, it was found that the spectra were quite similar to those obtained from microcrystalline tripalmitin. Our recent investigation8 showed that the oriented layers described in that work had already rearranged from the LB-state into the microcrystalline state. The spectrum of the microcrystals taken at a temperature close to the melting point, however, does resemble the spectrum of tripalmitin in the LB layers, as is demonstrated by Fig. 2a and8. Because of this similarity it can be concluded that the liquid crystalline state predominates in LB layers of tripalmitin.

d. Stability of LB layers

It was demonstrated that the LB state of tripalmitin layers can be conserved in its ultrastructural aspects by rapid freezing8. Other authors18,19 have found that it is possible under their conditions of preparation to obtain stable LB layers at ambient temperature.

It can be expected that mixed films result in a greater stability because their free energy of segregation works against the driving force of crystallization. Substances which exhibit bad crystallization properties, e.g., due to unsaturation or branching of their hydrocarbon chains or to the influence of side chains such as phosphorylcholine, may also preserve the LB state after deposition on a solid substrate. Thus, we have found that oriented layers of dipalmitoyl-phosphatidyl-choline and layers of egg lecithin remain stable whereas layers of lecithin derivatives with modifications in the fatty acid ester groups region show a tendency to crystallize.

A precondition for construction of predetermined arrangements of functional units in molecular dimensions, e.g. in models of biological membranes, is that the systems thus formed be stable. In light of our results we have come to the conclusion that any system built up from LB layers has to be checked thoroughly for the stability of its layer assembly.

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7 W. Baumeister and M. Hahn, Cytobiologie 7, 244 [1973].
13 W. Baumeister, Fette, Seifen, Anstrichm. 77, 109 [1975].
20 U. P. Fringeli, to be published.