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Miscibility in Binary Monolayers of Phospholipids and Linker Lipid

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We studied the miscibility in binary lipid matrixes made by the Langmuir−Blodgett (LB) technique. The components in the lipid matrix were *N*-(ϵ-maleimidocaproyl)-dipalmitoyl phosphatidylethanolamine (DPPE-EMC; biofunctionalized linker lipid) and a phospholipid. Three different matrix phospholipids were used: 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylethanolamine (DPPE), 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylethanolamine (DMPE), and 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC). The phase-transition temperature of the linker lipid as determined by Fourier transform infrared spectroscopy was 45 °C. The surface potential of the linker lipid, 290 mV at pH 6.8, was clearly smaller than the values observed for pure phospholipids. Clear evidence of the miscibility could not be obtained from the surface pressure−area isotherms. On the contrary, Brewster angle microscopy (BAM) enabled a visual investigation of the miscibility and domain morphology. The best miscibility was obtained for DPPC/DPPE-EMC matrixes but only to some extent for DPPE/DPPE-EMC and DMPE/DPPE-EMC matrixes. Atomic force microscopy on solid supported LB films showed domains similar to the BAM images of Langmuir monolayers.

Introduction

The Langmuir−Blodgett (LB) technique allows the transfer of a monolayer from the air−water interface onto a solid support and thus enables the preparation of novel organic assemblies.[1] Several groups have used the LB technique for biosensor design because the orientation and surface density of antibodies can be controlled.[2,3] Different approaches and molecules have been used to biofunctionalize a lipid monolayer, such as a biotinylated amphiphile−streptavidin system,[4] protein A,[5] *N*,*N*-dioctadecyl-*N*-methyl-N−(2-mercaptoethyl) ammonium bromide (DOMA),[6] lipid-tagged single-chain antibodies,[7] or linker lipids.[8] Linker lipids were first used in liposomes to make the liposomes suitable for targeted drug delivery.[9,10] Egger et al.[11,12] produced planar supported lipid matrixes with incorporated linker lipids by the vesicle-spreading method. Vikholm and Albers[13] studied ternary monolayers (lipid, cholesterol, and linker lipid) transferred by the Langmuir−Schaefer (LS) method with quartz crystal microbalance (QCM). Previous reports on binary and ternary systems have focused on the ability to transfer monolayers to solid substrates. This approach often neglects the miscibility of monolayer components. However, to obtain a good sensing surface a homogeneous distribution of linker lipids in the monolayer is desired. Miscibility in floating Langmuir films can be detected by Brewster angle micoscopy (BAM) and by atomic force microscopy (AFM) in LB films on solid substrates.[14] A recently developed tapping mode AFM[15] and especially phase-contrast imaging facilitate the analysis of different materials on solid supports.[16−19]

In this study the LB method was used to produce a lipid matrix monolayer for coupling of Fab′ fragments to be applied as a sensing surface for immunoassays. The immobilization matrix was made of a mixture of a linker lipid and a phospholipid. The linker lipid synthesized for the antibody immobilization was *N*-(ϵ-maleimidocaproyl)-dipalmitoyl phosphatidylethanolamine (DPPE-EMC), which is a phospholipid derivatized with a heterobifunctional cross-linking molecule. DPPE-EMC was characterized by Fourier transform infrared (FT-IR) spectroscopy and by a surface potential method to analyze the phase-transition temperature of the molecule and to obtain information about the dipole moments, respectively. Three

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(1) Ulman, A.; Ed. *An Introduction to Ultrathin Organic Films: From Langmuir−Blodgett to Self-Assembly;* Academic Press: San Diego, 1991.

(2) Petty, M. C. *J. Biomed. Eng.* **1991**, *13*, 209.

(3) Wang, H.; Brennan, J. D.; Gene, A.; Krull, U. J. *Appl. Biochem. Biotechnol.* **1995**, *53*, 163.

(4) Herron, J. N.; Müller, W.; Paudler, M.; Riegler, H.; Ringsdorf, H.; Suci, P. A. *Langmuir* **1992**, *8*, 1413.

(5) Turko, I. V.; Yurkevich, I. S.; Chashchin, V. L. *Thin Solid Films* **1992**, *210/211*, 710.

(6) Ahluwalia, A.; Carra, M.; De Rossi, D.; Ristori, C.; Tundo, P.; Bomben, A. *Thin Solid Films* **1994**, *247*, 244.

(7) Vikholm, I.; Györvary, E.; Peltonen, J. *Langmuir* **1996**, *12*, 3276.

(8) Heyn, S.-P.; Egger, M.; Gaub, H. E. *J. Phys. Chem.* **1990**, *94*, 5073.

(9) Martin, F. J.; Hubbell, W. L.; Papahadjopoulos, D. *Biochemistry* **1981**, *20*, 4229.

(10) Leserman, L. D.; Babet, J.; Kourilsky, F.; Weinstein, J. N. *Nature* **1980**, *288*, 602.

(11) Egger, M.; Heyn, S.-P.; Gaub, H. E. *Biochim. Biophys. Acta* **1992**, *1104*, 45.

(12) Fischer, B.; Heyn, S. P.; Egger, M.; Gaub, H. E. *Langmuir* **1993**, *9*, 136.

(13) Vikholm, I.; Albers, W. M. *Langmuir* **1998**, *14*, 3865.

(14) Solletti, J. M.; Botreau, M.; Sommer, F.; Tran Minh Duc; Celio, M. R. *J. Vac. Sci. Technol., B* **1996**, *14(2)*, 1492.

(15) Zhong, Q.; Inniss, D.; Kjoller, K.; Elings, V. B. *Surf. Sci. Lett.* **1993**, *290*, 688.

(16) Whangbo, M.-H.; Magonov, S. N.; Bengel, H. *Probe Microscopy* **1997**, *1*, 23.

(17) 17. Burnham, N. A.; Behrend, O. P.; Oulevey, F.; Gremaud, G.; Gallo, P.-J.; Gourdon, D.; Dupas, E.; Kulik, A. J.; Pollock, H. M.; Briggs, G. A. D. *Nanotechnology* **1997**, *8*, 67.

(18) Garcia, R.; Tamayo, J.; Calleja, M.; Garcia, F. *Appl. Phys. A* **1998**, *66*, S309.

(19) Whangbo, M. H.; Bar, G.; Brandsch, R. *Appl. Phys. A: Solids Surf.* **1998**, *66*, S1267.

10.1021/la981253x CCC: $18.00
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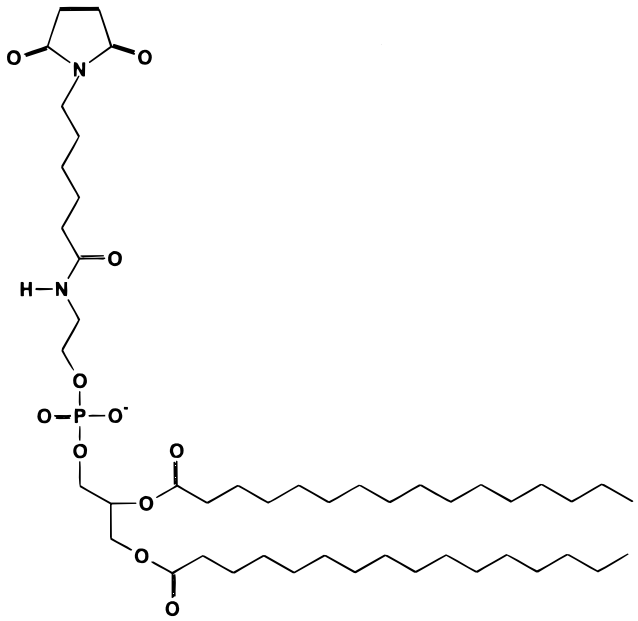

Figure 1. The structure of the linker molecule, DPPE-EMC.

different matrix phospholipids were used: 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylethanolamine (DPPE), 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylethanolamine (DMPE), and 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC). The miscibility of the binary monolayers was studied with surface pressure−area isotherms, surface phase rule, and excess area criterion. BAM enabled a visual investigation of the miscibility in the Langmuir monolayer, and AFM was applied to study the morphology of the corresponding LB films on solid substrates.

Experimental Details

Materials. DPPE, DMPE, and DPPC were obtained from Avanti Polar Lipids. Chloroform obtained from Merck was used as the spreading solvent. The synthesis of DPPE-EMC (Figure 1) was carried out with the method described by Heath[20] and Martin et al.[9] through a reaction of *N*-(ϵ-maleimidocaproyl)-succinimide (EMCS, Fluka >99%) with DPPE (>99%) using triethylamine (Baker >98%) as a homogeneous catalyst. DPPE-EMC was purified by chromatography on silica gel (Si 60) using a mixture of chloroform with methanol. The final product was analyzed with 600-MHz ^{1}H NMR.[21] *N*-(2-Hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES) (10 mM) (Sigma), 0.9% NaCl (Fluka) pH = 6.8 buffer was used as a subphase for monolayer studies. The distilled water was purified with a Millipore Milli-Q-filtering system, yielding a water resistance >18 MΩ·cm. NaOH used to adjust the pH was obtained from Merck and was *pro analysis* grade.

FT-IR Spectroscopy. The CH_2 asymmetric and symmetric stretching vibration bands of DPPE-EMC were studied by FT-IR spectroscopy. The temperature of the system was controlled by an external water thermostat (Haake F3C, West Germany). Spectra were recorded with a Bruker IFS 48 (Karlsruhe) FT-IR spectrometer.

Surface Pressure Measurements. The surface pressure−area (π-A) isotherms were measured with a computerized Langmuir trough manufactured by NIMA, Coventry. The experiments were carried out at 23 ± 0.5 °C using a thermostated Teflon barostat (125 × 600 mm^2). The surface pressure was monitored using a filterpaper as a Wilhelmy plate. The lipids were mixed in various ratios with the linker lipid and spread onto the subphase. The compression was started 15 min after spreading with use of a constant barrier speed of 2.5 Å^2 molecule^{-1} min^{-1}.

Surface Potential Measurements. The surface potential measurements for the pure lipids and linker lipid were performed with a KSV 5000 Langmuir trough (KSV Instruments, Helsinki). The experiments were carried out at 23 ± 0.5 °C using a thermostated Teflon trough (65 × 750 mm^2). The surface pressure was monitored using a Pt Wilhelmy plate. The surface potential, ΔV, of the monolayer was measured simultaneously with the surface pressure by the vibrating plate method. The upper, vibrating Pt electrode (ϕ, 43 mm) was positioned about 2 mm above the subphase surface, and it was perforated to minimize the noise.

LB Monolayers Deposited on the Au Substrate. Flat Au substrates were made by the technique described earlier by Wagner et al.[22] A gold layer is evaporated onto freshly cleaved mica sheets, and the Au surface is then glued onto a glass coverslip using Epo-tek 301−2 (Epoxy Technology, Billerica MA). The mica sheets were detached in tetrahydrofuran just before the Au substrates were used as LB supports.

The binary monolayer was prepared as described above (in surface pressure measurements) and it was compressed to 40 mN/m. A monolayer was deposited horizontally onto a Au substrate with the Langmuir−Schaefer (LS) technique, in which the substrate was pressed through the lipid film and placed in a glass holder in the subphase. The matrix-coated substrates were kept and handled in the glass holder filled with the subphase until further analysis.

BAM. For the morphological studies of the monolayer, a Brewster angle microscope 2 (BAM) manufactured by NFT, Göttingen, was mounted on a NIMA film balance. The BAM was equipped with a 30-mW laser diode, polarizer, analyzer, and a CCD camera. The lateral resolution of the BAM is about 2 μm, and the size of a BAM image is 280 × 210 μm. A vibration isolation system (MOD-2, JRS Scientific Instruments, Switzerland) was used, and the whole apparatus was installed in a cabinet to minimize dust and airflow.

AFM. A Nanoscope III multimode AFM (Digital Instruments, Inc., Santa Barbara) was used for the topographical studies of deposited films. The tapping mode in liquid was used to scan the surface of a lipid monolayer on Au substrate. The free oscillation amplitude (A_0) of the tip was 4−8 nm, and the damping ratio, $r_{sp} = A_{sp}/A_0$, was 0.5−0.9 (A_{sp} = setpoint amplitude). The scanner head J (125 × 125 μm^2 scan range) was used for imaging together with silicon cantilevers with a force constant of 2.9 N/m and a resonance frequency of about 70−90 kHz (in liquid).

Results and Discussion

FT-IR Spectroscopy of the Linker Lipid and the Choice of Mixed Lipids. The linker molecule (EMCS) used in this study is a heterobifunctional cross-linking molecule consisting of an *N*-hydroxysuccinimidyl ester group and a reactive maleimide group. The pH range of a stable linker largely depends on the maleimide group.[23] We have chosen EMCS because the aliphatic maleimide group is stable over a wide pH range (pH = 5−7.5).[23] The reaction of EMCS with DPPE gives a negatively charged linker lipid molecule because the phosphate moiety retains its negative charge. This cross-linker does not produce unstable disulfide bonds with the antibodies in serum, which is seen as a remarkable advantage in the development of biosensors.[24]

The linker lipid was analyzed with FT-IR to study the temperature dependence of the CH_2 asymmetric and symmetric stretching vibration bands. These bands shift in frequency when the lipid undergoes a phase transition, enabling the determination of the crystalline-isotropic phase-transition temperature (T_m).[25] The phase-transition

(20) Heath, T. D. *Methods Enzymol.* **1987**, *149*, 111.
(21) Viitala, T.; Albers, W. M.; Vikholm, I.; Peltonen, J. *Langmuir* **1998**, *14*, 1272.
(22) Wagner, P.; Hegner, M.; Güntherodt, H.-J.; Semenza, G. *Langmuir* **1995**, *11*, 3867.
(23) Kitagawa, T.; Shimozono, T.; Aikawa, T.; Yoshida, T.; Nishimura, H. *Chem. Pharm. Bull.* **1981**, *29*, 1130.
(24) Martin, F. J.; Papahadjopoulos, D. *J. Biol. Chem.* **1982**, *257*, 286.
(25) Blume, A. *Colloid Interface Sci.* **1996**, *1*, 64.

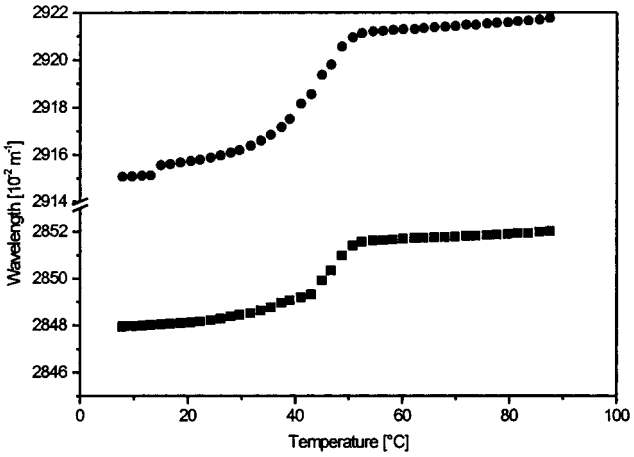

Figure 2. The CH_2 stretching vibration bands of DPPE-EMC.

temperature of DPPE-EMC was determined to be 45 °C (see Figure 2).

Matrix phospholipids were chosen with a T_m similar to that of DPPE-EMC to get a miscible lipid film. DMPE and DPPC have shown T_m values of 48 °C and 41 °C, respectively.[26] DPPE also was tested (although the T_m of DPPE was as high as 63 °C) because it forms the lipid base in the linker lipid. For example, DMPC was not used because of a relatively low T_m of 21 °C.

Pressure−Area and Surface Potential Isotherms of Pure Matrix Phospholipids and the Linker Lipid. There are numerous reports on the surface pressure−area (π-A) isotherms of phospholipids at the air/water interface.[27,28] In our study the isotherms were measured in a subphase of 10 mM HEPES, 0.9% NaCl pH = 6.8 buffer. The isotherms for pure lipids resembled earlier published isotherms and showed a pure liquid-condensed phase (LC) for DPPE (see Figure 3a) and a liquid-expanded to liquid-condensed phase transition (LE-LC) for DMPE (Figure 3b) and DPPC (Figure 3c). All three pure matrix phospholipid monolayers collapsed at surface pressures of 45−55 mN/m. As previously noticed different buffers have little effect on the π-A isotherms of zwitterionic phospholipids.[29,30] Between the pH 3 and 8, all the matrix phospholipids used are dissociated, which makes the polar group zwitterionic, whereas DPPE-EMC depends more on the pH and subphase content. The π-A isotherm of DPPE-EMC in Figure 3d shows that an expanded monolayer with a collapse pressure at approximately 40 mN/m was formed on the above-mentioned subphase.

The ΔV-A isotherms followed nicely the changes in the compression isotherm and were reproducible throughout the entire area range studied. The obtained maximum ΔV values of DPPE (590 mV) and DPPC (560 mV) fell within the range reported by most other workers.[31,32] As expected, the HEPES−NaCl solution did not affect the surface potentials of these zwitterionic lipids when compared with other buffered subphases or water, confirming that the double-layer contribution to ΔV is minimal.[32,33] The DMPE monolayer had a ΔV_{max} value of 580 mV. DPPE forms a condensed monolayer and undergoes a gas-to-liquid condensed phase transition (G-LC); thus the ΔV-A isotherm shows a sudden surface potential increase. The phase transitions of DMPE and DPPC are clearly seen in the ΔV-A curves: G-LE as a sudden initial surface potential increase and LE-LC as a broad gradual surface potential change.

The measured ΔV_{max} value for the DPPE-EMC monolayer at pH 6.8 was much lower (290 mV) than that of the pure matrix phospholipid ΔV_{max} values. Also, in the DPPE-EMC isotherm, the G-LE phase transition was observed as a sudden surface potential increase; however, this increase was smaller in magnitude than that observed for DMPE and DPPC monolayers. After the initial increase, the surface potential increased monotonically. The negatively charged phosphate group of the DPPE-EMC molecule and the complex formation, most probably with Na^+ in the buffer, yielded a negative contribution to the surface potential. The surface potential was measured also at pH 2.1, where the linker lipid should not be ionized. The measured ΔV_{max} of 455 mV indicates that at pH 6.8 the contribution of the double-layer potential is −165 mV. However, the negative charge also increases the solvatization of DPPE-EMC compared with the zwitterionic DPPE. The increased solvatization partly explains the decreased amphiphilicity of DPPE-EMC which appears as a more expanded monolayer than that of DPPE. Part of the decreased ΔV_{max} value is connected, therefore, to a decreased degree of order of the molecules within the monolayer. Hence, the differences between DPPE and DPPE-EMC in mean molecular area (orientational and packing effect), the size of the polar headgroup (amphiphilicity), and the charge of the polar headgroup (the dipole moment and double-layer potential) all contribute to the observed difference in the surface potential of 300 mV.

Figure 3 shows also the corresponding apparent total dipole moments calculated from the Helmholtz equation. However, the Helmholtz equation used is valid only for un-ionized monolayers at the air/water interface. We used neither the Vogel and Möbius[34] (VM) or the Demchak and Fort[35] (DF) model to estimate the local dipole moments of the studied lipids. For example, the local permittivities reported by Demchak and Fort or by Oliveira et al.[36] do not necessarily apply for the subphase that we used here and especially not for DPPE-EMC. The only clear conclusion is that the linker unit attached to the headgroup of DPPE decreases the total surface potential and increases the solvatization of the molecule.

Pressure−Area Isotherms of Binary Monolayers. π-A isotherms of binary mixtures of DPPE and DPPE-EMC at 23 °C are presented in Figure 4A. The binary mixtures did not show any clear phase transition. However, in the DPPE/DPPE-EMC (40:60) monolayer some irregularities were seen at a surface pressure of about 30 mN/m. The mixed monolayers collapsed at the same pressure as pure DPPE-EMC (45 mN/m), except the DPPE/DPPE-EMC (90:10) monolayer which showed some irregularity at a surface pressure of 45 mN/m, but collapsed at 53 mN/m.

As seen in Figure 4B, changing of the matrix phospholipid to DMPE led to completely different isotherms. The LE-LC transition point was visible in all binary

(26) Small, D. M. The Physical Chemistry of Lipids: From Alkanes to Phospholipids. In *Handbook of Lipid Research;* Hanahan D. J., Ed; Plenum Press: New York, 1986; Vol. 4.
(27) Tancrede, P., Parent, L., Paquin, P., Leblanc, R. M. *J. Colloid Interface Sci.* **1981**, *83*, 606.
(28) Gorwyn, D.; Barnes, G. T. *Langmuir* **1990**, *6*, 222.
(29) Robert, S.; Tancréde, P.; Salesse, C.; Leblanc, R. M. *Biochim. Biophys. Acta* **1983**, *730*, 217.
(30) Bos, M. A.; Nylander, T. *Langmuir* **1996**, *12*, 2791.
(31) Beitinger, H.; Vogel, V.; Möbius, D.; Rahmann, H. *Biochim. Biophys. Acta* **1989**, *984*, 293.
(32) Taylor, D. M.; Oliveira, O. N. Jr.; Morgan H. *J. Colloid Interface Sci.* **1990**, *139, 2*, 508.
(33) Cseh, R.; Benz, R. *Biophys. J.* **1998**, *74*, 1399.
(34) Vogel, V.; Möbius, D. *J. Colloid Interface Sci.* **1988**, *126*, 408.
(35) Demchak, R. J.; Fort, T. J. Jr. *J. Colloid Interface Sci.* **1974**, *46*, 191.
(36) Oliveira, O. N. Jr.; Taylor, D. M.; Lewis, T. J.; Salvagno, S.; Stirling, C. J. M. *J. Chem Soc. Faraday Trans. 1* **1989**, *85*, 1009.

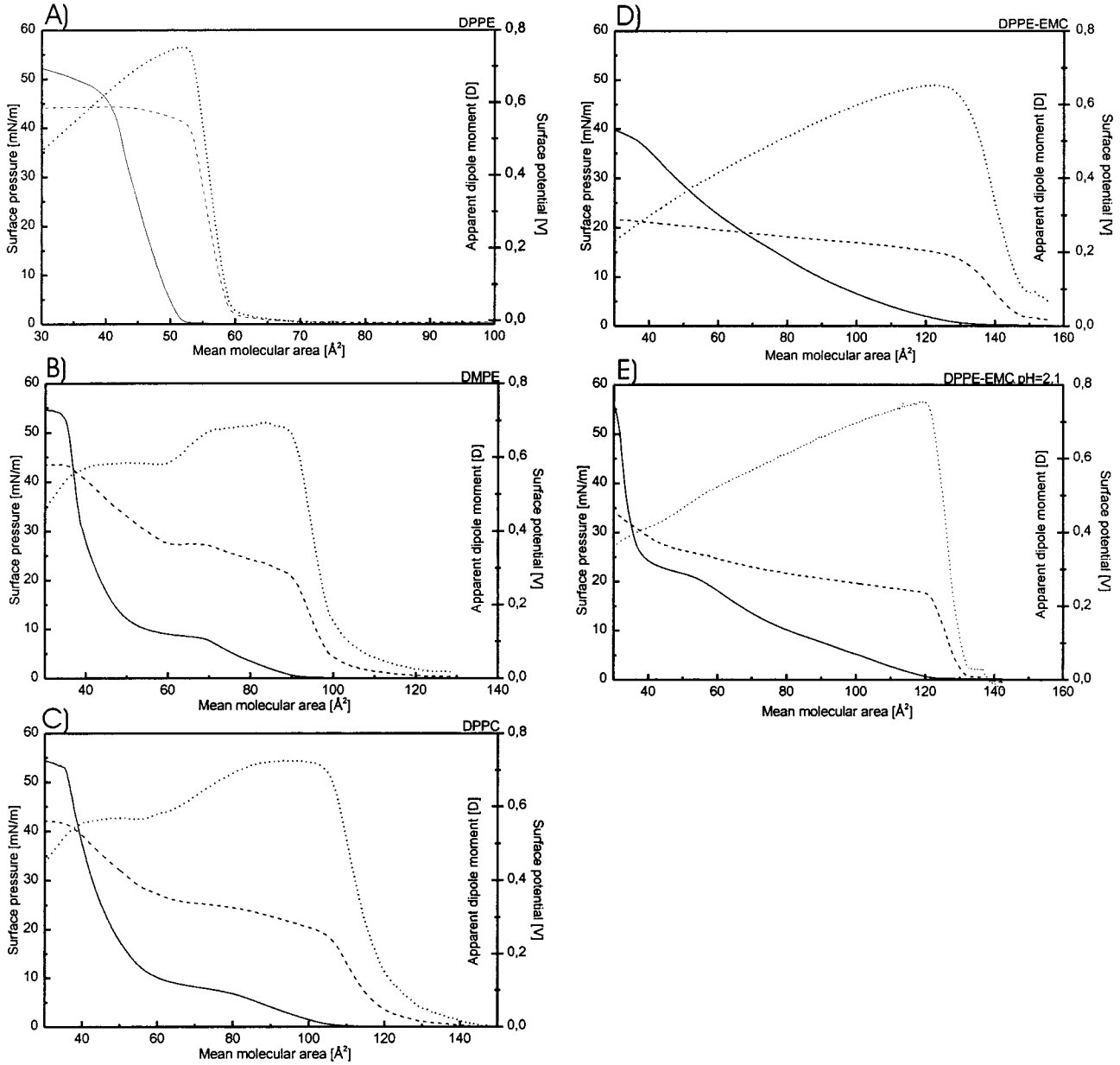

Figure 3. π-A (—) and surface potential (- - -) isotherms of pure (A) DPPE, (B) DMPE, (C) DPPC, (D) DPPE-EMC monolayers at a 10 mM HEPES, 0.9% NaCl pH = 6.8 subphase at 23 °C, and (E) DPPE-EMC at pH = 2.1. Also shown is the apparent dipole moment (···) calculated from ΔV using the Helmholtz equation.

monolayers, and it moved to a slightly larger area and higher pressure when increasing the DPPE-EMC amount. The collapse pressure (π_c) of DMPE/DPPE-EMC isotherms behaved similarly as in DPPE/DPPE-EMC mixtures, i.e., the monolayer collapsed mainly at the π_c of DPPE-EMC.

The DPPC/DPPE-EMC isotherms are presented in Figure 4C. In addition to pure DPPC, a clear LE-LC transition was visible only in the DPPC/DPPE-EMC (90:10) monolayer. Also π_c behaved differently from other binary mixtures. The monolayers of different amounts of DPPC did not collapse at the same surface pressure. The mixtures of DPPC/DPPE-EMC (90:10 and 60:40) have a π_c intermediate to that of the pure components. For the monolayer DPPC/DPPE-EMC (40:60) the π_c was difficult to determine.

Miscibility in Monolayers. The miscibility was tested by the surface phase rule.[37,38] The components are immiscible, if the equilibrium spreading pressure or the collapse pressure is independent of the monolayer composition. The π_c was clearly observed in DPPE/DPPE-EMC and DMPE/DPPE-EMC mixed monolayers, whereas it varied irregularly in the DPPC/DPPE-EMC monolayer. In the DPPE/DPPE-EMC and DMPE/DPPE-EMC monolayers the values of π_c were constant within experimental error with one exception. In a mixing ratio of 90:10 (matrix phospholipid:linker lipid), the monolayer collapsed at a pressure corresponding to an intermediate π_c value of pure monolayer components. However, a weak transition associated with a decrease in mean molecular area at 45 mN/m indicated that the linker component was partly compressed out of the monolayer, and the collapse pressure approached that of a pure matrix lipid film. On the contrary, the DPPC/DPPE-EMC monolayer seemed to be miscible as long as the linker lipid was in minority.

(37) Crisp, D. J. *Surface Chemistry (Supplement to Research)* 23; Butterworth: London, 1949; p 17.

(38) Gaines, G. L. *Insoluble Monolayers at Liquid−Gas Interaces*; Wiley-Interscience: New York, 1966.

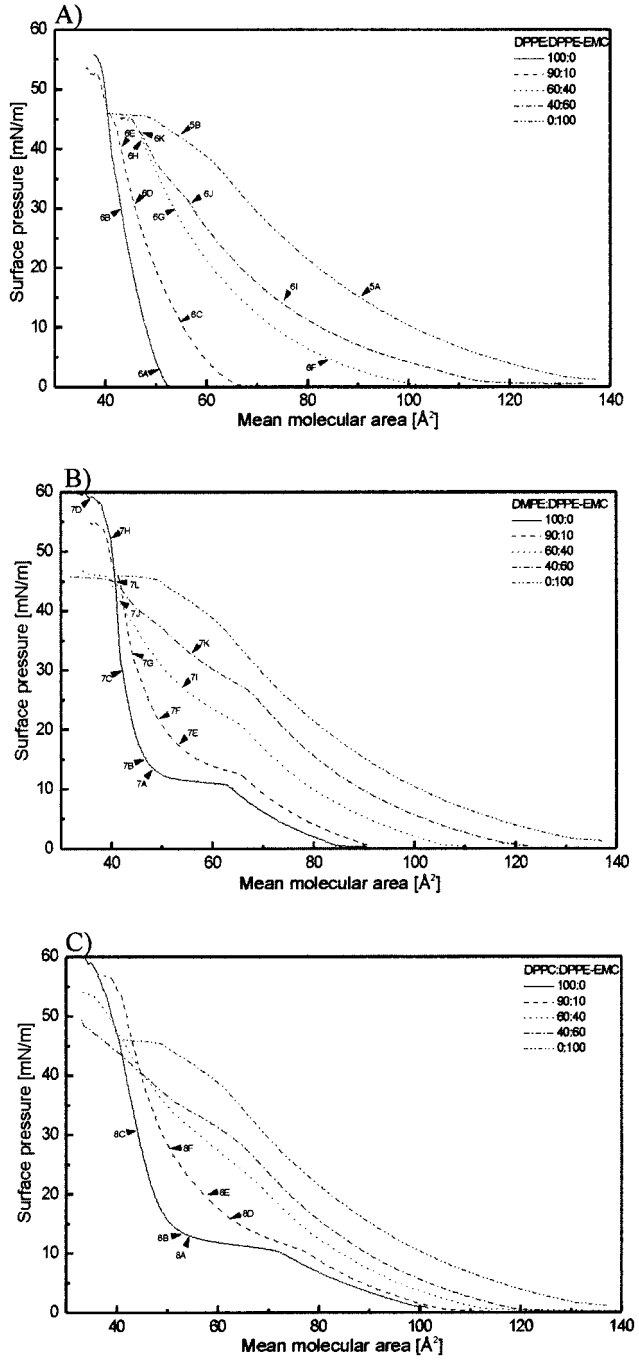

Figure 4. π-A isotherms of binary mixtures of (A) DPPE and DPPE-EMC, (B) DMPE and DPPE-EMC, and (C) DPPC and DPPE-EMC at a 10 mM HEPES, 0.9% NaCl pH = 6.8 subphase at 23 °C.

The excess area criterion[38,39] to the binary monolayers was tested but did not fully clarify the miscibility of the components in the monolayers. No large deviations from the additivity rule were observed; nor was this criterion exclusive, because a complete miscibility may also occasionally lead to matching of the additivity rule.[38] Therefore, some additional microscopic studies were carried out to better characterize the system.

BAM. Phase behavior of Langmuir monolayers and binary mixtures can be studied by BAM.[40] The compression rate has a remarkable effect on the shape and size of the lipid domains formed during a phase transition.[41] Higher compression rates lead to an increase in the irregularity of the domain shape. Furthermore, the shape of the growing domains is also affected by the molecular area, ratio of the phases, whereas the growth rates also depend on the supersaturation of the LE phase.[42] In our study the monolayer was compressed at an intermediate rate of 2.5 Å^2 molecule^{-1} min^{-1}, therefore some branching of the domains was observed.

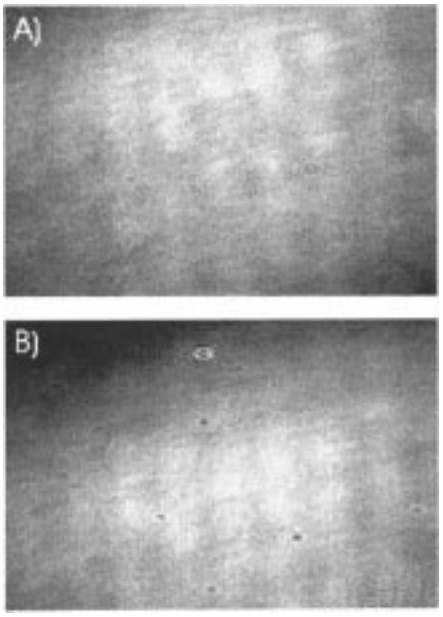

Figure 5. Brewster angle microscopy of a pure DPPE-EMC monolayer on 10 mM HEPES, 0.9% NaCl pH = 6.8 subphase at 23 °C. The images are taken at the points indicated in Figure 4A.

The pure LE DPPE-EMC monolayer did not show any domain formation, as expected for a one-phase system. As shown in Figure 5 the monolayer was homogeneous at both low and high surface pressures. However, some heterogeneities were observed before the collapse.

BAM images of pure DPPE and DPPE/DPPE-EMC binary monolayers are presented in Figure 6. In the pure DPPE monolayer the LC phase started to be visible at low surface pressures (see Figure 6A and B). The LC phase did not show any particular domain size or shape, and at 30 mN/m the monolayer became homogeneous. Even at higher surface pressures, when the monolayer turned into a solid state, there were no changes in the BAM images. DPPE-EMC (10 mol %) changed the morphology of the monolayer, and it showed a heterogeneous structure for the whole surface pressure range (see Figure 6C through E). However, the condensed phase appeared continuous throughout the compression. At 40 mol % DPPE-EMC, domain formation was visible (see Figure 6F through H). It started at low surface pressures, and during compression the domains packed closer but the monolayer did not reach a homogeneous structure. At a greater amount of DPPE-EMC (60 mol %) some heterogeneous structures started to build up at 6 mN/m, but clear domains were visible first at 14 mN/m (see Figure 6I and J). The domain size in the 40 mol % and 60 mol % DPPE-EMC monolayers was found to be approximately 8−11 μm in diameter in both monolayer compositions at low pressures. However,

(39) Birdi, K. S. *Lipid and Biopolymer Monolayers at Liquid Interfaces*; Plenum Press: New York, 1989.
(40) Hönig, D. Molekular organisierte Filmen: laterale Struktur und optische Eigenschaften. Ph.D. Thesis, Göttingen, 1994.
(41) Weidemann, G.; Vollhardt, D. *Colloids Surf. A* **1995**, *100*, 187.
(42) Siegel, S.; Vollhardt, D. *Thin Solid Films* **1996**, *284−285*, 424.

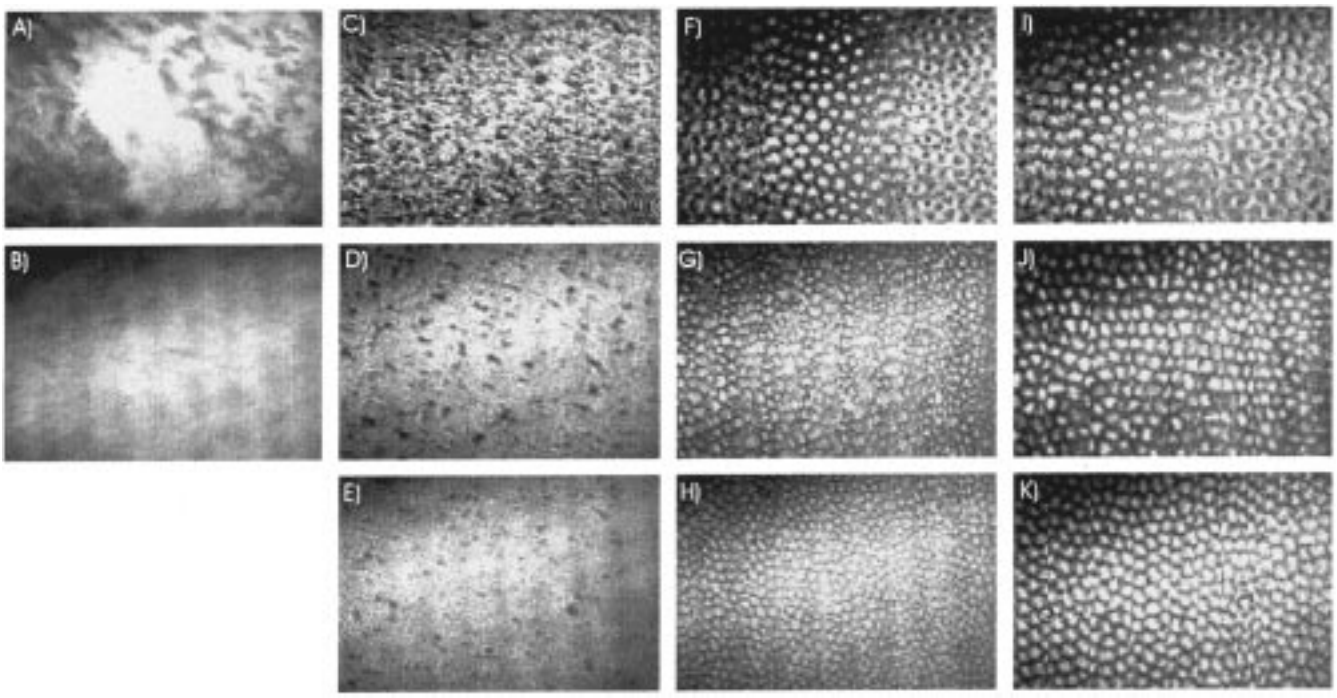

Figure 6. Brewster angle microscopy of DPPE/DPPE-EMC monolayers on 10 mM HEPES, 0.9% NaCl pH = 6.8 subphase at 23 °C. The images are taken at the points indicated in Figure 4A.

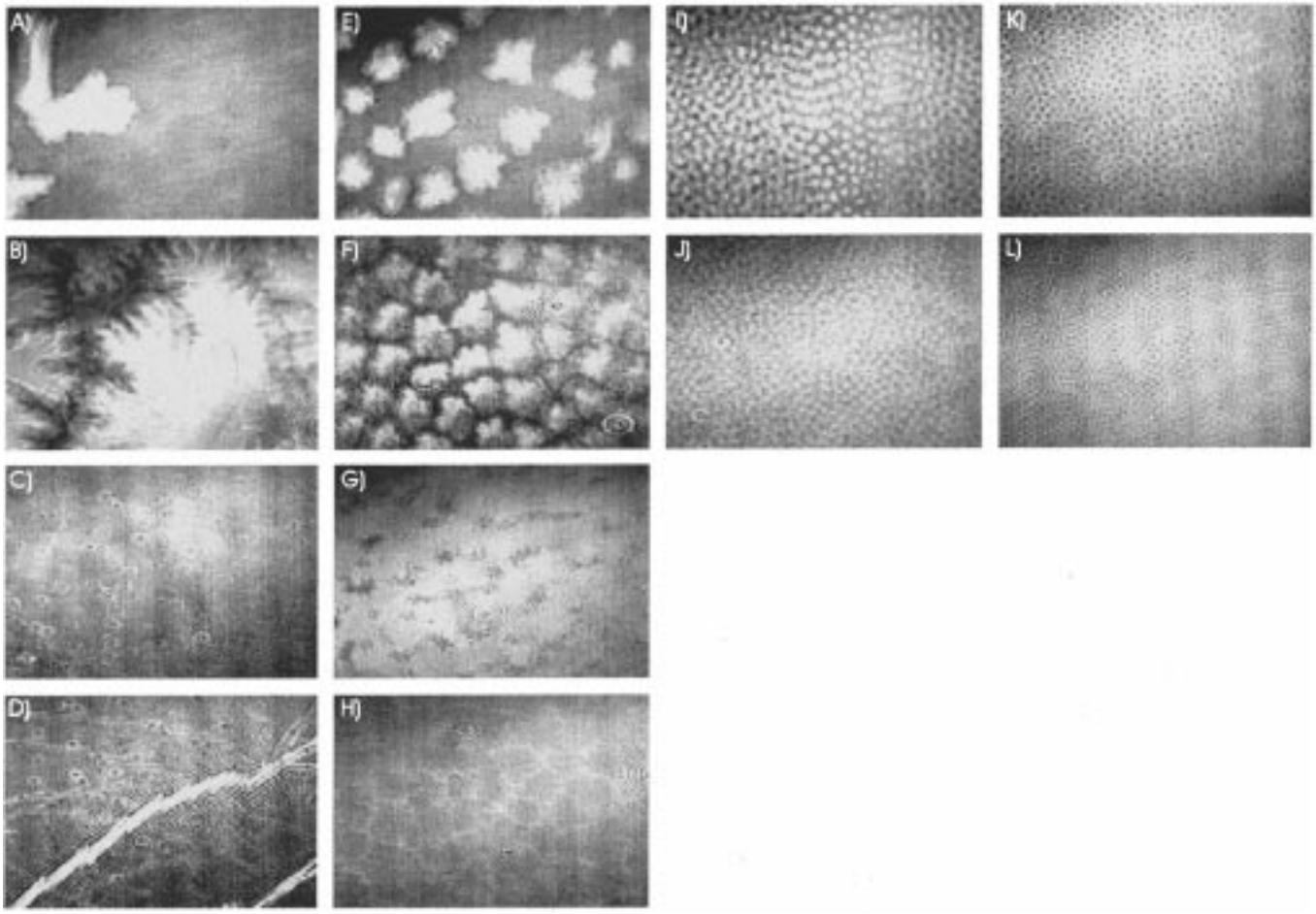

Figure 7. Brewster angle microscopy of DMPE/DPPE-EMC monolayers on 10 mM HEPES, 0.9% NaCl pH = 6.8 subphase at 23 °C. The images are taken at the points indicated in Figure 4B.

in the first monolayer the domain size decreased strongly during compression, whereas in the second monolayer the domains preserved the same size even at higher pressures.

The pure DMPE monolayer showed a clear phase transition at 10 mN/m, and in BAM images large LC domains also started to be visible at approximately 13

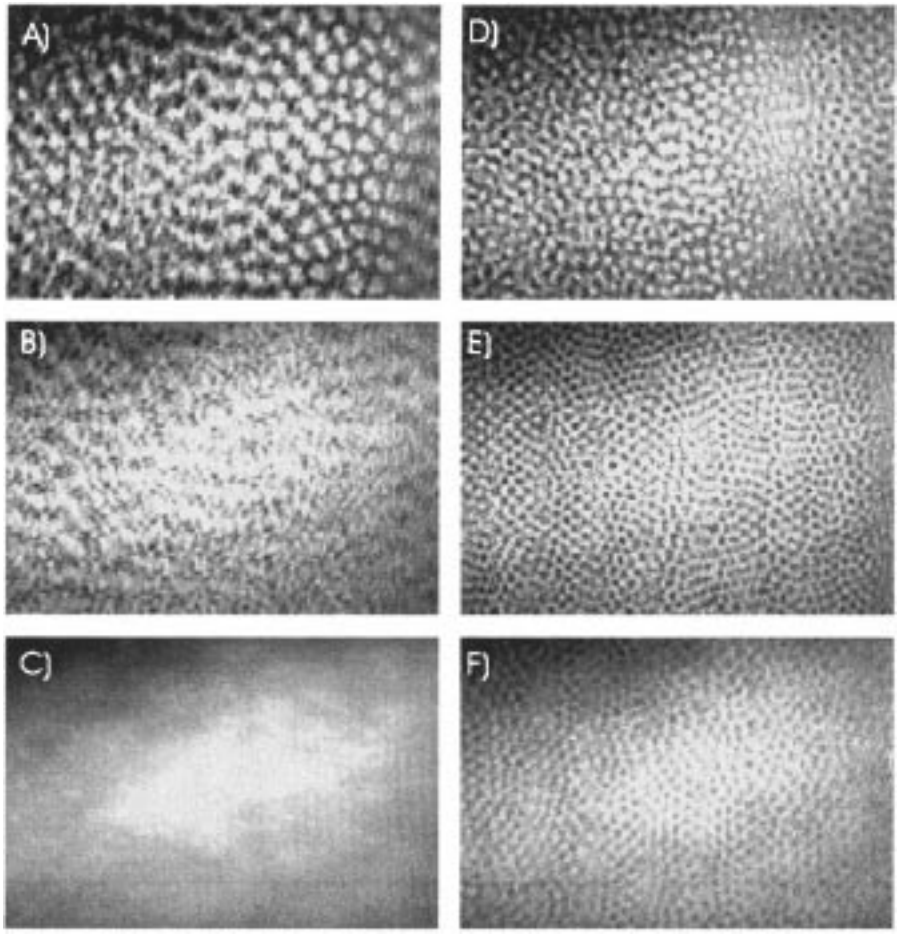

Figure 8. Brewster angle microscopy of DPPC/DPPE-EMC monolayers on 10 mM HEPES, 0.9% NaCl pH = 6.8 subphase at 23 °C. The images are taken at the points indicated in Figure 4C.

mN/m (see Figure 7A through D). The domain size and structure resembled those observed by other workers.[41,43] At higher surface pressure the monolayer became homogeneous and at the end of the compression the collapse of the monolayer is seen as a breakdown (multilayer formation). A small addition of DPPE-EMC (10 mol %) made the size of the domains smaller, 28−34 μm in diameter at 22 mN/m (see Figure 7E through H). The domains were also less branched than the LC domains in a pure DMPE monolayer. The first LC domains started to exist at 17 mN/m; however, the monolayer became homogeneous first at 46 mN/m. The monolayer collapse was started at the domain boundaries. The size of the LC domains decreased with the amount of DPPE-EMC in the binary monolayers (see Figure 7I and J) and at 60 mol % DPPE-EMC the domains could be seen as small spots (see Figure 7K and L). The monolayers with greater DPPE-EMC amounts (40 mol % and 60 mol %) did not reach a homogeneous structure during the whole compression.

The clear phase transition of pure DPPC at approximately 13 mN/m was observed in the BAM images (see Figure 8A through C). In addition, here the 10 mol % addition of DPPE-EMC reduced the domain size in the binary monolayer (see Figure 8D through F). However, further addition of DPPE-EMC inhibited the domain formation, and the monolayer stayed homogeneous during the whole compression.

We looked more closely at the BAM images of the binary monolayers at the normal LB transfer pressure of 30−40 mN/m. The only binary monolayer mixture that has a homogeneous structure at this pressure and in all mixture compositions was DPPC/DPPE-EMC. DPPE/DPPE-EMC monolayer was heterogeneous in 90:10 composition and had domains at 60:40 and 40:60 compositions. Also in DMPE/DPPE-EMC monolayer, domains were visible at those compositions.

Line Tension in Binary Monolayers. Lipid molecules exhibit permanent dipole moments that are partially aligned to the surface. Linker lipids as well as lipids with dissociable headgroups possess a net surface charge. Therefore the LC phase formed during compression exhibits an excess dipole moment and possibly an excess charge relative to the LE phase. The two-phase lipid monolayer exhibits domains with a myriad of shapes. The lipid domain shapes have been studied by various groups.[44,45] Keller et al.[44] observed that the domain shape results from a competition between interfacial line tension (which favors round domains) and long-range repulsive electrostatic forces (which favor elongation of the domains). The balance between these factors can lead to undulations and branching of domains. Domains can also be described by the elasticity theory of the liquid crystals.[46] In this case the inner order of the domains is studied instead of the domain perimeter.

Hönig[40] studied binary monolayers of molecules with dipole moments of opposite sign and observed that the domain shape elongates with increasing effective dipole

(43) Hönig, D.; Möbius, D. *J. Phys. Chem.* **1991**, *95*, 4590.

(44) Keller, D. J.; Korb, J. P.; McConnell, H. M. *J. Phys. Chem.* **1987**, *91*, 6417.

(45) Vanderlick, T. K.; Möhwald, H. *J. Phys. Chem.* **1990**, *94*, 886.

(46) de Gennes, P. G.; Ed. *The Physics of Liquid Crystals*; Clarendon Press: Oxford, 1974.

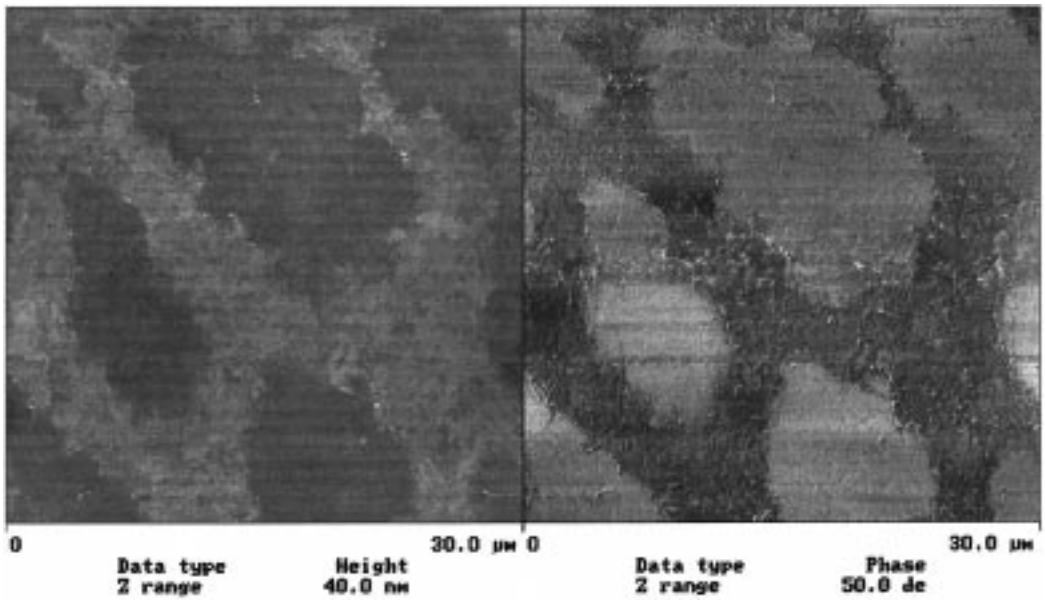

Figure 9. AFM height image (left) and phase image (right) of DPPE/DPPE-EMC (40:60) measured with tapping mode in subphase ($r_{sp} = 0.9$).

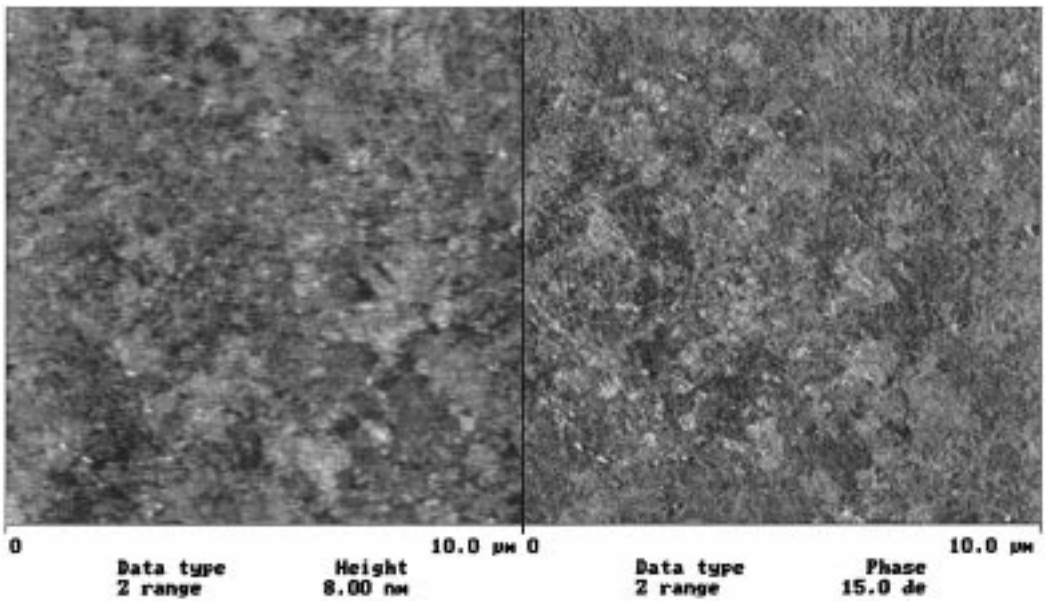

Figure 10. AFM height image (left) and phase image (right) of DMPE/DPPE-EMC (40:60) measured with tapping mode in subphase ($r_{sp} = 0.6$).

moment. In our study the dipole moment difference between linker lipid and matrix phospholipid was not that large, but the effective dipole moment decreased with an increasing amount of linker lipid in the binary monolayer. This effect could clearly be seen in the DMPE/DPPE-EMC monolayer where the large and branched domains changed their structure and became smaller and less branched by the addition of DPPE-EMC to the monolayer. Also the amount of the domains in the monolayer increased.

Monolayer Topography on Solid Substrate. AFM was used to study the correspondence between the domains in immiscible films at the air/subphase interface and those visible in deposited lipid matrixes on solid substrates. DPPE/DPPE-EMC monolayers showed clear immiscibility in BAM images at high DPPE-EMC content; therefore, a DPPE/DPPE-EMC monolayer in 40:60 composition was transferred onto a gold substrate for AFM studies. In Figure 9 darker appearing (i.e., lower lying) domains of 100−200 μm^2 are seen clearly. The domain size in this image typical of this sample is in the same range as that detected from the BAM images (see Figure 6K). This is clear evidence that domains not only exist in monolayers of the chosen composition, but they also retain their structure after transfer onto a solid substrate.

The domains are most probably DPPE in the LC state, and the higher areas around the domains represent DPPE-EMC where the linker is protruding from the lipid matrix. In the phase image the assumed DPPE domains appear brighter, i.e., the phase shift is larger. For phase imaging in air, such a contrast would refer to local differences in sample stiffness or adhesion. Imaging in liquid excludes the tip−sample capillary effects often disturbing imaging in air. However, the tip−sample mechanical interaction in liquid becomes more complicated because of viscoelastic effects. At the moment, therefore, we may only speculate that the bright domains in the phase image refer to areas of higher density compared with the surrounding phase.

For comparison, matrixes of 40:60 DMPE/DPPE-EMC and DPPC/DPPE-EMC were imaged. As expected the DPPC/DPPE-EMC film was homogeneous (data not shown), whereas the DMPE/DPPE-EMC film was heterogeneous with no clear domains visible (Figure 10). In the DMPE/DPPE-EMC matrix the contrast in height or phase image was not as strong as in DPPE/DPPE-EMC matrix.

Conclusions

The phase-transition temperature of the linker lipid was 45 °C, and the surface potential was 290 mV at pH 6.8 (clearly smaller than the values observed for pure phospholipids). The miscibility in binary monolayers of matrix phospholipids and the linker lipid was studied by the surface pressure rule and the excess area criterion. The surface pressure rule indicated miscibility in 90:10 composition of DPPE/DPPE-EMC and DMPE/DPPE-EMC and in all DPPC/DPPE-EMC matrixes; however, the excess area criterion did not unambiguously prove miscibility. By interpretation of the BAM images it was clear that the addition of linker lipid decreased the effective dipole moment and therefore also domain shape became less elongated. The addition of DPPE-EMC made the DPPC/DPPE-EMC matrixes more homogeneous and decreased the domain size in DMPE/DPPE-EMC matrixes; however, in DPPE/DPPE-EMC matrixes the effect was the opposite. The miscibility in the binary matrixes increased in the following order: DPPE/DPPE-EMC < DMPE/DPPE-EMC < DPPC/DPPE-EMC. The domains formed in immiscible matrixes were also visible in transferred films when studied with tapping mode AFM in liquid. The next step will be to use the studied matrixes as sensing surfaces and analyze the immunological activity and specificity, e.g., by surface plasmon resonance.

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