Spontaneous Formation of Interfacial Lipid-Protein Monolayers during Adsorption from Vesicles

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ABSTRACT Spread and adsorbed monolayers of lipid-protein mixtures have served as models for biomembranes and pulmonary surfactant, but their similarity was unclear. Epifluorescence microscopy of monolayers spontaneously adsorbed from vesicles of dipalmitoylphosphatidylcholine or dipalmitoylphosphatidylcholine plus surfactant protein C (SP-C) showed gas, liquid expanded, and liquid condensed (LC) domains. The shapes and distribution of LC domains in the adsorbed and solvent-spread monolayers were quite similar. Labeled SP-C adsorbed into the air-water interface in the company of the lipids. In both forms of monolayers, SP-C occupied the fluid phase and reduced the size and amount of the LC domains. The properties suggest that these adsorbed and spread monolayers are analogous to one another.

INTRODUCTION

Monomolecular lipid films can serve as good models for biological membranes and pulmonary surfactant. Although lipids are easily spread in monolayers and yield some information on biological membranes, lipid-protein monolayers are difficult to assemble (Schindler, 1989) because of the insolubility of most proteins in volatile nonpolar solvents. Monolayers formed by adsorption from lipid-protein vesicles can have proteins incorporated in them (Schindler, 1989). Planar lipid-protein bilayers formed from adsorbed monolayers have served as tools in studying biomembrane lipid-protein and lipid-lipid interactions (Pattus et al., 1981; Schürholz and Schindler, 1991). Adsorbed monolayers have also been used in studying processes such as biomembrane assembly (Gershfeld, 1989), lipid exchange between membranes (Jähnig, 1984), antibody-antigen recognition (Fischer et al., 1993), and pulmonary surfactant biophysics (Goerke and Clements, 1986; Keough, 1992). Monolayers spontaneously formed from vesicles may be similar to their solvent-spread counterparts (Gershfeld, 1976; Schindler, 1989), but limited direct experimental evidence exists on this issue (Salesse et al., 1987). In studying adsorption processes of some pulmonary surfactant components, we have found direct evidence for some significant similarities of monolayers formed by both processes.

Pulmonary surfactant, a complex lipid-protein mixture in the lining layer of the alveolar fluid, reduces surface tension in the lung and consequently decreases the work of breathing (Goerke and Clements, 1986). It undergoes a complex set of transformations from the bilayer form in which it is secreted into a putative monolayer at the air-water interface. Its principal component is dipalmitoylphosphatidylcholine (DPPC). The adsorption of the material is promoted by surfactant proteins including the very hydrophobic surfactant protein SP-C (Curstedt et al., 1987; Simatos et al., 1990; Shiffer et al., 1988; Takahashi and Fujiwara, 1986; Yu and Possmayer, 1990). SP-C also serves as a good model for membrane-spanning α-helical protein segments in bilayers (Morrow et al., 1993).

Over the last decade, powerful microscopic techniques have allowed for visual observation of lipid phase transitions, domain structures, and molecular arrangements in monolayers (Knobler and Desai, 1992; McConnell, 1991; Stine, 1995). Fluorescence microscopy of solvent-spread lipid and lipid-protein monolayers has been used to visually observe monolayer structures at the air-water interface (Möhwald, 1990; Stine, 1995). Phase domains with dimensions ranging from tens to hundreds of microns have been observed in monolayers due to partitioning of fluorescent lipids and proteins in any one particular phase (Grainger et al., 1989; Möhwald, 1990; Stine, 1995). Gas, liquid expanded (LE), and liquid condensed (LC) phase domains can be seen in these monolayers during compression-induced isothermal phase transitions (Knobler and Desai, 1992; McConnell, 1991). Such domains have also been observed using Brewster angle microscopy of pure monolayers not containing fluorescent probes (Honig and Möbius, 1991). Recently, using fluorescence microscopy, Heyn et. al. (1990) showed that, during isothermal compression, some monolayers formed by adsorption of lipid vesicles showed similar surface pressure-area profiles and LC domain geometry during compression compared with the ones spread from organic solvents. These monolayers were formed from vesicular suspension that had been transferred by a wet bridge onto an air-water interface to yield monolayers without attached vesicles (Heyn et al., 1990). We report here on the phase characteristics as a function of time and changing

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surface pressure (π) of monolayers of DPPC and DPPC/SP-C that were formed by adsorption from vesicles injected below the air-water interface. Some of the DPPC/protein systems are compared with the properties of monolayers spread from organic solvent. The location of the protein SP-C in DPPC monolayers was determined with fluorescently labeled protein (F-SP-C).

MATERIALS AND METHODS

Materials

DPPC was purchased from Sigma Chemical Co. (St. Louis, MO), and the fluorescent lipid probes *N*-lissamine rhodamine B sulfonyl-1,2,hexadecyl phosphatidylethanolamine (R-PE) and fluorescein isothiocyanate (FITC) were from Molecular Probes (Eugene, OR). The DPPC and R-PE each showed a single spot on thin layer chromatography and was used as received.

Pulmonary surfactant protein SP-C was extracted from porcine lung surfactant by methods discussed in detail elsewhere (Perez-Gil et al., 1993).

The vesicle solvent and monolayer subphase buffer was prepared by dissolving 0.15 M NaCl, 10 mM MOPS (Sigma), and 5 mM $CaCl_2$ in doubly glass-distilled water, the second distillation being performed with dilute $KMnO_4$, and the pH adjusted to 6.9 with 0.1 N HCl.

Fluorescent labeling of SP-C

The extracted SP-C was labeled by reacting it with FITC by the following methods. Purified SP-C (200 μg) in 2 ml of chloroform/methanol (2:1, v/v) was adjusted to pH 7.8 by the addition of appropriate amounts of 50 mM Tris in methanol. SP-C was incubated overnight at 4°C after addition of 20 μl of 10 mM FITC. The pH of the incubated mixture was adjusted to 2, and this solution was applied to an LH-20 Sephadex (Pharmacia, Uppsala, Sweden) column and eluted with chloroform/methanol (2:1, v/v) to remove the unreacted FITC. The profile of the LH-20 effluent was followed by absorbency at 250 nm (unreacted SP-C) and at 450 nm (F-SP-C). F-SP-C showed a single band on sodium dodecyl sulfate gel electrophoresis with a mobility corresponding to approximately 5000 Da.

The molecular weight of F-SP-C was determined using matrix-assisted laser desorption/ionization mass spectrometry (MALDI) by techniques discussed elsewhere (Hillenkamp et al., 1991). SP-C has been previously shown using mass spectrometry to be a dipalmitoylated protein of 4210 Da, and the deacylated form is 3734 Da (Curstedt et al., 1990). F-SP-C was detected as a major peak at 4110 Da (3734 (SP-C) + 376 (fluorescein) = 4110) in MALDI spectrometry, indicating that the protein was deacylated and labeled with a single fluorescein.

Preparation of vesicles

Liposomal suspensions of DPPC and DPPC plus F-SP-C were prepared by two methods to yield multilamellar vesicles (MLVs) and sonicated vesicles (SVs). DPPC, F-SP-C, and probe R-PE were individually dissolved in chloroform/methanol (3:1, v/v) and mixed in desired proportions. The solvents were dried under a stream of nitrogen in small 1-ml chromosulfuric-acid-cleaned glass vials. SVs were prepared by resuspending the dried materials in a 0.9% buffered saline and sonicating the suspensions on ice. Sonication was performed with 60 medium-powered individual bursts lasting 1 s with a microprobe sonicator (Bronson Ultrasonics, Ontario, Canada). MLVs were made by incubating the resuspended material at 45°C for 30 min (above T_c of DPPC) and vigorously vortexing the suspensions until a milky homogeneous suspension was achieved. These methods and their liposomal-type yields are discussed in detail by Woodle and Papahadjopoulos (1989).

Epifluorescence microscopic adsorption apparatus

Adsorption was observed on a modified version of an epifluorescent microscopic surface balance (Nag et al., 1990). The original surface balance consisted of a custom-designed Langmuir trough with epifluorescence microscopic attachments. Modification was done by attaching an adsorption chamber to the original balance. The adsorption apparatus was made of Teflon and contained two circular chambers connected by a short channel to maintain subphase and monolayer continuity. The total volume of the subphase was 10 ml and the total surface area was 15 cm^2. The vesicles in desired amounts were injected into the buffer under the air-buffer interface at the center of the channel. Surface fluorescence was monitored by epifluorescence microscopy from one of the circular chambers and surface pressure by a Wilhelmy dipping plate suspended from a pressure transducer from the second chamber.

Monolayer formation and visual observation

Vesicular suspensions of DPPC or DPPC plus protein were injected under the air-buffer interface of the surface balance discussed above. The change of surface pressure (π) during adsorption from vesicles was monitored by a roughened platinum Wilhelmy dipping plate attached to a pressure transducer connected to a personal computer, as discussed in detail elsewhere (Nag et al., 1990, 1991). Experiments were performed at a temperature of 22 ± 2°C. After injection of vesicles under the buffer interface, the surface of the solution was brought into focus in the epifluorescence microscope. The formation of the monolayer was observed from R-PE or F-SP-C fluorescence emission. As the vesicles adsorbed, the change in π was monitored as a function of time in minutes, at a fixed interfacial surface area (15 cm^2). As the surface area of the adsorption chamber was fixed, the adsorbing lipids to the interface increased the packing density of the film with time, and this allowed for observation of different microscopically visible phases (gas, LE, and LC), which had previously been observed in solvent-spread films during lateral isothermal compression. In the adsorbed films, the packing of the molecules at the air-water interface was induced by transfer of material from the vesicles into the surface film instead of mechanical compression with a barrier as is done with solvent-spread films. The spontaneous adsorption from vesicles did not allow for measurements of area per molecule of lipid in the monolayer, as the number of molecules at the surface at any π during the adsorption process could not be determined. Typical monolayer images were recorded on a video tape with an image intensifier and charge couple device (CCD) camera attached to the microscope, and the images were analyzed by methods discussed in detail elsewhere (Nag and Keough, 1993).

The vesicular subphase was not stirred, as stirring (30–50 cycles/minute) aggregated the monolayer phase domains and made the monolayers extremely inhomogeneous in visual appearance. The general shapes of the condensed domains forming the aggregates, however, were the same in stirred and unstirred systems.

RESULTS AND DISCUSSION

Adsorption from lipid vesicles

Surface pressure versus time (π-t) isotherms of DPPC plus 1 mol% R-PE formed by adsorption from vesicles are shown in Fig. 1 *a*, and the typical images observed at the π indicated by letters in the isotherms are displayed in Fig. 1 *b*. SVs of DPPC adsorbed more rapidly (reaching a $\pi \approx 9$ mN/m in 30 min) than MLVs (0.1 mN/m in 30 min). As shown in Fig. 1 *b*, image *A*, the surface monolayer initially formed by adsorption from MLVs had gas-phase domains (dark areas) coexisting with fluorescent LE phase (light areas). Typical images of adsorbed monolayers formed

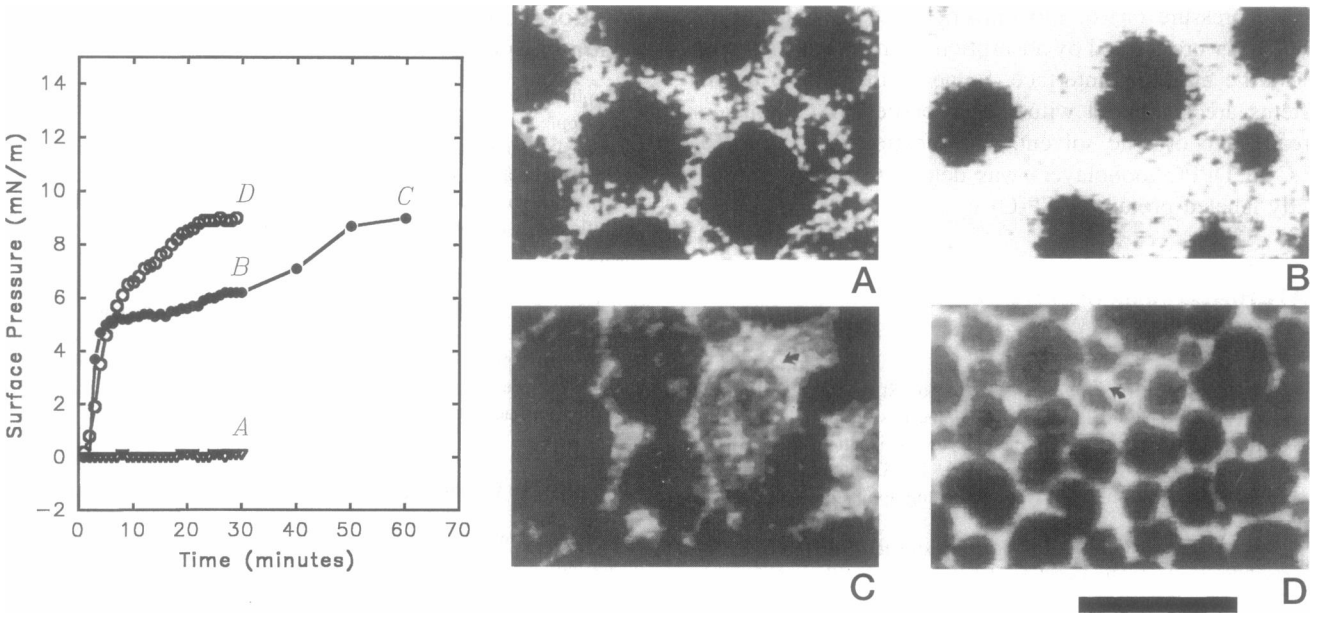

FIGURE 1 π-t isotherms of DPPC plus 1 mol% R-PE adsorbing from vesicles (a) and typical images from the surface monolayers formed (b). Letters A–D represent the π at which the images in b were obtained. The light regions indicate the LE phase into which the probe R-PE partitioned. The bright areas indicated by the arrows in b (C and D) may be vesicles in close proximity to the interface. Scale bar, 25 μm. MLVs of DPPC (▽) adsorbed only to a $\pi \approx 0.1$ mN/m in 30 min and the surface monolayer formed showed gas phase (dark) domains coexisting with LE phase (A). SVs of DPPC at 0.04 mg/ml (●) adsorbed more rapidly than MLVs but slowly enough to show typical kidney-bean-shaped LC phase (dark) domains (B), which increased in size with time and came in contact around $\pi \approx 9$ mN/m (C). SVs of DPPC at 0.06 mg/ml (○) adsorbed to reach a similar π as in C but in a shorter time and showed smaller and more numerous LC domains (D).

from SVs are shown in Fig. 1 b (B, C, and D). These images show distinct condensed (LC) domains (dark areas) coexisting with the fluorescent LE phase. Images B and C are taken from a monolayer formed by adsorption from a SV suspension containing 0.04 mg/ml DPPC, and image D is from a monolayer adsorbed from SVs at 0.06 mg/ml. The bright areas indicated by the arrows in images C and D might be fluorescent vesicles attached to or near the surface monolayer.

It is apparent from images in Fig. 1 b that DPPC monolayers undergo gas to LE to LC phase transitions because of increasing surface pressure during adsorption. Gas and LE phase domains, which were seen previously in highly expanded solvent-spread DPPC monolayers at $\pi \approx 0$ mN/m also appear in the spontaneously adsorbed ones (Fig. 1 b, A) at similar π. The increase in surface pressure during adsorption (between points B and C in Fig. 1 a) occurred due to increased packing density of the lipids and is equivalent to the increase in π seen during compression of solvent-spread monolayers. The LC domains of DPPC in solvent-spread monolayers that have been compressed relatively slowly display a kidney bean shape (Flörsheimer and Möhwald, 1989; McConnell, 1991; Nag et al., 1990; Weis and McConnell, 1985), and these are seen at similar π in the adsorbed monolayers (Fig. 1 b, B).

Spread monolayers of DPPC and of other lipids, when compressed relatively rapidly, showed a different LC domain distribution compared with that seen in slowly compressed ones (Chi et al., 1993; Nag et al., 1991; Shimomura et al., 1992). Fast compression leads to large numbers of small-sized LC domains compared with those seen in monolayers that have been slowly compressed to equivalent π (Nag et al., 1991; Shimomura et al., 1992). This pattern of dependency of size and number of condensed domains on compression rates is also seen here in adsorbed monolayers. The condensed domains in Fig. 1 b, C (slower adsorption) are larger in size and less numerous per image compared with those shown in D (faster adsorption). The monolayer formed from higher concentration of lipid in the subphase (Fig. 1 b, D) had more material adsorbed at a shorter time (analogous to fast compression of a spread monolayer) than the one seen in C (analogous to slow compression of spread monolayers).

Studies by Heyn et al. (1990) have indicated that the π-A profiles of selected adsorbed films, and the geometry of the domains observed in such films, are similar to the ones obtained from solvent-spread ones. They interpreted their results to suggest that the composition of the films adsorbed from liposomes containing DPPC, dimyristoylphosphatidic acid, and small amounts of cholesterol were the same as the initial liposomal composition. This conclusion was based on the appearance of the adsorbed film in comparison with the spread film of similar composition. In the present study, the geometry of the LC domains (kidney shape) and their sizes (10–30 μm) in adsorbed DPPC films indicated that the composition of the DPPC vesicles (DPPC plus 1 mol% lipid probe) and the adsorbed films were quite similar.

Schindler (1979) proposed a model sequence for adsorption from lipid vesicles that agrees with our experimental observations. His model proposes that bilayer vesicles disintegrate at an open air-water interface at $\pi \approx 0$ mN/m. This disintegration leads to monolayer formation at the interface and an initial rapid increase in π. Once the monolayer is formed, no further vesicular disintegration occurs due to the absence of an open interface, and a layer of vesicles can form under the monolayer, which may come in contact with the monolayer (Schindler, 1979). Slow exchange of material then occurs between the outer leaflet of bilayer vesicles and the surface monolayer with more material moving from the bilayer to the monolayer than in the opposite direction. The exchange occurs due to exclusion of free water between monolayer and vesicles as polar lipid head-group contacts are transiently formed. This absence of free water decreases the free energy and allows the hydrophobic chains to transfer from one layer to the other, until a steady-state or equilibrium π is reached.

DPPC vesicular adsorption seems to follow this sequence. A surface monolayer was formed relatively quickly (a rapid rise in π in the first few minutes). Once the initial DPPC monolayer was formed, possible transfer of material from the bilayer occurred more slowly (a decreased slope in the π-t curve after 10 min) and was accompanied by growth of LC domains. LC domains grew relatively slowly, allowing for tilt and orientational order to set in, thus producing the shapes and sizes of LC domains that are typical of a slowly formed condensed monolayer (Fig. 1, *a* and *b*, *C*). With the higher concentrations, the rate of transfer was greater and the condensed monolayer was achieved faster (Fig. 1, *a* and *b*, *D*). Once the condensed monolayers were formed (as evidenced by the high amounts of LC phase lipids in Fig. 1, *a* and *b*, *C* and *D*), the bilayer-monolayer lipid transfer process appeared to be approaching directional equivalence or a steady state.

Adsorption from lipid-protein vesicles

Monolayers made by adsorption of vesicles containing lipids and proteins from biological membranes have been used to study lipid-protein interactions and protein function (Pattus et al., 1981; Schindler, 1989; Schürholz and Schindler, 1991). The lipid-protein ratio of such adsorbed monolayers was found to be close to that of the vesicles from which they were formed (Pattus et al., 1981; Schürholz and Schindler, 1991). Such adsorbed monolayers have been used to form black lipid (or lipid-protein) membranes by special techniques (Schindler, 1989). Ion channel proteins in such reconstituted black lipid membranes were found to be functionally fully active (Schindler, 1989). Formation of such adsorbed lipid-protein monolayers has shown that the adsorption of lipids was enhanced in the presence of membrane proteins. The transbilayer parts of most membrane proteins are α-helical in their structure, and some of these proteins can be incorporated in the monolayers (Pattus et al., 1981; Schindler, 1989). Pulmonary surfactant protein SP-C is mainly α-helical in lipid environments (Morrow et al., 1993) and promotes the adsorption of lipids to the air-saline interface (Curstedt et al., 1987; Simatos et al., 1990; Takahashi and Fujiwara, 1986; Yu and Possmayer, 1990). Incorporating 3 and 7 wt% F-SP-C in MLVs of DPPC enhanced the rate of adsorption of DPPC as shown in Fig. 2 *a*. The surface monolayers formed by adsorption from DPPC/F-SP-C vesicles after 30 min are shown in Fig. 2 *b*. The fluorescent (light) part of the images in Fig. 2 *b* reflect F-SP-C emission and indicate that the protein accompanies the lipid into the monolayer at the air-water interface and that it is in the LE or fluid phase of the monolayer.

Increasing amounts of F-SP-C not only enhanced the rate of adsorption of DPPC (*A* to *B* in Fig. 2 *a*) but also decreased the average size of LC domains in the corresponding monolayers (*A* to *B* in Fig. 2 *b*). The fact that F-SP-C induced a decrease in the size of condensed domains is consistent with previous results from spread monolayers of DPPC that contained similar amounts of unlabeled SP-C (Keough et al., 1992; Perez-Gil et al., 1992). This effect of proteins on lipid distribution between LE and LC phases has also been observed by others in different lipid-protein monolayers (Möhwald, 1990). The changing LC domain distribution in the adsorbed DPPC/F-SP-C films indicated that the adsorbed films have similar lipid to protein ratios as was present in their vesicular counterparts.

SP-C has been reported to produce discoidal structures in certain lipid dispersions (Williams et al., 1991). Defects produced in the bilayer structure or the presence of the discoidal structures may promote rapid monolayer formation. Also, SP-C seems to behave in a lipid environment in a similar fashion to some transmembrane proteins (Pattus et al., 1981; Schürholz and Schindler, 1991) in enhancing monolayer formation over that seen for pure lipid vesicles.

Spread and adsorbed monolayers

To further analyze the similarities between solvent-spread and adsorbed monolayers, films of DPPC plus 3 wt% F-SP-C were spread from solvent and compressed at a rate to achieve the same π over the same time as a monolayer adsorbed from MLVs of a similar mixture (e.g., Fig. 2 *a*). Fig. 3 *a* shows typical images from an adsorbed monolayer (*A*) at a $\pi \approx 15$ mN/m and a solvent-spread monolayer (*B*) after compression to the same π. The shapes of LC domains were similar in both monolayers. The total amounts of LC phase (black areas in the images) at $\pi = 12$ and 15 mN/m for such monolayers are shown in Fig. 3 *b*. The relative amounts of LC phase in adsorbed and spread monolayers of DPPC plus F-SP-C were similar, suggesting that the process by which monolayers were formed led to complementary distributions at the interface.

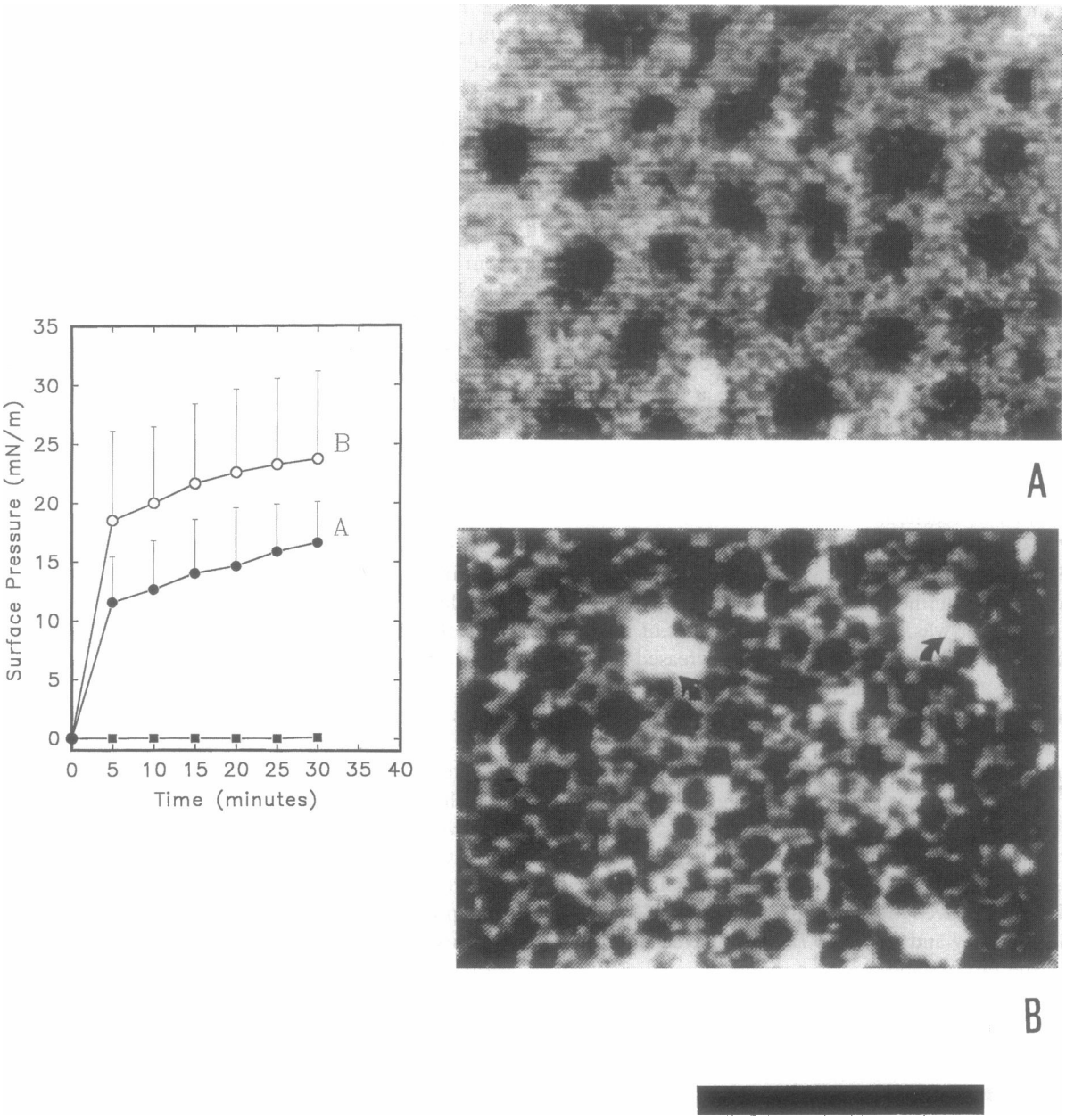

FIGURE 2 π-t isotherms obtained from adsorption of MLVs of DPPC and DPPC plus F-SP-C (*a*) and typical images from the surface monolayers formed (*b*) at π values indicated in the isotherms. MLVs of DPPC (■) or DPPC plus 3 wt% (●) and 7 wt% (○) F-SP-C were used. Error bars indicate standard deviation from three sets of experiments. Scale bar, 25 μm (*b*). The bright part of the images (*b*) represents the fluorescence observed from the labeled protein obtained at points *A* and *B* noted on the adsorption isotherms in *a*. The highly fluorescent regions (*arrow*) in image *B* indicate possible protein aggregates. These were not likely vesicles, as the fluorescence intensity in these spots was much higher than that seen in DPPC vesicles adsorbing to or near the surface monolayer (Fig. 1, *C* and *D*, *arrows*).

Hydrophobic proteins of pulmonary surfactant alter the π-A isothermal characteristics of some spread lipid monolayers (Cochrane and Revak, 1991; Longo et al., 1993; Pastrana-Rios et al., 1995; Perez-Gil et al., 1992; Taneva and Keough, 1994a,b, 1995). SP-C has been shown to expand spread DPPC monolayers (Pastrana-Rios et al., 1995; Perez-Gil et al., 1992; Taneva and Keough, 1994a,b, 1995) and broaden the range of gel to liquid crystalline melting temperature (T_c) of the DPPC bilayers (Morrow et al., 1993; Shiffer et al., 1993), which is consistent with a decrease of the cooperativity of the bilayer gel to fluid phase transition (Gershfeld, 1976; Simatos et al., 1990). Adsorption of fluid lipid bilayers at an air-water interface is more rapid than that from gel state bilayers (Gershfeld, 1976, 1989; Heyn et al., 1990). F-SP-C may increase adsorption of DPPC bilayers by fluidizing the chain packing of such lipids or producing defects in the packing of the system. In spread monolayers of DPPC containing similar amounts of SP-C to

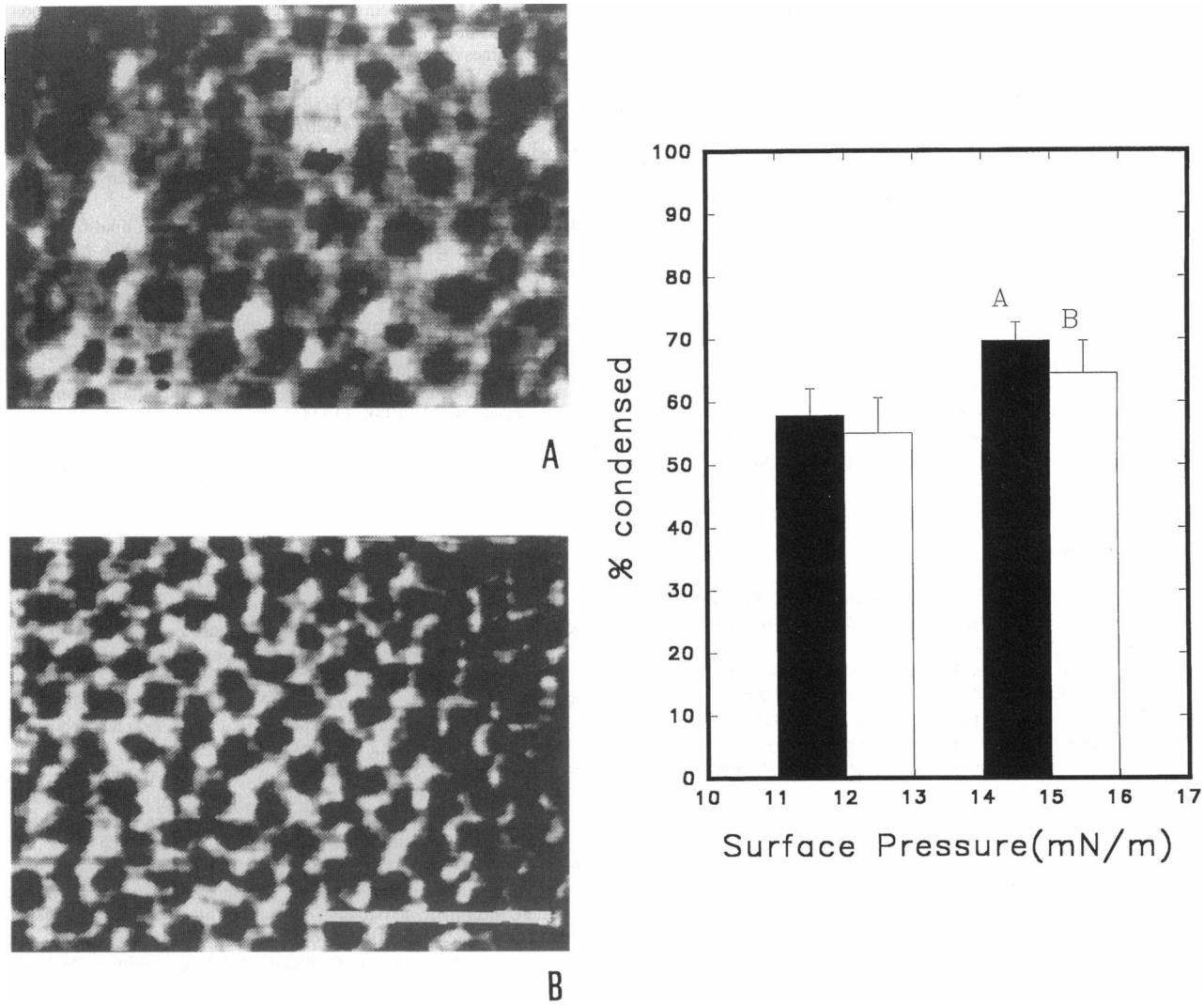

FIGURE 3 Typical images observed in monolayers formed by solvent spreading and adsorption from vesicles each containing DPPC plus 3 wt% F-SP-C (*a*) and the percentage of LC phase observed in such monolayers at $\pi \approx 12$ and 15 mN/m (*b*). Image *A* is from an adsorbed monolayer and *B* is from a solvent-spread monolayer compressed to a $\pi \approx 15$ mN/m. Scale bar (*B*), 25 μm. In *b*, the bars indicate the types of monolayers analyzed as adsorbed (black) and spread (white). The total percentage of condensed domains was calculated by estimating the total amount of LC phase observed per image. An average of 10 images were analyzed at each π from both types of monolayers, and the error bars indicate +1 SD.

those in this system, the protein increased the amount of LE phase at any given π in comparison with that in pure DPPC (Keough et al., 1992; Perez-Gil et al., 1992). In adsorbed monolayers, the decreased size of the condensed (LC) domains (Fig. 2 *b*, *A* to *B*) seen when the amount of F-SP-C was increased is consistent with a decrease in the cooperativity of an isothermal phase transition.

Solvent-spread films of DPPC/SP-C have been studied recently by Taneva and Keough (1994b) using a leak-free surface balance apparatus. These studies indicated that, above the collapse surface pressure of the protein ($\pi >$ 65 mN/m), some SP-C remained in the lipid monolayer. Those solvent-spread DPPC/SP-C films were also capable of achieving low surface tension or high π upon high compression, behavior as seen in films of pulmonary surfactant (Goerke and Clements, 1986). Upon repeated dynamic compression-expansion cycling of DPPC/SP-C monolayers, some of the protein may get squeezed out of such monolayers at high π but reenters the monolayers upon expansion (Taneva and Keough, 1994b). This process can occur over a number of cycles, with no substantial irreversible loss of material from such lipid-protein monolayers, indicating possibly that SP-C is quite stable in such DPPC/SP-C films. Some of these studies were also substantiated using fluorescently labeled SP-C (as used in this study) in fluorescence microscopy of such monolayers (Nag et al., 1996).

CONCLUSIONS

The results of the direct visualization presented here taken with previous findings in other systems suggest that these

adsorbed and solvent-spread monolayers are analogous. The protein SP-C enhanced monolayer formation and is excluded from the LC regions of DPPC monolayers. The latter finding confirms the influence of SP-C in DPPC monolayers as observed through the use of a phospholipid probe. Establishing the similarity of adsorbed and spread monolayers reinforces the use of either system to study monolayer characteristics as they pertain to pulmonary surfactant or as models for biological membranes.

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