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Anomalous pH dependence of the coexistence pressure of the polymerizable two-chain N-lipid methyl-bis(pentacosadiinoyl-oxyethyl)-amine

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Abstract Molecular films from polymeric materials play an important role in basic research as well as in technology. We have synthesized the double chain diacetylenic ammonium lipid N-bis-(10, 12-pentacosadiinoyl)-oxyethyl)-N-methyl-amine (ONCO). We have characterized monomolecular films at the air/water interface by means of microfluorescence filmbalance techniques and by atomic force microscopy (AFM). ONCO forms stable monomolecular films that exhibit a fluid-solid phase transition with a transition enthalpy of 90 kJ/mol at 10 °C and neutral pH. The coexistence pressure was found to decrease with decreasing protonation, which is in contrast to the commonly found Coulomb mechanism. A change in the chain packing due to a different nitrogen bond angle is discussed as a possible mechanism. This model is corroborated by the finding that crystals at high and at low pH differ in their polymerization properties as measured by microfluorescence.

Key words Diacetylenic lipids · Polymerization · Microfluorescence · Filmbalance · AFM

Abbreviations Abs. absolute · $CHCl_3$ chloroform · DCC dicyclohexylcarbodiimide · DEMA N-methyl-N-bis-ethanolamine · DPPE: Dipalmitoyl-sn-glycerol-phosphatidyl-ethanolamine · HOBT hydroxy-benzotriazole · MeOH methanol · NHS N-hydroxy-succinimide · NEt_3 triethyl ammonia · ONCO N-bis-((10, 12-pentacosadiynoyl)-oxyethyl)-N-methyl-amine · PCA 10, 12-Pentacosadiynoic acid · HEPES [4-(2-hydroxyethyl)-1-piperazine-propane-sulfonic acid monohydrate] · Net_3 Triethylamine · FAB Fast atomic bombardment · TLC Thin layer chromatography · MS Mass spectroscopy

Introduction

Ultrathin polymeric lipid films have been at the focus of interest of basic research for nearly two decades because of their unique properties as low dimensional model systems (Sackmann 1996; Roth 1995; Ulmann 1991; Hub et al. 1980; Tieke et al. 1976). They also have a broad range of technical applications ranging from biocompatible surface coatings to tribology (Swalen et al. 1987; Novotny et al. 1989; Landman and Israelachvili 1995). Molecular films with a polydiacetylenic polymer backbone show a several unique properties. In addition to their high mechanical stability they exhibit unique optical effects such as chemically triggered fluorescence, a high third order nonlinear response and a strong dichroism (Lochner et al. 1978; Greene et al. 1990; Wu and Chen 1991; Suzuoki et al. 1991; Charych et al. 1993).

Films made from several different diacetylenic lipids have been the subject of extended microfluorescence and AFM investigations, that have revealed many details of molecular packing as well as hydrocarbon chain rearrangements (Göbel et al. 1987; Radmacher et al. 1991; Goettgens et al. 1992; Tillmann et al. 1993; Tillmann et al. 1994).

In a previous study we investigated the single chain diacetylenic lipid EMPDA (Sullivan et al. 1994). We wanted to increase the mechanical stability of such films by introducing a second hydrocarbon chain. Here we present a new synthesis protocol for the two chain amine lipid ONCO (Hub et al. 1980), a thermodynamic characterization of monomolecular films at the air/water interface, microfluorescence investigations of the morphologies of the phases and the polymerization characteristics of the crystalline

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films, followed by a brief AFM investigation of the films after they had been transferred to silicon oxide. Particular emphasis was put on the charge and conformational interactions of the head groups since it is commonly believed that these play an important role in the biological function of membranes (Sackmann 1996).

Materials and methods

Chemicals

PCA was purchased from ABCR (Karlsruhe, Germany), Thin-Layer-Chromatography (TLC) plates (Kieselgel 60 F_{254}), and silical gel (40–63 mm, 230–400 mesh) were obtained from Merck (Darmstadt, Germany). All other chemicals and solvents were from Fluka (Neu-Ulm, Germany) and were reagent grade. NEt_3 was refluxed over CaH_2 and distilled prior to use. Texas Res-DPPE (Molecular Probes, Eugene, Alabama, USA) was used to label the fluid phase for the monomer microfluorescence investigations.

CACO buffer consisted of 10 mM sodium cacodylic acid titrated with HCl. HEPES buffer consisted of 10 mM HEPES and 10 mM NaCl titrated with NaOH. GLYCINE buffer consisted of 10 mM glycine titrated with NaOH. di-SODIUM buffer consisted of 10 mM disodium-hydrogen-phosphate titrated with NaOH.

Synthesis of ONCO

Reactions were monitored by TLC and visualized by bromocresol green (acids and bases) or iodine. Solvent ratios are given in volume/volume. 1.123 g (3 mmol) PCA were dissolved in 40 ml abs. $CHCl_3$ and after the addition of 414 mg (3.6 mmol) DCC in 10 ml abs. $CHCl_3$ and 486.4 mg (3.6 mmol) HOBT in 10 ml abs. $CHCl_3$ the solution was stirred overnight in the dark at room temperature. The precipitated dicyclohexyl-urea was filtered off and 172.2 ml (1.5 mmol) DEMA and 432 ml (3 mmol) abs. NEt_3 were added. After stirring for 24 h the precipitated triethylammonium chloride was filtered and the organic layer was extracted twice with 1 N HCl and twice with saturated $NaHCO_3$, dried over anhydrous sodium solution and the solvent was removed in vacuo. ONCO was purified by silica column flash chromatography. After eluting unreacted PCA with $CHCl_3$, the product was eluted with $CHCl_3$/MeOH 9:1.

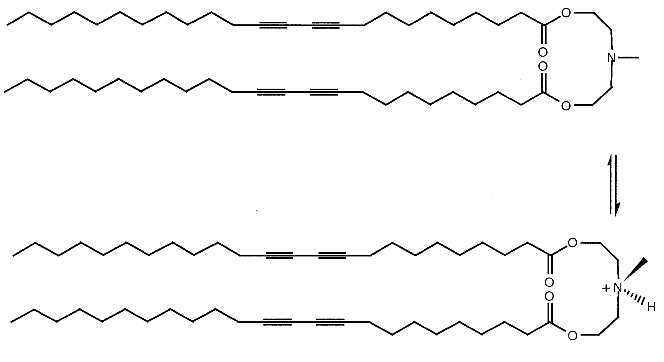


Fig. 1 Chemical structure of the lipid ONCO

Yield: 30%
TLC ($CHCl_3$/MeOH 9:1) = 0.35

^{1}H-NMR (400 Mhz, CD Cl_3): d = 0.85 (t, 3 H, $C\mathbf{H}_3(CH_2)$), d = 1.2–1.25 (m, 56 H, CH_2), d = 1.5 (m, 4 H, $C\mathbf{H}_2(CH_2)_2C{\equiv}C$), d = 1.6 (m, 4 H $C\mathbf{H}_2CH_2COO$), d = 2.15 (m, 8 H, $C\mathbf{H}_2CH_2C{\equiv}C$), d = 2.3 (t, 4 H, $C\mathbf{H}_2COO$), d = 2.35 (s, 3 H, $NC\mathbf{H}_3$), d = 2.6 (t, 4 H, $CH_3\ (CH_2)_{10}C\mathbf{H}_2C{\equiv}C$), d = 2.7 (t, 4 H, $C{\equiv}CC\mathbf{H}_2\ (CH_2)_8COO$), d = 3.6 (t, 4 H, $COOCH_2C\mathbf{H}_2N$), d = 4.2 (t, 4 H, $COOC\mathbf{H}_2CH_2N$).
^{13}C-NMR (100 Mhz, CD Cl_3): d = 14.79, 19.88, 23.36, 25.56, 29.02, 29.45, 29.56, 29.76, 30.03, 30.16, 30.31, 32.61 ($\mathbf{C}H_3(CH_2)$, CH_2), d = 34.92 (N-$\mathbf{C}H_3$), d = 43.12 ($CH_2\mathbf{C}H_2COO$), d = 56.1 ($COOCH_2\mathbf{C}H_2N$), d = 59.34 ($COO\mathbf{C}H_2CH_2N$), d = 62.31, 62.65, 65.94, 66.01 ($C{\equiv}C$), d = 176.32 ($CH_2\mathbf{C}OOCH_2$).

MS (FAB pos., $C_{55}H_{93}NO_4$): $M + H^+ = 832.9$ g/mol.

Substrates

Silicon (100) p-type wafers (Wacker Burghausen) with a thermally grown oxide layer of about 150 nm thickness were cleaned according the following procedure: the samples were ultrasonicated in 2% Hellmanex (Mühlheim, Germany) for 30 min at 30 °C, followed by 10 times rinsing with pure water (Milli-Q-System, Molsheim, France) and ultrasonication in pure water for 30 min at room temperature. The samples were then rinsed in MeOH for 5 min and dried overnight at 80 °C. Square centimeter sized pieces of the wafers were then glued to the metal sample holders with an epoxy resin and stored in a dessicator (Hofmann et al. 1995).

Film balance

A custom-built film balance, with a 400 cm^2 surface area, was used to record the pressure area (π, A) diagrams. UV polymerisation was carried out at a constant surface pressure (25 mN/m) on the subphase by irradiating the surface with a Hg pen ray lamp (Oriel, type 6035) kept at a distance of 3 cm for 15 min. Polymerized films were transferred by standard Langmuir-Blodgett (LB) techniques. The fluorescence micrographs were recorded in a small trough with a 30 cm^2 surface area (Tillmann et al. 1994; Sullivan et al. 1994).

Atomic Force Microscopy

AFM images were recorded on a Nanoscope III (Digital Instrument, Santa Barbara, CA) using silicon nitride cantilevers (120 mN/m spring constant) with sharpened oxide tip (Digital Instrument).

Results and discussion

Monomolecular films from ONCO were spread at the air/water interface of the combined microfluorescence filmbalance through following standard protocols (Goettgens et al. 1992; Tillmann et al. 1993). The compression isotherms exhibited a slight compression rate dependent overshooting at the onset of the fluid solid transition. This is a well known effect common to most diacetylenic lipids (Göbel et al. 1987; Goettgens et al. 1992; Tillmann et al. 1993; Tillmann et al. 1994) and is interpreted as a seed density dependent super cooling of the two-dimensional fluid. A set of isotherms was recorded at different temperatures (see Fig. 2). It showed a well pronounced temperature dependence of the coexistence pressure. The transition enthalpy was derived with a Clausius Clapeyron analysis (Adam et al. 1988) as 90 kJ/Mol at 10 °C. This value is comparable to the ones previously reported for double chain diacetylenic lipids (Albrecht et al. 1978).

Since the amine headgroup of the lipid was expected to undergo a deprotonation upon increase of the subphase pH, a set of compression isotherms was recorded on buffers of different pH at 5 °C. A selected set is shown in Fig. 3a. The coexistence pressure showed a marked pH dependence but much to our surprise this was opposite to that commonly shown by lipids with titratable headgroups. As shown in Fig. 3b, the coexistence pressure increases with increasing pH. At low pH values (<pH 5) the amine is assumed to be protonated and thus positively charged. This assumption is justified since at this pH, the coexistence pressure of ONCO is comparable to that of EDIPAB, which is the same lipid but with a quarternary amine instead of the ternary amine head group (Tillmann et al. 1994). Also, the macroscopic domain morphology, as seen in the microfluorescence images (Fig. 4a), is very similar to that of EDIPAB (Tillmann et al. 1994). With increasing pH the amine headgroup becomes neutral, which generally results in a reduction of the coexistence pressure due to a reduced Coulomb repulsion. In the case of ONCO, however, we found that the coexistence pressure increased significantly with increasing pH. This means that an additional mechanism must work against the crystallization at higher pH values and overcompensate the reduced Coulomb repulsion. One possible mechanism could be that upon deprotonation, the bond geometry of the chains at the nitrogen atom changes from the tetrahedral to the pyramidal configuration (Streitwieser and Heathcock 1986). This may result in an orientation of the chains which makes crystallization less faborable and thus increase the coexistence pressure.

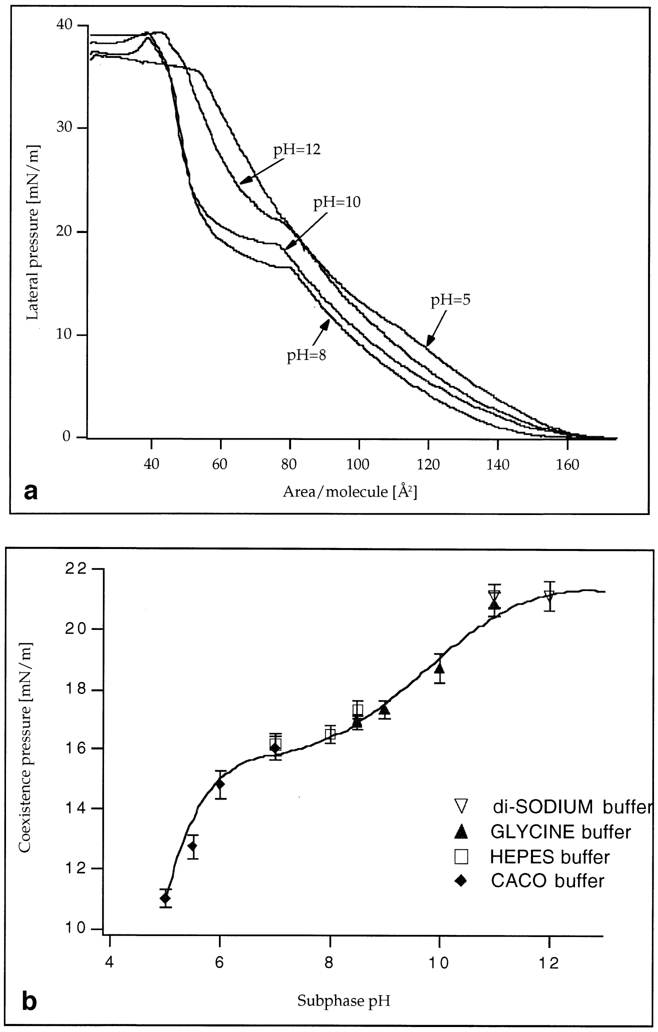


Fig. 3 Monomeric ONCO films at the air/water interface (T = 278 K). **a** Pressure-area isotherms at various values of the subphase pH. **b** Fluid-solid coexistence pressure plotted as a function of the pH in different subphases

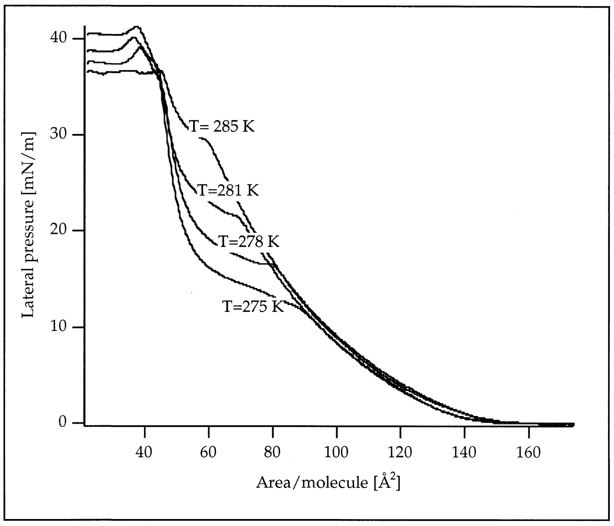
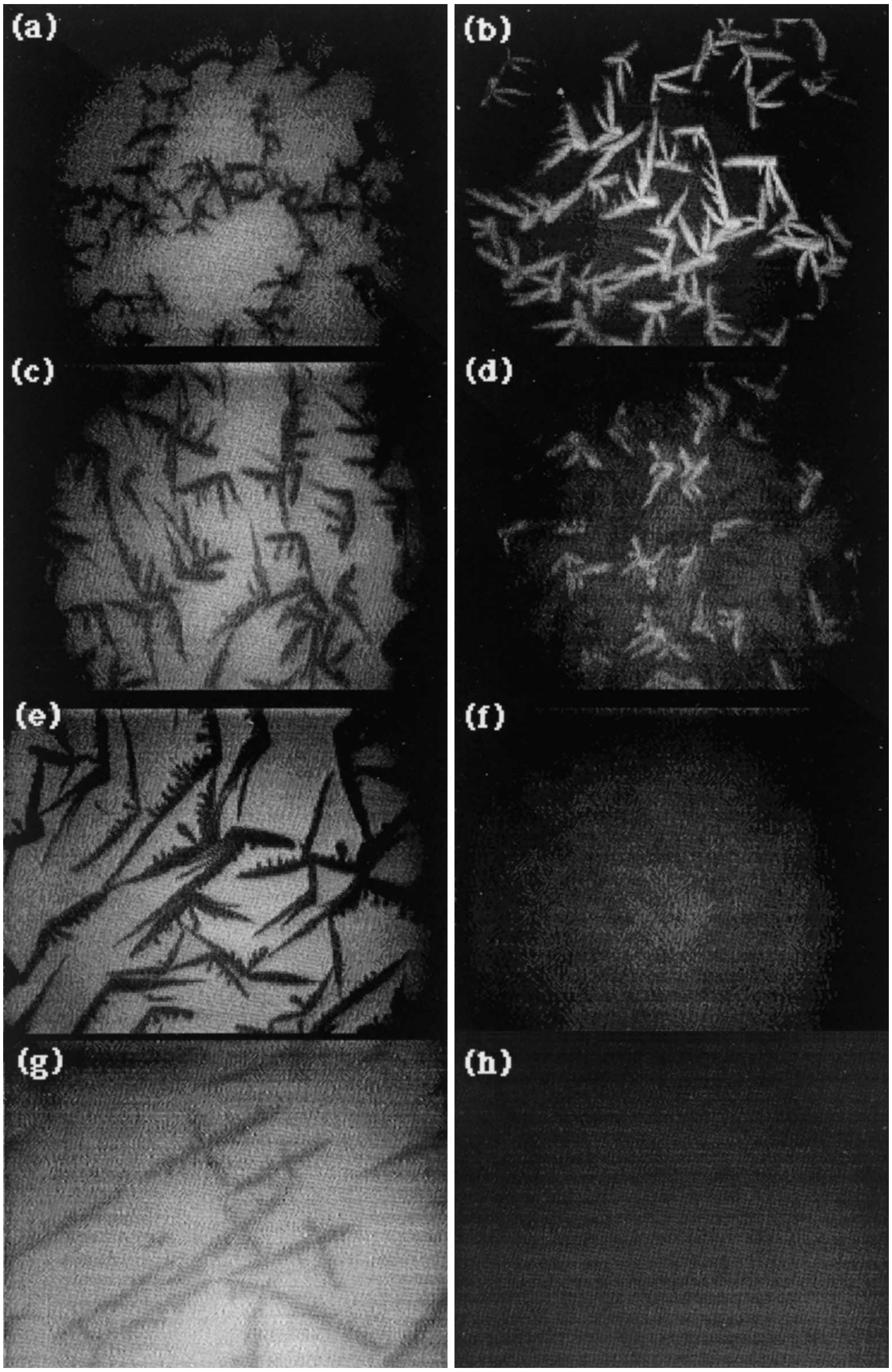


Fig. 2 Pressure-area isotherms of monomeric ONCO films for various temperatures in HEPES buffer pH 8.0

This assumption was corroborated by the results from a series of microfluorescence images recorded at different pH values before and after UV-polymerization (see Fig. 4). Before polymerization the solid-lipid domains appear dark as they exclude the fluorescence labelled lipid that was

Fig. 4 Fluorescence micrographs of a ONCO monolayer at different subphase pH, before (**a, c, e, g**) and after (**b, d, f, h**) polymerisation (T=278 K). **a**, **b** pH 5, CACO buffer; **c, d** pH 6, HEPES buffer; **e, f** pH 8, GLYCINE buffer; **g, h** pH 12, di-SODIUM buffer. Image size: 200 μm×250 μm



added in trace amounts (<0.2‰). During UV polmyerization the fluorescence label in the fluid phase is irreversible photobleached. At low pH values (Fig. 4b) the solid domains polymerized rapidly and exhibited the well known bright intrinsic fluorescence of the polymer backbone. At pH 6, only a fraction of the solid domains became fluorescent after polymerization and finally at higher pH values the solid domains did not turn fluorescent any more even after prolonged UV exposure.

Two possible explanations may account for this finding: either the domains did not polymerize at higher pH values or, they did polymerize, but the resulting polymer backbone is not fluorescent. Both explanations would suggest, that the solid domains at different pH, although macroscopically not distinguishable, differ in their packing.

In order to distinguish between the two possibilities, we transferred films after polymerization onto silicon wafers and images them with the atomic force microscope. Figure 5 shows a typical example of a film that was polymerized at pH 5. The more elevated structures gave faint molecular resolution (data not shown) at higher magnification with chain spacings comparable to those found for other lipids of this kind (Goettgens et al. 1992; Tillmann et al. 1993; Tillmann et al. 1994; Sullivan et al. 1994), identifying these areas as ordered polymeric domains. Films that were polymerized even for prolonged times at higher pH values did not show any domains in the AFM. Since during prolonged polymerization the pressure drops below the coexistence pressure, the most likely explanation for this finding is, that the domains had not polymerized and either dissolved or degraded. This means that at all pH values, the lipid forms solid domains that exhibit long range order as evidenced by the regular macroscopic shape of the domains. At high pH values, however, the molecular packing of the chains is such, that the polymerization reaction, which is known to be topochemical, is not possible. This again would support our hypothesis, that the pyramidal bond geometry at the nitrogen head forces the chains into a different packing. This packing must be incompatible with the packing of the tetragonal bond geometry since in the crossover pH range (pH 6) two populations of domains occur which are either fully polymerized or not polymerized at all. Future high resolution AFM investigations should directly reveal this difference in molecular packing.

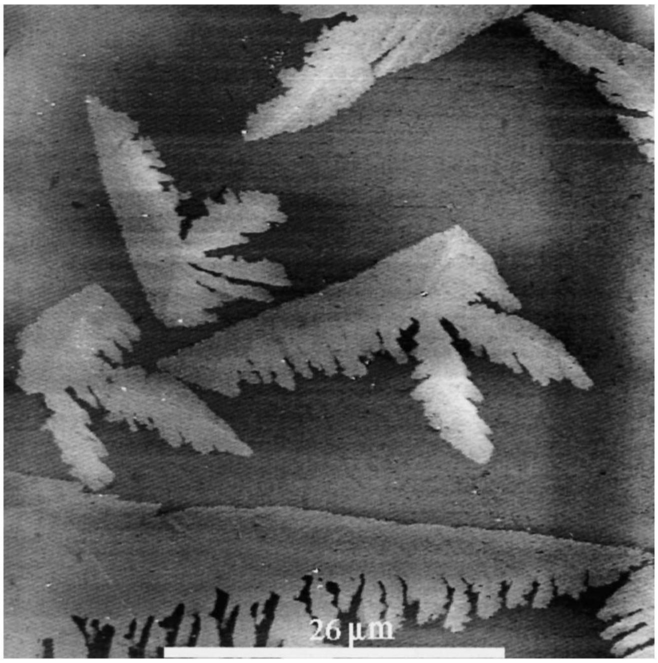


Fig. 5 AFM image of a polymerized ONCO single crystal deposited on silicon oxide. Obtained in air with a Nanoscope using a silicon nitride cantilever with an oxide sharpened tip

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