Apparatus for the Continuous Monitoring of Surface Morphology via Fluorescence Microscopy during Monolayer Transfer to Substrates

Ka Yee C. Lee, Michael M. Lipp, Dawn Y. Takamoto, Evgeny Ter-Ovanesyan, and Joseph A. Zasadzinski*

Department of Chemical Engineering, University of California, Santa Barbara, California, 93106-5080

Alan J. Waring

Martin Luther King Jr.−Drew University Medical Center and Perinatal Laboratories, Harbor−UCLA, Los Angeles, California 90059

Received April 24, 1997. In Final Form: February 23, 1998

Langmuir monolayers can be characterized at the air−subphase interface with fluorescence microscopy at the micron scale, but must be transferred to solid substrates for higher resolution imaging using atomic force microscopy. To utilize both techniques to characterize the same region of interest or to ensure that the deposition process is artifact-free, we have developed a transfer technique that allows for the continuous optical imaging of surface morphology as the monolayer is transferred from the air−subphase interface to the substrate. The advantages of this deposition technique are that it is simple and inexpensive and can be implemented in almost all Langmuir troughs and that artifacts associated with nonideal transfer can readily be identified. This new transfer technique is ideal for transferring rigid and viscous films that are difficult to deposit using the traditional Langmuir−Blodgett technique. This technique can also be used for transferring monolayers to germanium attenuated total reflection (ATR) crystals for Fourier transform infrared (FTIR)−ATR spectroscopic measurements of protein conformation and lipid orientation while maintaining the desired monolayer morphologies.

Introduction

Surfactant and protein monolayers at the air−subphase interface are important model systems and also have a variety of practical applications. Life scientists study phospholipid and protein monolayers as simplified models of biological membranes, while physicists and chemists are interested in monolayers as models for self-assembly in two-dimensions.[1−3] For over a decade, fluorescence microscopy has been one of the primary tools used to study the morphology and phase behavior of Langmuir monolayers.[4,5] The ability to directly visualize monolayer morphology allows for the direct observation of coexisting phases and domain structures that were previously inferred from pressure−area isotherms, surface potentials, and other techniques.[6] The resolution of fluorescence microscopy is similar to that of any type of optical microscopy and is limited to about 1 μm. Higher resolution images of monolayers and multilayers are possible with atomic force microscopy (AFM), but this requires that the film be transferred to a solid substrate.[7−9] Only after the monolayer is transferred to a substrate by Langmuir−Blodgett or similar deposition techniques[10] can high-resolution characterization of the film on the molecular level be carried out with AFM.

The conventional method of transferring a monolayer onto a solid substrate is via the Langmuir−Blodgett (LB) technique,[11] in which the substrate, aligned perpendicular to the interface, is alternately pulled out from, or pushed into, the monolayer-covered subphase. Ideally, each pass transfers a single monolayer from the air−water interface to the substrate with little or no change in the local packing or phase state of the monolayer. The transfer process, however, does not work above a certain critical velocity or for viscous and rigid films. An alternative deposition process, known as the Langmuir−Schaefer (LS) technique,[12] is a horizontal deposition process in which the substrate is aligned parallel with the monolayer and the monolayer is transferred when the substrate is pushed through the monolayer into the subphase. This method can be used to transfer viscous films as well as mixed monolayers of lipids and proteins. Fundamental limitations of the Langmuir−Schaefer method are that a hydrophobic substrate is required and the transferred monolayer is only stable when immersed in a fluid subphase.[12] An important limitation of both LB and LS techniques is that the transfer process cannot be followed directly; in many circumstances, the local molecular organization, the distribution or size of domains, the fraction of various phases, and so forth can change during the transfer and the relationship between the structure of the film on the substrate and at the air−water interface is lost.[7−9]

(1) Knobler, C. M. *Adv. Chem. Phys.* **1990**, *77*, 397.
(2) Möhwald, H. *Annu. Rev. Phys. Chem.* **1990**, *41*, 441.
(3) McConnell, H. M. *Annu. Rev. Phys. Chem.* **1991**, *42*, 171.
(4) von Tscharner, V.; McConnell, H. *Biophys. J.* **1981**, *36*, 409.
(5) Lösche, M.; Sackmann, E.; Möhwald, H. *Ber. Bunsen-Ges. Phys. Chem.* **1983**, *87*, 848.
(6) Gaines, G. L. *Insoluble Monolayers at Liquid Gas Interfaces*; Wiley: New York, 1966.
(7) Zasadzinski, J. A.; Viswanathan, R.; Madsen, L.; Garnaes, J.; Schwartz, D. K. *Science* **1994**, *263*, 1726.
(8) Schwartz, D. K.; Garnaes, J.; Viswanathan, R.; Zasadzinski, J. A. *Science* **1992**, *257*, 508.
(9) Viswanathan, R.; Zasadzinski, J. A.; Schwartz, D. K. *Nature* **1994**, *368*, 440.
(10) Special Issue: Organic Thin Films, *Adv. Mater.* **1991**, *3*.
(11) Blodgett, K.; Langmuir, I. *Phys. Rev.* **1937**, *51*, 964.
(12) Langmuir, I.; Schaefer, V. J. *J. Am. Chem. Soc.* **1938**, *57*, 1007.

S0743-7463(97)00421-6 CCC: $15.00
Published on Web 04/11/1998

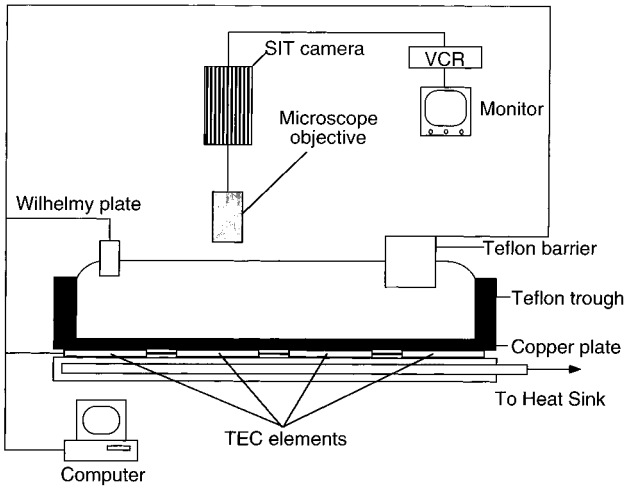

Figure 1. A schematic of the Langmuir trough with fluorescence microscopy attachment. The home-built Teflon Langmuir trough is attached to a copper plate, which in turn is in contact with nine TEC elements and a reservoir for temperature control. A fluorescence microscopy attachment equipped with a SIT camera is used for observing the surface morphology of the monolayer, and the images are recorded with a VCR. The measurement of the surface pressure via a Wilhelmy plate, the motion of the barrier, and the temperature of the trough are all controlled by a computer interface.

To address this problem, it is necessary to develop a transfer process compatible with commonly used optical microscopy techniques, especially fluorescence microscopy. We have built a simple device to do an inverse Langmuir–Schaefer transfer technique, which allows us to monitor, via fluorescence microscopy, the morphology of the monolayer at the air–subphase interface during the transfer process to the substrate of choice. Combined with a similar fluorescence microscope interfaced to the AFM, it also makes possible the identification of regions of interest for AFM scanning and a direct correlation between structure at the air–subphase interface and on the substrate. We can also quickly identify any problems encountered during the transfer process such as changes in domain shape or size, or changes in local phase state. The new technique is also capable of transferring viscous monolayers that cannot be deposited by conventional LB techniques. The transfer unit can be modified for deposition onto different substrates for other characterization techniques including electron diffraction or spectroscopy of the monolayers; we present FTIR–ATR spectroscopic measurements on monolayers transferred to germanium ATR plates.

Experimental Section

Langmuir Trough with Fluorescence Microscopy Attachment. A home-built Langmuir trough with an attached fluorescence microscope is used for the manipulation and visualization of the Langmuir monolayers as well as the transfer process; a schematic of the setup is shown in Figure 1.[13] The system consists of a Langmuir trough milled from a solid piece of Teflon with a working surface area of 112 cm^2 and a subphase volume of 150 mL. A Teflon barrier, driven by a UT100 motorized translation stage (Newport-Klinger, Irvine, CA), runs linearly along the top edge of the trough and controls the surface area available to the monolayer. The barrier is spring-loaded against the trough, and the ends of the barrier in contact with the well edges are beveled at an angle of approximately 10° to prevent leakage of the surfactant. The subphase temperature is controlled through the use of nine thermoelectric cooling (TEC) elements (Marlow Industries, Dallas, TX) connected in series and located between a constant temperature reservoir and a copper plate on which the trough is mounted. The surface pressure is measured by a Wilhelmy plate-type transducer (R&K, Wiesbaden, Germany) and can be controlled via a feedback loop for constant pressure measurements.

(13) Lipp, M. M.; Lee, K. Y. C.; Zasadzinski, J. A.; Waring, A. J. *Rev. Sci. Instr.* **1997**, *68*, 2574.

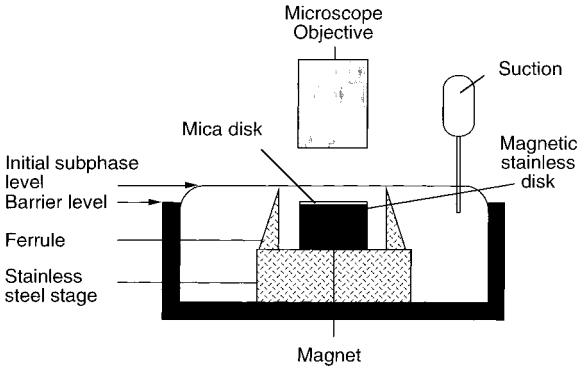

Figure 2. A schematic of the inverse Langmuir–Schaefer deposition system. The desired substrate is attached to a magnetic stainless steel disk. The disk is held by a magnet at the center of a stainless steel base fitted with a sharp-edged stainless steel ferrule. The whole transfer unit is placed directly below the microscope objective. When the subphase level is lowered, the sharp edge of the ferrule cuts the film, thus preserving its surface area and pressure. The deposition process of the cut film can then be observed directly.

Fluorescence microscopy is used to visualize the surface morphology of the spread film by incorporating a small percentage (<1 mol %) of fluorescent probe into the spreading solution. As the probe molecules preferentially partition into the less-ordered phase, contrast is generated, with the less-ordered phase bright and the more highly ordered phase dark. A Nikon Optiphot microscope with a 40× power long-working distance objective is used for visualization (Nikon Inc. Instrument Group, Garden City, NY). To eliminate condensation on the objective and to reduce convective air currents, a resistively heated glass cover (Delta Technologies, Stillwater, MN) is placed between the objective and the air–subphase interface. A 100-W high-pressure mercury lamp is used for excitation, and a dichroic mirror/barrier assembly is used to direct the excitation light onto the monolayer and to filter the emitted fluorescence. The fluorescence is detected via a silicon intensified target (SIT) camera from Dage MTI (VE-1000 SIT system, Michigan City, IN). Images are recorded by a JVC super VHS VCR (Elmwood Park, NJ) and digitized via a Scion frame grabber (Frederick, MD).

Transfer Unit and Procedures. The inverse LS transfer module is shown in Figure 2. A magnetic, stainless steel base measuring $^3/_4$ in. × $^3/_4$ in. is machined to fit a $^3/_4$ in. sharp-edged stainless steel ferrule (Swagelok, Solon, OH) directly on top. The four sides of the base are partially shaved off, leaving it in a cross shape to allow water to drain under the ferrule. At the center of the base is a housing for a cylindrical magnet used to hold the substrate assembly. The substrate assembly is made from a magnetic, stainless steel disk, $^1/_2$ in. in diameter, with the desired substrate surface (mica, glass, etc.) cut to the same size glued on top. The substrate assembly can be placed directly onto the piezoelectric scanner of a NanoScope III (Digital Instruments, Santa Barbara, CA) for AFM imaging.

The transfer unit is submerged in the subphase before the monolayer is spread. For optimal performance, the edge of the ferrule has to lie just below the subphase surface (<0.5 mm), and the top of the substrate has to be slightly below the ferrule's edge. To deposit the film onto the solid substrate, the monolayer is compressed to the desired surface pressure. The barrier is then stopped, and the subphase is aspirated out of the trough using a tapered pipet to slowly lower the subphase level, eventually reaching the ferrule's edge. The ferrule then cuts through the monolayer, locking in a fixed surface area, and hence a fixed surface pressure. It is important to have the ferrule lying just below the initial subphase level to ensure that the surface pressure of the film to be deposited is preserved; otherwise, the

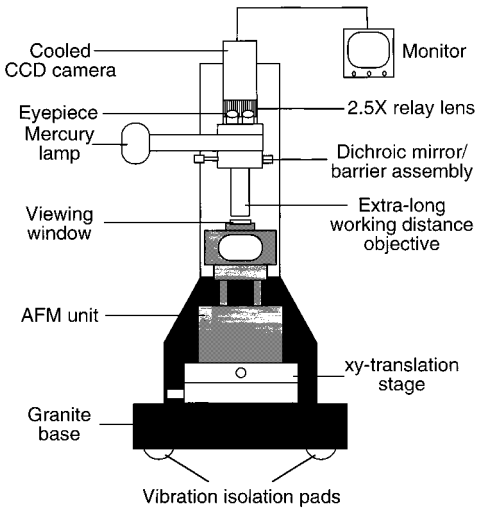

Figure 3. A schematic of the AFM with a fluorescence microscopy attachment. The NanoScope III force microscope is placed on a *xy*-translation stage that sits on the granite base of the fluorescence microscopy attachment. Due to the limitations posed by the dimensions of the force microscope, an extra-long working distance objective is needed for the fluorescence setup. The images are captured using a cooled CCD camera.

monolayer might leak under the barrier during subphase drainage, which could alter the surface density of the monolayer. Further suction (hence lowering of the subphase level) results in the deposition of the film onto the substrate. The entire transfer process is monitored using fluorescence microscopy to ensure that the monolayer morphology and phase state are not changed during the deposition. As the monolayer is deposited horizontally, viscous films can be deposited successfully that could not be transferred by the LB method.

AFM with Fluorescence Microscopy Attachment. One difficult aspect of AFM imaging is the lack of an easily accessible "low-magnification" mode for surveying the sample and identifying areas of interest. In principle, this can be done by exchanging the scanners—progressing from a large-scale scanner for survey work to a small-scale scanner for molecular resolution. In practice, however, it is virtually impossible to mechanically exchange the scanners while maintaining a position over a particularly interesting area. Hence, correlating the macroscopic features of a sample with the microscopic features visible with AFM is quite difficult. As a result, many modern atomic force microscopes incorporate a viewing window that allows for an optical microscope to focus on the cantilever tip and the sample and helps locate interesting areas on which to scan.

To correlate domains observed at the air−subphase interface, we have attached an optical microscope to our AFM system (NanoScope III) and have adapted it to work with fluorescence detection to help identify regions of interest on the transferred monolayers with sufficient resolution. Figure 3 shows a schematic. The fluorescence microscope is mounted onto a granite base supported by vibration isolation pads (A. G. Heinze, Irvine, CA). The microscope is similar to the one used with the Langmuir trough, with a high-pressure mercury lamp for illumination and a dichroic mirror/barrier assembly for directing the excitation light and filtering the emitted fluorescence. However, a 10× extra-long-working distance (48 mm) objective is used (Nikon Inc. Instrument Group, Garden City, NY); this extra-long-working distance is necessary for operation with NanoScope III due to the limitations posed by the apparatus' dimensions. For higher magnification, a 2.5× relay lens can be placed between the objective and the cooled CCD camera (Optronics Engineering, Goleta, CA). The AFM unit sits on a *xy*-translational stage which allows easy lateral positioning; the stage is in turn placed on the granite base with the viewing window of the AFM directly below the microscope objective. The fluorescence microscope makes it possible to view the surface morphology of the transferred film and engage the cantilever tip on areas of particular interest. Figure 4 shows the AFM tip approaching a fluorescent domain. With these combined instruments we can follow an individual

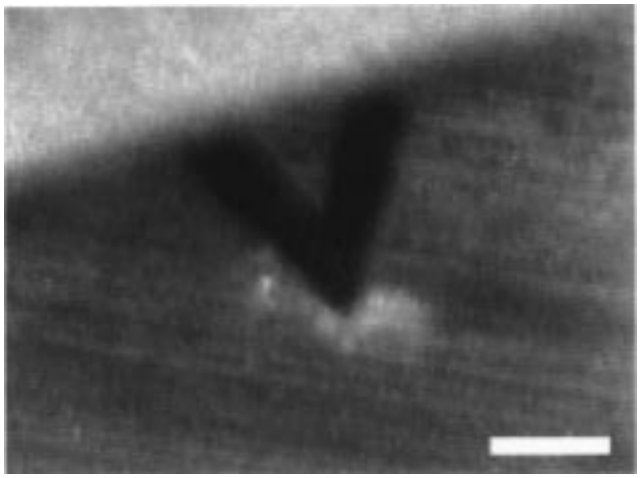

Figure 4. Image showing how the fluorescence microscopy attachment enables the user to locate a fluorescent domain for engaging the cantilever tip of the AFM; the bar denotes 100 μm.

domain from micrometer-resolution imaging with fluorescence microscopy at the air−subphase interface to near atomic resolution imaging with AFM on substrate-supported, transferred films.

Results and Discussion

To demonstrate the capabilities of this new apparatus, we chose to examine well-characterized monolayers of fatty acid and fatty acid salts.[7] Three different experiments have been carried out to show that the apparatus is capable of (1) successful transfer of multiphase monolayers, (2) distinguishing between successful and unsuccessful transfers, and (3) depositing rigid long-chain fatty acid salts onto solid substrates.

We first examined the transfer of collapsed domains of cadmium palmitate from the air−subphase interface onto a solid substrate. Palmitic acid (PA, $CH_3(CH_2)_{14}COOH$, 99% pure; Sigma Chemical Co., St. Louis, MO) with 0.5 mol % of NBD-HDA (*N*-7-nitrobenzo-2-oxa-1,3-diazol-4-yl-hexadecylamine, Molecular Probes, Eugene, OR) was spread quantitatively from a chloroform solution (Fisher Spectranalyzed) onto a subphase of 18-MΩ water (Milli-Q UV Plus, Millipore, Bedford, MA) that contained 6×10^{-4} M $CdCl_2$. The pH was adjusted to 6.9 by the addition of $NaHCO_3$. The transfer device with a freshly cleaved $^1/_2$-in.-diameter mica substrate assembly was placed in the subphase at the beginning of the experiment. The film was compressed past collapse, which occurred at about 40 mN/m. A fluorescence micrograph of the collapsed film at the air−subphase interface is shown in Figure 5A; the bright domains are the collapse structures. To transfer the film, the subphase was slowly drained from the trough. The monolayer was first "cut" by the sharp edge of the stainless steel ferrule; the subphase was further drained from inside the transfer unit through outlets formed by the shaved edges of the stainless steel base. Finally the monolayer was deposited onto the solid substrate. Figure 5B is the fluorescence micrograph of the transferred film showing the collapse structures are preserved; the inset shows a different focal plane where the air bubbles entrapped between the top surface of the stainless steel disk and the mica substrate are visible. After all the subphase liquid was drained, the mica substrate assembly was removed from the transfer unit and was placed on the piezoelectric stage of the AFM for higher resolution imaging. To ensure that a region with collapse structures was imaged, the FM attachment to the AFM was used to locate the region of interest before engaging the cantilever

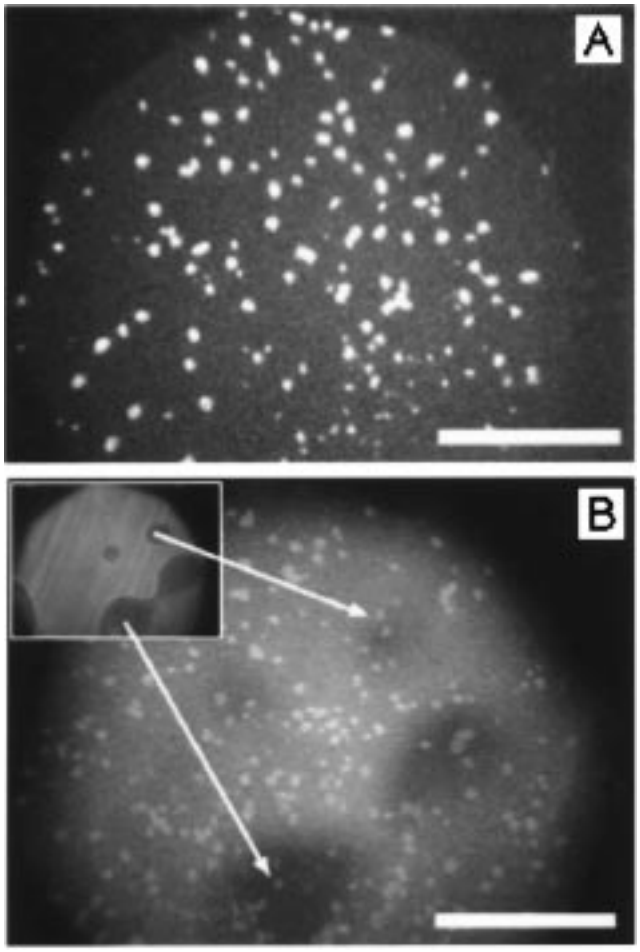

Figure 5. Fluorescence micrographs of (A) a collapsed film of cadmium palmitate at the air−cadmium subphase interface at room temperature, and (B) the same collapsed cadmium palmitate film after being transferred onto a mica substrate; the bar denotes 100 μm. The inset in (B) shows a different focal plane where the air bubbles entrapped between the mica substrate and the stainless steel disk are visible.

tip of the AFM onto the sample. Figure 6 shows the AFM image of the same film with the collapse structures that are of bilayer and multilayer thickness; Figure 7 shows the high-resolution AFM image of the collapsed terraces, detailing the molecular packing; the collapse structure is identical to multilayer films of cadmium arachidate deposited by LB deposition.[7]

An even more important application of the new transfer method is to identify those situations in which transfer is not successful. To demonstrate that this technique can distinguish between transfers in which the surface morphologies of the films are preserved from those where the morphologies are altered, we have deposited palmitic acid monolayers from the air−water interface onto mica substrates at low surface pressures. Figure 8A shows the fluorescence micrograph of a PA monolayer on a pure water subphase at 28 °C. The surface pressure is 4.9 mN/m; the dark domains are the liquid-condensed phase, and the bright background is the liquid-expanded phase. The film was transferred onto a mica substrate in a similar fashion as described above. Unlike the transfer of the collapsed cadmium palmitate film, the surface morphology of this low-pressure PA monolayer is not preserved upon transfer. Figure 8B shows how the morphology of the film is altered drastically upon contact with the solid substrate; the solid-phase domains are stretched and distorted and the fluid domains are transformed into extended stringy structures.

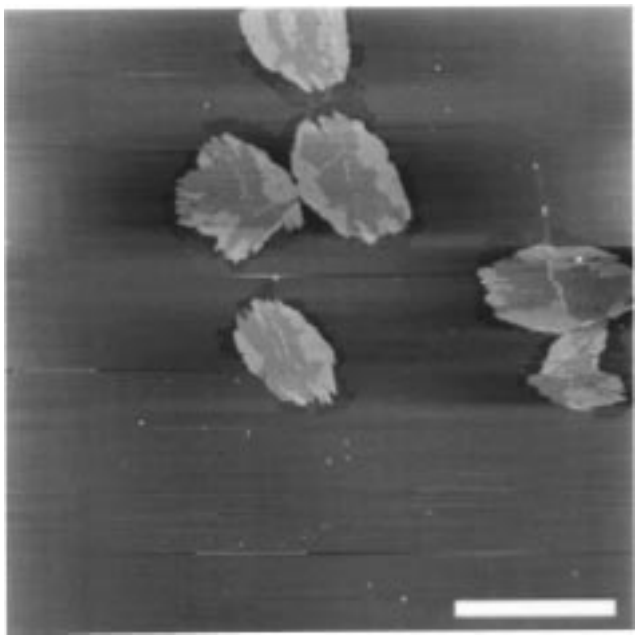

Figure 6. Low-resolution AFM image of the transferred film shown in Figure 5. Regions of lighter color are the raised collapsed structures; the variation in colors on the domains are bilayer and multilayer steps. The bar denotes 10 μm.

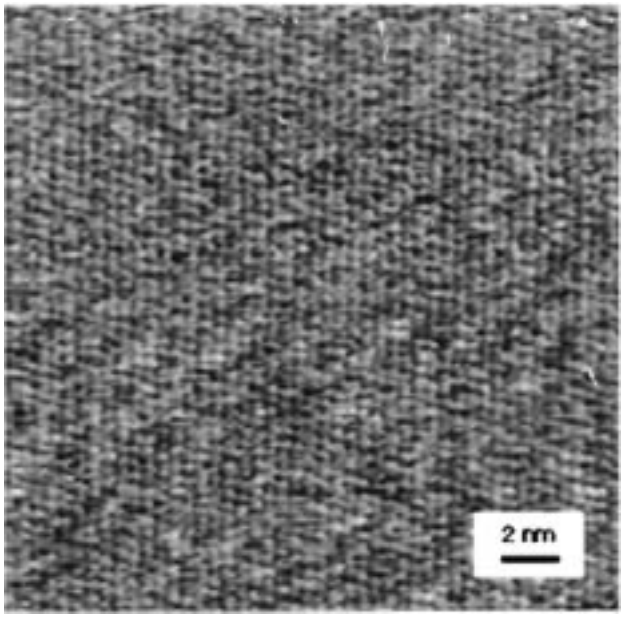

Figure 7. High-resolution AFM image of the transferred film in Figure 5. The packing of the molecules in the collapsed terraces are revealed and is similar to that of multilayer films of cadmium arachidate deposited by LB deposition.[7]

Clearly, the correlation between the structures at the air−water interface and on the substrate have been lost. The ability to continuously monitor the surface morphology of the film throughout the deposition process thus allows one to identify transfers with artifacts from artifact-free transfers.

Another advantage of this technique is that it is capable of transferring rigid films, a task that is difficult to achieve using other deposition methods. For instance, it is known that long-chain fatty acid salts form rigid monolayer films that are difficult to transfer onto solid substrates. Using this new apparatus, however, we have demonstrated that such transfers are possible. We have successfully trans-

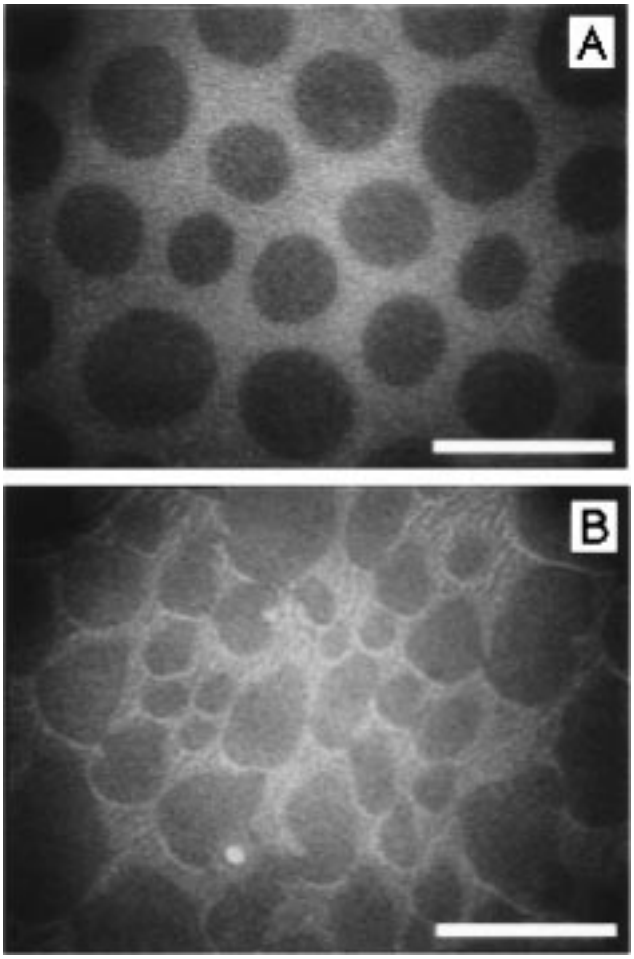

Figure 8. Fluorescence micrographs of (A) a PA monolayer on a pure water subphase at 4.9 mN/m and 28 °C, showing the coexistence between the dark liquid-condensed and the light liquid-expanded phase, and (B) the same film after being transferred onto a mica substrate; the bar denotes 100 μm. Comparison of the two images shows that the transfer process drastically alters the surface morphology of the film. The solid-phase domains are distorted and stretched, and the fluid phase has been transformed into extended, stringy structures. Any correlation between the monolayer structure at the air−water interface and on the substrate has been lost.

ferred a triacontanoic acid ($CH_3(CH_2)_{28}COOH$) monolayer from the air−lead ion subphase interface onto a mica substrate at a surface pressure of 30 mN/m and a temperature of 40 °C. Figure 9 shows the AFM image of the transferred lead triacontanoate monolayer, giving information on the molecular packing of the film. Previously, we had been limited to films of fatty acids of 20 carbons or less using the LB technique.[7]

Transfer for FTIR Study. The transfer technique described above can also be used to deposit monolayers onto germanium ATR crystals for FTIR−ATR studies. We deposited a PA monolayer at 30mN/m from the air−water interface onto a 50 × 20 × 3 mm^3 45° germanium ATR crystal (Spectra-Tech Inc., Stamford, CT). The ATR crystal with the transferred monolayer was then mounted onto a continuously variable ATR assembly (model 304, Spectra-Tech Inc.). The ATR assembly was fitted into the sample compartment of a Nicolett 850 FTIR Spectrometer (Nicolett Analytical Instruments, Madison, WI). The crystal acts as a waveguide for the infrared radiation, and the evanescent wave penetrating into the deposited monolayer makes FTIR spectroscopic measurements possible. Figure 10 shows a FTIR−ATR spectrum of a

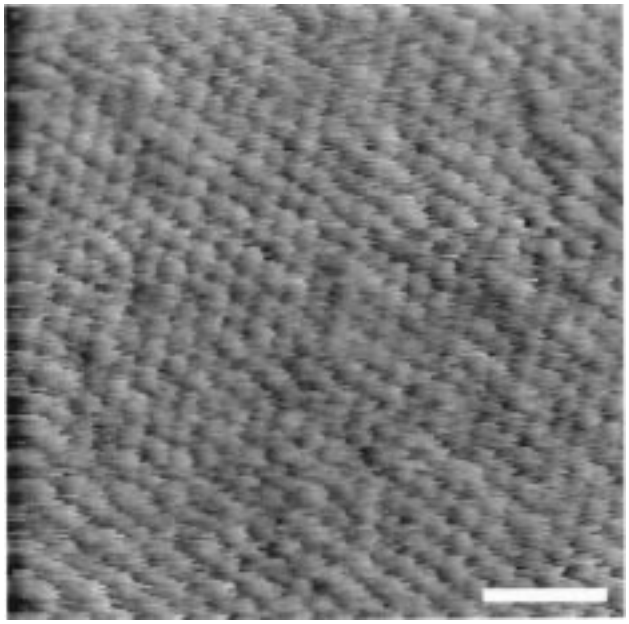

Figure 9. High-resolution AFM image of a lead triacontanoate (30-carbon fatty acid) monolayer on a mica substrate transferred at 30 mN/m and 40 °C. The new technique described allows for the transfer of this rigid film from the air−subphase interface onto a solid substrate without disturbing the integrity of the film. Typical LB deposition is limited to fatty acid salts less than 20 carbons long.[7] The bar denotes 2 nm.

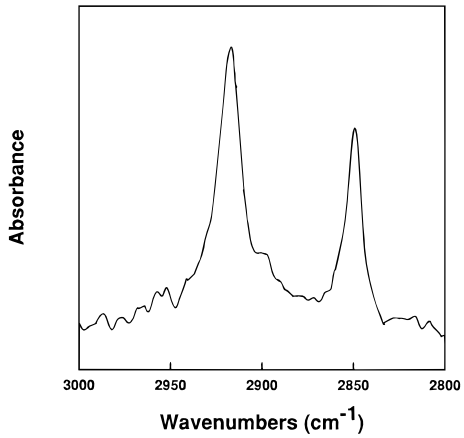

Figure 10. FTIR spectrum of a PA monolayer transferred onto a 45° germanium ATR crystal at a surface pressure of 33 mN/m. The infrared spectral range is from 4000 to 500 cm^{-1} wavenumbers, which includes the lipid acyl chain stretching modes shown above. The spectrum shows that the molecules are oriented nearly normal to the substrate as expected from the AFM images.

monolayer of PA obtained after background subtraction and averaging over 256 scans at a spectral resolution of 4 cm^{-1}. The CH_2 symmetric and asymmetric stretching modes of the fatty acid acyl chain can be seen near 2850 and 2920 cm^{-1}, respectively.

We have also carried out polarized FTIR−ATR spectroscopic measurements on the transferred film. The ATR crystal with the transferred PA film was mounted in the FTIR spectrometer as described above, and the orientation of lipid acyl chains was assessed by placing a gold wire grid polarizer (Perkin-Elmer, Norwalk, CT) in the infrared beam parallel and perpendicular to the ATR crystal surface. The orientational order of the PA acyl chains can be estimated by monitoring the dichroic ratio

$$R_{ATR} = A_{||}/A_{\perp} \quad (1)$$

of the symmetric CH_2 stretching frequency at 2850 cm^{-1}, with $A_{||}$ the absorption polarized parallel to the plane of incidence and $A_{\perp}$ the absorption polarized perpendicular. Assuming uniaxial orientation of the acyl chains around the surface normal, and knowing that the transition moments of the vibration are oriented at a right angle to the acyl chain, the order parameter is given by[14,15]

$$S = \frac{-2(E_x^2 - R_{ATR}E_y^2 + E_z^2)}{E_x^2 - R_{ATR}E_y^2 - 2E_z^2} \quad (2)$$

where E_z is the electric field amplitude perpendicular to the crystal surface and E_x and E_y are parallel to the crystal surface, with E_x pointing in the direction of the beam's propagation. For a film transferred at a surface pressure of 33 mN/m, the polarized FTIR has a dichroic ratio of 1. Using refractive indices $n_1 = 4.0$ for the germanium crystal, $n_2 = 1.5$ for the thin film and $n_3 = 1$ for air;[16] an order parameter of 0.59 was found for our system. As $S = -0.5$, $S = 0$ and $S = 1.0$ imply completely horizontal, random, and completely normal orientation of the acyl chains, respectively; our results indicate that at the given surface pressure, the acyl chains are nearly normal to the film surface, consistent with the lattice structures we observe with AFM.[7]

Conclusions

We have demonstrated that a simple inverse Langmuir−Schaefer transfer technique can be used to successfully deposit monolayers from the air−subphase interface to solid substrates while monitoring the surface morphology with optical microscopy. The ability to visualize the entire transfer process allows us to ensure the deposition of artifact-free films that accurately reflect the structure at the air−subphase interface, or to identify alterations due to the deposition process. This technique also makes possible the transfer of rigid films from the air−subphase interface onto solid substrates. The simplicity of the method makes it easy to incorporate into any Langmuir trough system. With the fluorescence optical attachment to the AFM, regions of interest (collapsed structures, domains in a coexistence region, tagged proteins in a lipid matrix, etc.) can be identified for high-resolution imaging. This allows for the direct correlation of the information obtained via AFM with the structures or morphology observed with fluorescence microscopy. We have also demonstrated that molecular information on lipid and protein conformation and orientation in the monolayer can be obtained using FTIR−ATR spectroscopic techniques on films transferred in this manner.

Acknowledgment. The authors wish to thank R. Stuber and A. Weinberg for the construction of the apparatus, and L. M. Bull for her help with the FTIR measurements. The work was funded by NIH Grant HL51177 (K.Y.C.L., J.A.Z., M.M.L.) and NSF Grant CTS-9622506, and made use of the MRL Central Facilities supported by the NSF under Award No. DMR-96-32716. K. Y. C. Lee was supported by a University of California President's Postdoctoral Fellowship and a National Research Service Award (1 F32 HL09812-01). M. M. Lipp is grateful for the support from a University of California Regents Graduate Fellowship and a Corning Graduate Fellowship. A.J.W. was supported by NIH Grant HL55534.

LA9704213

(14) Fringeli, U. P.; Gunthard, H. H. In *Membrane Spectroscopy*; Grell, E., Eds.; Springer-Verlag: Berlin, 1981; p 270.
(15) Frey, S.; Tamm, L. *Biophys. J.* **1991**, *60*, 922.
(16) Fringeli, U. P. In *Biologically Actice Molecules*; Schlunegger, U. P., Eds.; Springer-Verlag: Berlin, 1989; p 241.