Molecular Mobility in the Monolayers of Foam Films Stabilized by Porcine Lung Surfactant

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ABSTRACT Certain physical properties of a range of foam film types that are believed to exist in vivo in the lung have been investigated. The contribution of different lung surfactant components found in porcine lung surfactant to molecular surface diffusion in the plane of foam films has been investigated for the first time. The influence of the type and thickness of black foam films, temperature, electrolyte concentration, and extract composition on surface diffusion has been studied using the fluorescence recovery after photobleaching technique. Fluorescent phospholipid probe molecules in foam films stabilized by porcine lung surfactant samples or their hydrophobic extracts consisting of surfactant lipids and hydrophobic lung surfactant proteins, SP-B and SP-C, exhibited more rapid diffusion than observed in films of its principal lipid component alone, L-α-phosphatidylcholine dipalmitoyl. This effect appears to be due to contributions from minor lipid components present in the total surfactant lipid extracts. The minor lipid components influence the surface diffusion in foam films both by their negative charge and by lowering the phase transition temperature of lung surfactant samples. In contrast, the presence of high concentrations of the hydrophillic surfactant protein A (SP-A) and non-lung-surfactant proteins in the sample reduced the diffusion coefficient (D) of the lipid analog in the adsorbed layer of the films. Hysteresis behavior of D was observed during temperature cycling, with the cooling curve lying above the heating curve. However, in cases where some surface molecular aggregation and surface heterogeneity were observed during cooling, the films became more rigid and molecules at the interfaces became immobilized. The thickness, size, capillary pressure, configuration, and composition of foam films of lung surfactant prepared in vitro support their investigation as realistic structural analogs of the surface films that exist in vivo in the lung. Compared to other models currently in use, foam films provide new opportunities for studying the properties and function of physiologically important alveolar surface films.

INTRODUCTION

Investigations of the foam films (FFs) composed of artificial or natural mixtures of lipids and proteins are of increasing interest, because such films are found widely in nature, both in vitro and in vivo. An example of the functional importance of lipid-protein foam films in vivo is found in lung surfactant (e.g., Scarpelli, 1978; Lalchev et al., 1979; Scarpelli and Mautone, 1994). The surface activity and properties of the lipid-protein mixture at the alveolar hypophase/air interface in the alveoli and its role in alveolar and lung integrity during the breathing process have been intensively discussed over several decades. Following Von Neergaard's work some 66 years ago, it has become generally accepted that the stabilization of the alveoli and anticollapse phenomena in the lung are due to a combination of tissue and surface forces. The latter forces are manifest in the different types of surface lipid-protein films (mono-, bi- and multilayer) that exist in vivo in the lung. A hypothesis that proposed the formation of foam films in vivo in the alveoli with both bilayer (Newton black FFs) and multilayer (stratified FFs) structures was proposed some time ago (Exerowa and Lalchev, 1986). This hypothesis arose from the fact that if the existing conditions in the lung (pressures, electrolyte concentration, pH, phospholipid and protein concentrations, etc.) were reproduced in in vitro model systems, very stable black foam films were formed spontaneously (Lalchev, 1984; Exerowa et al., 1984, 1986). In addition, direct observation of foam in the lung (Scarpelli et al., 1979) lends credence to this hypothesis. It follows from the latter that FFs are also present, because they are the generic structural element of foam. Observations of bubble formations in the lungs of newborns (Scarpelli, 1988), film formations across the peripheral air spaces (Frazer et al., 1985), structures formed in the contact areas between the alveolar epithelial cells and the surface monolayer film (Hills, 1988) and bilayer (Newton black) or multilayer (stratified) FF formations (Exerowa and Lalchev, 1986; Scarpelli and Mautone, 1994) provide persuasive evidence for the existence of foam films of different types in the lung. However, little is known about the physical properties (e.g., surface diffusion) of FFs stabilized by lung surfactant lipids and proteins, and such phenomena could play a significant role in the anticollapse properties of the lung.

The experimental determination of surface diffusion in foam films was first described by Clark et al. (1990a,b). This was applied to sodium dodecyl sulfate (SDS) and protein FFs and involved combination of the FF method with fluorescence recovery after photobleaching (FRAP) measurements. FRAP measurements on phospholipid thick equilibrium FFs, common black films (CBF), and Newton

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black films (NBF) have been also reported (Lalchev et al., 1991, 1995a). Recently FFs stabilized by single synthetic phospholipids alone and by some mixtures composed of the lung surfactant lipid fraction have been systematically studied by FRAP (Lalchev et al., 1994, 1995b). The role of the film type and some molecular characteristics of the lung surfactant phospholipids, including acyl chain length, headgroup charge, unsaturation, polar group size, etc., on surface diffusion were examined. Observations that foam films and ordered structures like foam films exist in vivo (e.g. Scarpelli, 1988; Exerowa and Lalchev, 1986; Hils, 1988; Scarpelli and Mautone, 1994, etc.) have increased the interest in their properties, including surface diffusion, for the purpose of correlation with their function in the alveoli. The mobility of the lung surfactant molecules in the plane of the surface films must play an essential role in the regulation of surface tension and stabilization of the alveoli during the change of alveolar area in respiration. However, according to the monolayer theory invented to explain the stabilizing and antiedema effects of the lung surfactant, the adsorbed surface film in the alveoli is thought to be monomolecular and to be able to decrease the surface tension γ to near zero or zero dyn/cm during expiration (e.g., Pattle, 1955; Clements, 1962), a proposition that is contentious and is not widely accepted (e.g. Hills, 1981; Bangham, 1991, 1995). Similar contention surrounds the existence of monolayer films of very low surface tensions (e.g., King and Clements, 1972; Clements, 1962) defined as $\gamma < 5$ dyn/cm. Taking into account the existence of ordered structures like foam films in the lung, the alveolar stability and integrity could be explained without the doubtful concept of zero or near-zero surface tension. Systematically studied phospholipid foam films obtained from samples of pure phospholipids (Lalchev, 1984; Naydenova et al., 1990; Nikolova et al., 1994), animal lung surfactant lavages (Lalchev et al., 1983, 1987), human amniotic fluids, and tracheal aspirates (Exerowa et al., 1984, 1986; Gerginova et al., 1994) display stable ordered structures of two lipid monolayers, with the lipid polar groups oriented adjacent to each other. These are stabilized by both lateral (within the monolayers) and normal (between the monolayers) molecular interactions (Exerowa and Lalchev, 1986), and are sufficiently stable to provide additional support to the alveoli against their collapsing during expiration. It was also shown that the formation of stable in vitro foam films from pure phospholipids, amniotic fluids, and lung surfactant samples do not require zero or near-zero surface tension at the monolayer air/liquid interface. Surface tensions of about 24–30 dyn/cm, corresponding to almost-packed lipid monolayers, were found to be necessary for stable black film formation. This surface tension, provided by the surfactant in the bulk (alveolar hypophase), is a more realistic representation of in vivo conditions.

The goal of the work described here is to measure the lateral diffusion coefficients in different types of foam films stabilized by natural lung surfactant samples and to address the contribution of lung surfactant ingredients to molecular surface diffusion in the plane of FFs. Thick equilibrium foam films, together with two types of black (thinner) FFs stabilized by porcine lung surfactant lavages, their hydrophobic extracts (consisting of the lung phospholipids and hydrophobic apoproteins SP-B and C), reconstituted samples of hydrophobic extract plus the hydrophilic surfactant protein SP-A, and the major surfactant phospholipid L-α-phosphatidylcholine dipalmitoyl have been examined in the study. In addition, the temperature dependence of diffusion both below and above physiological temperatures has been examined to gauge the role of the phospholipid phase state on the diffusion coefficients in the lung surfactant foam films.

MATERIALS AND METHODS

L-α-Phosphatidylcholine dipalmitoyl (DPPC, P-6267), L-α-phosphatidylglycerol dimiristoyl (DMPG, P-6412), and bovine albumin (A-7030) were obtained from Sigma Chemical Co. and used without further purification. The fluorescent anionic lipid analog 5-*N*-(octadecanoyl)aminofluorescein (ODAF) was obtained from Molecular Probes (O-322).

Isolation of lung surface active material (LSAM)

Isolation of porcine LSAM (sample 1; LSAM-1)

Porcine lung from freshly killed animals was lavaged with 0.154 M NaCl solution (~0.2 ml/g lung). The procedure was repeated three times. The washings from three or four lungs were collected and pooled (~2400 ml) and kept on ice. Cell debris was removed by centrifugation (220 × *g* for 10 min). The supernatant was carefully removed and centrifuged again (16,000 × *g* for 60 min). The resulting pellet was resuspended in double-distilled water (15 ml), lyophilized, and stored at −20°C. Surface tension measurements (γ-t and γ-c) showed that this sample was highly surface active at the air/water interface and is referred to as crude highly surface active lung surfactant (Hall et al., 1992). This sample mainly consists of a mixture of (phospho)lipids and proteins. Polyacrylamide gel electrophoresis of the protein fraction showed that this sample consisted of specific lung surfactant proteins plus a large amount of non-lung surfactant proteins (Fig. 1).

Isolation of porcine LSAM (sample 2; LSAM-2)

The LSAM-2 sample was isolated by a modification of the method of Ng et al. (1983). Briefly, LSAM-1 was resuspended in buffer 1 (0.154 M NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 7.5) and overlayed on a discontinuous gradient consisting of 3.0 ml of 0.68 M sucrose and 3.0 ml of 0.25 M sucrose. The gradient was centrifuged (78,000 × *g* for 60 min) at 4°C. The white layers at the 0.25 M/0.68 M sucrose interface were collected, diluted with 3 volumes of buffer 1, and centrifuged (48,000 × *g* for 30 min). The resulting pellet was resuspended in double-distilled water and lyophilized. This sample is purified in respect to the non-lung surfactant proteins in LSAM-1 sample. Polyacrlyamide gel electrophoresis revealed that the LSAM-2 sample consisted mainly of specific lung surfactant proteins and much lower amounts of non-lung surfactant proteins compared to LSAM-1 (Fig. 1). Thus, LSAM-1 and LSAM-2 differ in their composition, mainly with respect to their protein content and the ratio of protein to total phospholipids. The latter ratio was higher in LSAM-1 (~1.6) than in LSAM-2 (0.5).

Isolation of hydrophobic fractions of lung surfactant

Hydrophobic fractions of lung surfactant (HFLSs) were isolated from LSAM-1 and LSAM-2 by using organic solvents as follows.

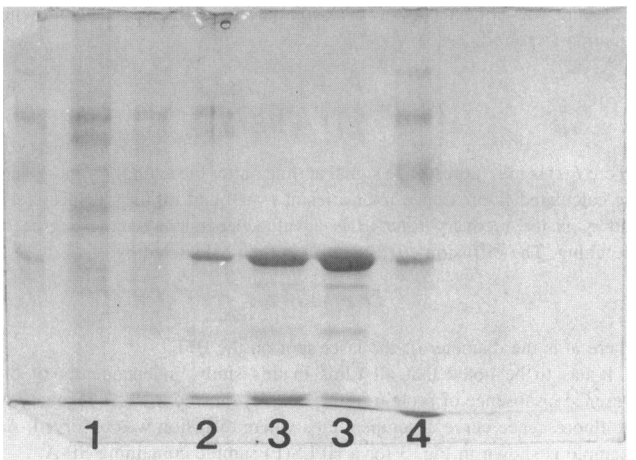

FIGURE 1 Electrophoretic analysis of protein content of LSAM-1 (*Lane 1*), LSAM-2 (*Lane 2*), and bound fraction after Con-A Sepharose chromatography (*Lane 3*). (*Lane 4*) Molecular weight markers. Proteins were analyzed by SDS-PAGE and stained with Coomassie brilliant blue. Molecular mass of markers (kDa): 29, 45, 67, 92.

HFLS(F) was prepared by extraction of LSAM-1 with chloroform/methanol/saline according to the method of Folch et al. (1957). The organic phase was gently removed and evaporated under nitrogen. The solid film in the tube was resuspended in double-distilled water and lyophilized. The resulting fraction was highly surface active and consisted of (phospho)lipids and the chloroform-soluble specific lung surfactant apoproteins SP-B and SP-C.

HFLS(B) was prepared by extraction of LSAM-1 according to the method of Bligh and Dyer (1959). After extraction, the organic phase was treated in the same manner as HFLS(F). HFLS(B) also contained (phospho)lipids, but with a profile different from that of HFLS(F) and the specific surfactant apoproteins SP-B and SP-C.

HFLS(C) was prepared by the extraction of LSAM-2 using a modification of the method of Cham and Knowles (1976). Briefly, 1-ml aliquots of LSAM-2 were extracted with 9.6 ml isopropyl ether, 6.4 ml tertiary butanol, and 5 ml water. The mixture was shaken vigorously and left at room temperature for 30 min with shaking every 5 min. The mixture was then centrifuged (1000 × *g* for 10 min). The upper organic phase was collected and evaporated under nitrogen. The dry residue was resuspended in distilled water and lyophilized. HFLS(C) had no detectable protein content, i.e., it is an apoprotein-free fraction (Liau and Ryan, 1988) consisting of the same (phospho)lipids but with a profile different from that of HFLS(F) and (B).

The lipid profiles of the three HFLS preparations differed as detected by thin-layer chromatography (Fig. 2). The lower phase left in the tube after extraction of HFLS(C) was used for isolation of the hydrophilic lung surfactant protein SP-A.

Isolation and purification of surfactant protein A

The aqueous phase after isopropyl ether/butanol extraction was mixed with 5 volumes of 100% ethanol at −20°C and left at 4°C overnight. It was subsequently centrifuged (18,000 × *g* for 30 min), and the pellet was resuspended in buffer 2 (0.02 M Tris-HCl, 0.5 M NaCl, pH 7.5) in the presence of 0.5% SDS and applied to a Con-A Sepharose column for chromatography. The column was washed with 10 volumes of buffer 2, and the bound material was collected after elution with 1 M methyl-D-mannoside in buffer 2. Ethanol (5 ml at −20°C) was added to the collected bound fraction, and the protein was allowed to precipitate overnight at 4°C. The protein precipitate was centrifuged (12,000 × *g* for 20 min) at 4°C, resuspended in water, and lyophilized. A SDS-polyacrylamide electrophoresis gel of LSAM samples and the protein fraction bound and subsequently eluted from the Con-A column is shown in Fig. 1. The mobility of the major protein band eluted from the Con-A column is consistent with that of the specific hydrophilic lung surfactant protein SP-A (M_r = 28–32 kDa) and is significantly purified compared to that in LSAM samples, although high loadings of the SP-A sample reveal the presence of some minor low-molecular-weight protein contaminants.

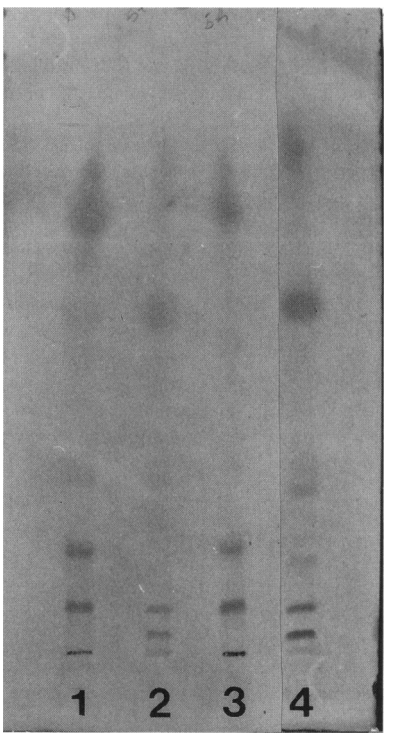

FIGURE 2 A thin-layer chromatogram of different hydrophobic fractions of lung surfactant. (*Lane 1*) HFLS (F); (*Lane 2*) standards; (*Lane 3*) HFLS (B); (*Lane 4*) HFLS (C).

Electrophoresis of surfactant proteins

SDS-polyacrylamide gel (SDS-PAGE) electrophoresis was performed on protein fractions according to the method of Laemmli (1970). Gels were stained with Coomassie brilliant blue 250.

Phospholipid profile of HFLS samples

Thin-layer chromatography of phospholipids was performed as previously described (Tonchstone et al., 1980), using silica gel plates (10 × 20 cm) obtained from Merck. Phospholipid spots were visualized by mineralization with $(NH4)_2SO_4$ and scraped into test tubes. Both the total and individual phospholipids were quantified by a lipid phosphorus assay (Kahovkova and Odavic, 1969).

Foam film formation

The foam films were formed in a glass annulus (0.4-cm internal diameter; 0.4 cm high) according to the method of Scheludko and Exerowa (1959). The annulus was housed in a specially constructed chamber suitable for the FRAP measurements, and reproducible FF formation in this apparatus was described in previous papers (Lalchev et al., 1994, 1995a,b). The temperature of the chamber was controlled through connection to a thermostated circulating water bath. The temperature in the chamber was monitored

using a thermometer located in a socket in the housing. The precision was ±0.1°C.

Preparation of the lipid/protein dispersions in distilled water (for thick FFs) or in the presence of different electrolyte (NaCl) concentrations (up to 0.1 M NaCl for CBF and 1.0 M NaCl for NBF) and the incorporation of the fluorescent label (ODAF) for FRAP measurements were as described previously (Clark et al., 1990b). Briefly, the lipid or lipid fraction was dispersed in water containing NaCl and vortexed (3 × 10 s). Complete hydration of the lipid was achieved by heating the dispersion to 35°C (for HFLS) or to 45°C (for DPPC) for 1 h before experiments were initiated. ODAF was incorporated into the lipid dispersions in the following way. Approximately 1 mg of the probe was allowed to dissolve in 100 μl of ethanol. This was made up to 1 ml distilled water. The concentration was then assayed using a Perkin-Elmer λ-9 spectrophotometer and application of a molar extinction coefficient of 87,000 M^{-1} cm^{-1} at 496 nm. The probe solution was added to the sample to make a final probe concentration of 1 μM. The volume of the added probe solution was never greater than 1% of the sample solution.

Film thickness measurement

The FF thicknesses were determined from the intensity of reflected light from both film surfaces using an interferometric apparatus described in several papers (e.g. Scheludko, 1967; Clark et al., 1990a). The equivalent water thickness was calculated using the triple-layer model, with the conventional assumption that the film is optically homogeneous with a refractive index equal to that of the aqueous dispersion from which the film was formed.

Measurement of surface lateral diffusion

The surface lateral diffusion of the fluorescent lipid analog ODAF in the adsorbed layer of black foam films (BFFs) was measured by the FRAP technique with an apparatus described previously (Lalchev et al., 1994, 1995b; Clark et al., 1990a,b). The method of beam modulation used involved the attenuation of the beam by reflection from glass flats. The split bleach and monitoring beams were carefully recombined at the sample. Exposure of the sample to the bleach beam was achieved using a computer-controlled electronic shutter (UniBlitz SD 1000). The $1/e^2$ diameter of the Gaussian profile spot at the sample was 2.85 μm. The intensity of the attenuated laser beam was adjusted such that it was below the bleaching threshold of the fluorescent probe. The duration of the bleach pulse was kept below 10% of the recovery time of the sample under test. These measures ensured that the recovery time was not artificially prolonged by overbleaching the sample. The fluorescence recovery curve after bleaching was collected by a microcomputer and fitted to the following equation (Axelrod, 1985):

$$F(t) = \frac{F_0 + (F_\infty\, t/\beta\tau_d)}{(1 + t/\beta\tau_d)},$$

where $F(t)$ is the fluorescence signal at time t after bleaching. F_0 and F_∞ are the calculated fluorescence intensities at $t = 0$ and infinity, respectively, and τ_d is the recovery time. β is a value dependent on the degree of bleaching. The diffusion coefficient D is then calculated by

$$D = \omega^2/4\tau_d,$$

where ω is the diameter of the laser spot on the BFF.

It has to be noted that all films in this study, independently of the presence or absence of protein in the sample, showed a 100% recovery of the fluorescence curve at temperatures where diffusion was observed. An example is shown in Fig. 3 for a HFLS(F) sample containing SP-A.

Capillary pressure determination

FFs formed in the thin-film apparatus from two parallel monolayers that sandwich a liquid core. The circular FF is suspended in the center of the annulus and is connected to the annulus by peripheral menisci (the Plateau border). In this model FF system, the capillary pressure (P_c) is the driving force for the spontaneous drainage of the bulk liquid from the core toward the Plateau border, as it constitutes the pressure difference between the bulk (liquid) and air phases. When the film is at equilibrium, the sum of the components of the disjoining pressure (mainly electrostatic and van der Waals) in the FF is equal to the P_c (Deryaguin et al., 1985; Exerowa et al., 1987; Platikanov et al., 1971). The capillary pressure can be calculated from the equation $P_c = 2\gamma/r$ (mN/m^2), where γ is the surface tension at the air/water interface (that is measured independently) and r is the radius of curvature of the surface, which in the model system used here, corresponds to the radius of the glass annulus used to support the FF. Alternatively, the capillary pressure can be measured in situ with the sample in the annulus (Exerowa et al., 1979). The P_c (usually expressed in the FF model system in dyn/cm^2 or N/m^2) can be transformed to pressure in centimetres in a water column (cm H_2O), which are the conventional units used in physiological studies of lung function. This is achieved by multiplying P_c in the FF by a factor of 10^{-3} or 10^{-2}, respectively. Our experiments in this study were performed with a P_c of 300 dyn/cm^2 = 30 N/m^2 = 0.3 cm H_2O. This P_c was chosen because it relates directly to that which exists in vivo in the lung during the respiration/expiration cycle (e.g., Scarpelli and Mautone, 1994). The FFs in the model system can be subjected to the entire range of driving pressures that are realized in the alveolus during the respiratory

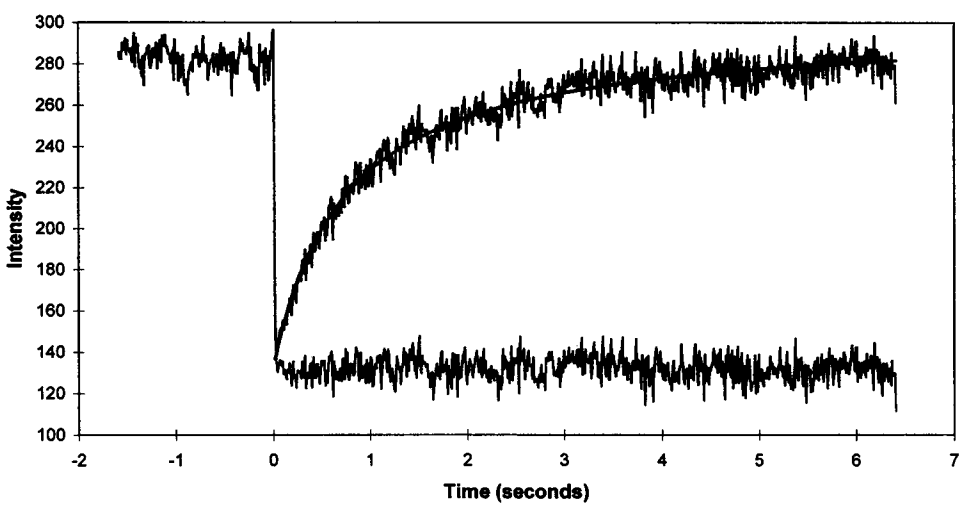

FIGURE 3 A typical FRAP curve showing 100% recovery of the fluorescence for a HFLS(F) sample containing SP-A. Radius of the film $r = 2 \times 10^{-2}$ cm. T = 50°C. Also shown is the least-squares fit to the equation for relating fluorescence intensity $F(t)$ and D, and the offset residuals for this fit.

cycle (~0-30 cm H_2O) and thus can accurately model the hydrodynamic properties of the alveolar surface layer. The model permits the P_c to be changed in three ways: by changing the surface tension by the surfactant concentration, by changing the radius of the glass annulus in which the foam film was formed, or by changing the air pressure inside the hermetically sealed chamber in which the FF was formed.

RESULTS AND DISCUSSION

The effect of film type on surface molecular diffusion in foam films of crude lung surface active materials (LSAM-1)

The surface lateral diffusion of adsorbed molecules in the plane of FFs stabilized by a crude preparation of lung surfactant (LSAM-1) was investigated. This is a novel measurement, and the absence of data in the literature describing surface mobility (flow or diffusion) in FFs of LSAM prompted an initial study of the role of the electrostatic interactions between the charged surfaces of FFs composed of lung surfactant lipids and proteins on the lateral molecular diffusion coefficient (D). The effect of electrostatic interactions on film thickness is well established and can be explained in terms of classical DLVO theory (Deryaguin and Landau, 1941; Verwey and Overbeek, 1948). The temperature dependence of D was investigated at low bulk electrolyte concentrations, where thick FFs were formed (appearing as gray in color by observation under reflected light) and, at elevated electrolyte concentrations up to 125 mM NaCl, where black (thin) FFs, referred to as common black films (CBFs), were formed.

The temperature dependence of D as a function of electrolyte concentration in LSAM-1 stabilized FFs is shown in Fig. 4. It is evident that at temperatures less than 30°C, the values of D observed were very low irrespective of film type. Indeed, inspection of the FRAP curves revealed zero recovery or anomalous behavior inconsistent with surface diffusion, which has previously been attributed to surface flow phenomena (Lalchev et al., 1994). As the temperature was increased to above 30°C, diffusion behavior was observed and D values increased. This effect was more pronounced in FFs formed from solutions made up in double-distilled water or in the presence of 5 mM NaCl, where D increased monotonically with temperature. According to DLVO theory, increasing the electrolyte concentration results in a shrinkage of the diffuse double layer between film surfaces and a subsequent decrease in film thickness. Observations of the FFs by microscopy under conditions of epi-illumination revealed that the thick films formed from samples in bidistilled water in the absence or presence of 5 mM NaCl appeared gray and dark gray in color, respectively. The measured electrical conductivities of the two dispersions (data not shown) were practically the same, obviously because of the high level of electrolyte "impurities" in the crude lung surfactant preparation LSAM-1. FFs formed from solutions containing 30 or 125 mM NaCl were common black, and the temperature dependences of D for these FFs differed significantly—both from each other and from that of the thick films (Fig. 4). Specifically, the increase in D was delayed in the sample containing 125 mM NaCl, and D in general showed a much reduced dependence on temperature compared to the 30 mM, 5 mM, and NaCl-free samples. (It should be noted that at physiological electrolyte concentration and 37°C, the LSAM-1 sample showed no diffusion.)

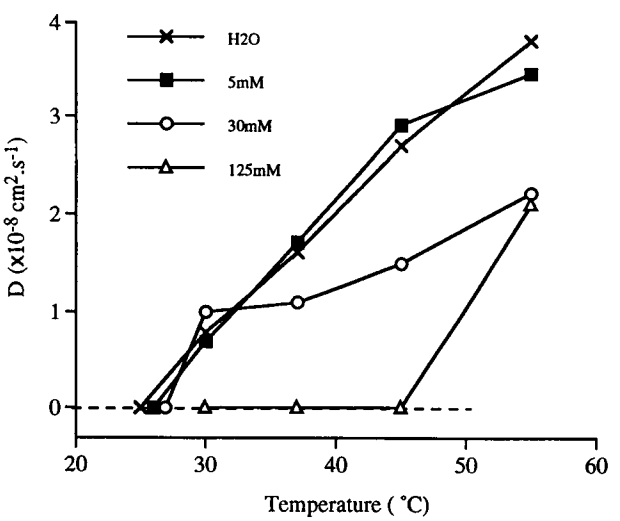

FIGURE 4 Temperature dependence of the diffusion coefficient (D) of surface-adsorbed 5-*N*-(octadecanoyl)aminofluorescein in foam films stabilized by LSAM-1. Solution conditions: 2.4 mg/ml LSAM in distilled water (×, gray films), 5 mM NaCl (■, dark gray films), 30 mM NaCl (○, CBFs), and 125 mM NaCl (△, CBFs). Capillary pressure, P_c = 0.3 cm H_2O. Radius of the film $r = 2 \times 10^{-2}$ cm.

These results show some agreement with but also some differences from our previously published data derived from a systematic study of the effect of FF thickness on surface diffusion in FFs stabilized by individual and mixtures of synthetic phospholipids (Lalchev et al., 1994, 1995b). First, in this study as with the previous studies, the magnitude of D was greatest in the thicker foam films (gray films) formed from LSAM-1 and decreased significantly under conditions where CBFs were formed. However, D values in black FFs stabilized by LSAM-1 in 30 and 125 mM NaCl were approximately one order of magnitude smaller than that measured in FFs of equivalent type formed from protein-free natural phospholipid mixtures or mixtures of synthetic phospholipids (Lalchev et al., 1995b). Indeed, FFs composed of mixtures of synthetic phospholipids (e.g., dimyristoylphosphatidylcholine (DMPC):dimyristoylphosphatidyl glycerol (DMPG), 9:1) in 150 mM NaCl gave values of D of approximately 1×10^{-7} cm^2/s at 30°C compared to complete immobility observed in the FFs formed from LSAM-1 in 125 mM NaCl. The origin of the reduced diffusion in the LSAM-1-stabilized FFs may be linked to the significant levels of non-lung surfactant protein present in this sample impeding lipid diffusion at the air/water interface of the FF. Surface diffusion in FFs stabilized by LSAM-2 samples, which contained a significantly reduced

level of non-lung surfactant proteins (Fig. 1, *Track 2*), was measured to test this hypothesis.

The effect of non-lung surfactant protein on surface molecular diffusion in foam films of lung surface active materials

The temperature dependence of D for the two different black film types (CBF and NBF) stabilized with LSAM-2 samples, which were relatively free of non-lung surfactant proteins (Fig. 1), is shown in Fig. 5. Measurable diffusion was detected above 30°C in both film types, but all D values in the temperature range studied were lower for the NBFs than the corresponding CBFs. We assume this effect to be due to the lack of a free liquid core between the interfaces of NBFs, as they are considered to be the thinnest (bilayer) foam film structures (e.g., Scheludko, 1967; Exerowa et al., 1987). This concurs with our earlier observations of D in CBFs and NBFs stabilized by pure synthetic phospholipids (Lalchev et al., 1994, 1995b).

Foam films stabilized by LSAM-2 and LSAM-1 are also compared in Fig. 5. First, considering the temperature dependence of D in CBFs formed from LSAM-1 (Fig. 4) and LSAM-2 at similar electrolyte concentrations (125 and 140 mM, respectively), we observe that the shapes of the two curves show some differences and that the onset of surface diffusion in the CBF of LSAM-2 is shifted down in temperature by 15–20°C. Second, the NBFs formed from LSAM-1 displayed no surface lateral diffusion in the temperature range studied. In contrast, NBFs of LSAM-2 show

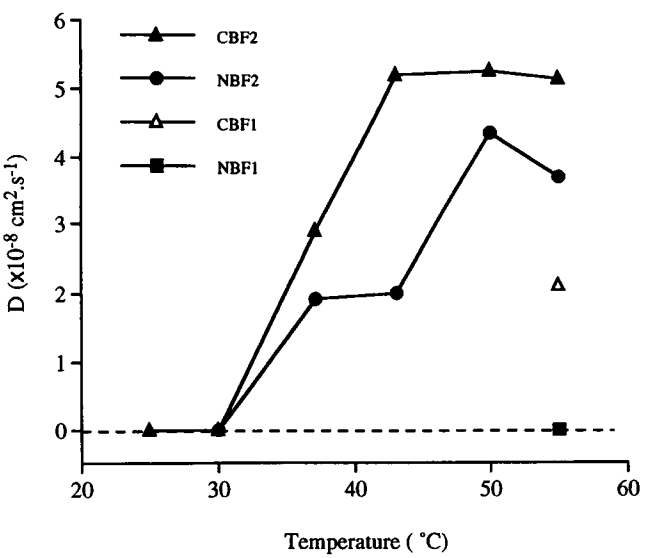

FIGURE 5 Temperature dependence of the diffusion coefficient (D) of surface-adsorbed 5-*N*-(octadecanoyl)aminofluorescein in black foam films stabilized by LSAM-2. Solution conditions: 1.0 mg/ml LSAM-2 in 0.14 M NaCl (▲) for common black foam film (CBF) and 0.5 mg/ml LSAM in 1.0 M NaCl (●) for Newton black foam film (NBF). Two points at 55°C for films of LSAM-1 are given for comparison: CBF (△) and NBF (■). Capillary pressure, P_c = 0.3 cm H_2O. Radius of the film $r = 2 \times 10^{-2}$ cm.

a surface diffusion of ODAF at temperatures above 30°C, reaching values between 1 and 4 × 10^{-8} cm^2/s. Keeping in mind that the protein/total phospholipid ratio in LSAM-1 was 1.6 and 0.5 in LSAM-2, we suspect that the non-lung surfactant proteins present in LSAM-1 are the components that cause a significant reduction in D in LSAM-1 samples in both CBFs and NBFs comparable to those in LSAM-2. So we conclude that the non-lung surfactant proteins act as "inhibitors" or "barriers" to molecular surface diffusion of ODAF in these FFs.

In summary, the results in Figs. 4 and 5 show that lateral molecular diffusion at the interfaces of both thick (gray) and thin (black) foam films composed of LSAM samples is observed at temperatures above 30°C. With increasing temperature, up to and above 37°C, D increases in different ways for LSAM-1 and LSAM-2, depending upon 1) the film characteristics as defined by ionic strength and its effect on FF thickness and 2) the presence of non-lung surfactant proteins in the former sample. The dependence on ionic strength (with respect to film thickness h) is clearly demonstrated for both LSAM-1 (Fig. 4) and LSAM-2 (Fig. 5) samples. As seen in Fig. 4, the thicker gray films have a higher D than the black films at $T > 30$°C, and there is no doubt that it is an effect of the decreased h of the black films upon D. For the sample LSAM-2, there was a decrease in measured h of some 4 nm in the CBFs as the ionic strength was increased from 30 mM to 125 mM NaCl (Table 1). In the presence of added albumin, this decrease in h increased to 8 nm. So we concluded that the different behavior of FFs with 30 and 125 mM NaCl (Fig. 4) was due to the decrease in film thickness. The decrease in h again explains the reduction in D, going from CBFs to NBFs for the LSAM-2 sample (Fig. 5). The comparison of results between equivalent film types of both samples (Fig. 5) shows that the increased content of non-lung surfactant proteins in LSAM-1 compared to LSAM-2 leads to lower D values across the temperature range 30–55°C. To check whether the effect is due to h changes, we measured the h of LSAM-2 alone and with the addition of albumin (as a representative non-lung surfactant protein) (Table 1). It can be seen in Table 1 that the addition of a non-lung surfactant protein leads to an increase in h that could result in an increase in D, i.e., LSAM-1 could have higher D than LSAM-2. Because the experimental results showed the opposite, we concluded that the decreased D in LSAM-1 compared to LSAM-2 samples was not due to the change in the film thickness and that non-lung surfactant proteins could decrease D by some other mechanism.

Furthermore, we assume that there is a relationship not only between the non-lung surfactant proteins and D, but also between the surfactant proteins and D. One reason for this arises from the comparison of data obtained from CBFs and NBFs stabilized by LSAM-2 with FFs of equivalent type stabilized by synthetic DMPC alone or by mixtures of DMPC:DMPG (9:1). The comparison shows that the CBFs of LSAM-2 samples, consisting of phospholipids and predominantly specific lung surfactant proteins, are character-

TABLE 1 Thickness *h* (nm, mean ± SD) and type of foam films stabilized by solutions of LSAM-2, HFLS, and DPPC:DMPG (7:3) mixture in distilled water and in water with different electrolyte (NaCl, mM) concentrations

Sample	Dist. H_2O	5 mM	30 mM	125 mM	500 mM
LSAM-2	36.6 ± 2.0 (gray film)	22.2 ± 1.1 (CBF)	20.6 ± 0.9 (CBF)	16.6 ± 0.9 (CBF)	16.8 ± 1.0 (CBF)
LSAM-2 + Alb (5:1 w/w)	42.5 ± 2.1 (gray film)	25.8 ± 1.2 (CBF)	24.8 ± 1.0 (CBF)	17.3 ± 1.1 16.9 ± 0.8# (CBF)	—
HFLS (B) (12%)*	29.7 ± 1.8 (gray film)	24.5 ± 0.9 (CBF)	25.8 ± 1.2 (CBF)	27.2 ± 1.0 (CBF)	23.1 ± 1.2 (CBF)
HLFS (F) (20%)*	88.3 ± 2.5 (gray film)	—	—	26.7 ± 0.7 (CBF)	21.7 ± 1.8 (CBF)
PC:PG (30%)*	84.8 ± 2.9 (gray film)	26.4 ± 1.0 (CBF)	25.5 ± 0.9 (CBF)	29.2 ± 1.2 (CBF)	28.1 ± 2.0 (CBF)

Concentration of the samples C = 1 mg/ml. T = 33°C.
*The numbers in the brackets show the percentage of charged lipids compared to the total lipid content in the sample.
#The value is obtained with 2:1 weight ratio of LSAM-2:albumin.

ized by D values that are 2–3-fold lower than those of CBFs of the phospholipids only (Lalchev et al., 1995b). This suggests that the specific lung surfactant proteins also influence the surface diffusion properties of the phospholipids. Another reason is the common presumption that this effect could derive both from the increased viscosity in the film surface (and liquid core) in the presence of proteins and from the influence of the proteins on the lipid phase state in the bulk phase and at the film interfaces. There are some reported data for the correlation between the surface phase transitions in FFs formed from phospholipids (e.g., Nikolova et al., 1994) and their bulk phase transitions measured by differential scanning calorimetry, but in the absence of proteins. Our previous studies by differential scanning calorimetry (DSC) are consistent with data from the literature, where bulk lipid (Tenchov, 1991) or lung surfactant (Dluhy et al., 1989; Tchoreloff et al., 1991) phase transitions were investigated by DSC. Although isolated fractions of the major phospholipids contained within the LSAM samples show sharp cooperative gel to liquid-crystalline transitions in the temperature range studied here (our DSC data, not shown here), the presence of specific lung surfactant proteins in the LSAM samples results in a broad temperature range transition from about 20°C to 39°C (Dluhy et al., 1989; Tchorelof et al., 1991). Therefore not only the non-lung surfactant proteins (which could be considered contamination of the LSAM-2 sample), but also the specific lung surfactant lipid-binding proteins could influence D in FFs, either by their effect on the phospholipid phase state or by increasing the viscosity in the film liquid core, i.e., the subphase of the film monolayers. Because the specific lung surfactant proteins represent a unique combination of highly hydrophillic (SP-A and -D) and highly hydrophobic (SP-B and -C) proteins (e.g., Kuroki and Voelker, 1994), it is important to study the effect of these two classes of proteins on the phospholipid diffusion in FFs. The next stage of our study was to investigate this possibility by examining the effect of specific lung surfactant proteins on the surface diffusion at the air/liquid interface of FFs stabilized by fractions of LSAM of differing protein content.

Thickness and molecular mobility in foam films composed of extracted hydrophobic fractions of lung surfactant

Three widely studied, compositionally distinct hydrophobic fractions extracted by established methods (see Materials and Methods section) were investigated 1) to determine whether the fractions as a whole differed in their surface diffusion properties and 2) to estimate the contribution of the individual surfactant ingredients to the surface lateral diffusion properties in FFs of these fractions. Comparison of composition and properties may help in our understanding of the contribution of the various components to molecular surface diffusion in these extremely complicated lipid-protein lung surfactant complexes. Such information could help to clarify the mechanism(s) of the effect of individual components (or fractions) relevant to lung surfactant function in vivo.

The fractions used were extracted with organic solvent systems of either chloroform:methanol (HFLS(F) and HFLS(B) fractions) or butanol:isopropyl ether (HFLS(C) fraction). The average phospholipid profile from thin-layer chromatography experiments for the three fractions is presented in Table 2. It has been reported that the latter solvent system allowed isolation of the lipids to the complete exclusion of detectable protein, i.e., HFLS(C) is a hydrophobic surfactant apoprotein-free fraction (Bates et al., 1992; Liau and Ryan, 1988). In contrast, it is well known that the chloroform extracts of the lung surfactant contain up to 1–2 wt% of specific hydrophobic surfactant apoproteins SP-B and -C (e.g., Revak et al., 1988). The in vitro surface properties of the latter and the correlation with their function in vivo in the lung, especially their capacity to enhance the phospholipid adsorption and spreading at the air/liquid interface, have been the subject of many investigations by

TABLE 2 Phospholipid profile in hydrophobic fractions of lung surfactant (HFLS)

HFLS*	Sm	PC	PS	PE	PG	DPG
(F)	4.0	71.0	4.5	4.5	11.0	4.6
(B)	1.8	82.0	1.8	1.5	8.2	1.8
(C)	2.9	78.1	2.0	0.4	12.4	3.2

*(F), Extraction according to the method of Folch; (B), extraction according to the method of Bligh and Dyer, which both employ chloroform/methanol/saline; (C), extraction according to the method of Cham and Knowles, which employs isopropyl ether/tertiary butanol/water.
Sm, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol.

different methods and have been debated intensively (e.g., Hall et al., 1992).

The influence of electrolyte concentration on the thickness of FFs formed from HFLS(F), HFLC(B), and a mixture of DPPC/DMPG was investigated. Regarding the HFLC(F) sample, it can be seen that (Table 1) in the absence of added electrolyte, samples of HFLS(F) in distilled H_2O produced thick equilibrium films with a characteristic thickness h = 88.3 nm. The presence of 140 mM NaCl resulted in drainage of the films to the common black state, with characteristic equivalent water thicknesses of 26.7 nm. Elevating the electrolyte concentration still further to 500 mM NaCl, which is above physiological levels, resulted in slightly thinner films that were 21.7 nm thick at equilibrium. The HFLC(B) sample showed much thinner h in distilled water (29.7 nm) than the (F) sample (88.3 nm), but above the physiological electrolyte concentration the thicknessses showed no statistically significant differences. The difference between the thicknesses of the thick films could be attributed to the lower content of charged lipids in the HFLC(B) sample (12%) than in the (F) sample (20%). This interpretation was confirmed with the measured h of the model mixture DPPC/DMPG (30% charged lipid), which showed h = 84.8 nm. The results of our investigation of film thicknesses of HFLC(F), (B), and PC:PG mixture showed that only at low ionic strength did the content of charged lipid in the samples give rise to statistically significant differences in the film thicknesses. Above C_{el} = 5 mM, all films were black and the thicknesses remained essentially the same (Table 1).

Having established that under physiological conditions of electrolyte concentration FFs of HFLS were of the CBF type of equivalent thickness, we extended the surface characterization of these fractions by measuring the surface molecular diffusion in CBFs by the FRAP technique. Surface diffusion data for HFLS(F), (B), and (C) fractions as a function of temperature are shown in Fig. 6. The CBFs formed from HFLS(C) yielded the lowest D values in the temperature range 35–60°C of all the HFLS samples. The D values from HFLS(B) showed behavior broadly similar to that of HFLS(C), but the values were consistently slightly higher at any given temperature. In addition, diffusion in FFs of HFLS(B) was first measurable at 30°C, which was significantly lower than the HFLS(C) sample. The data from the HFLS(F) sample were clearly distinguishable from the other two samples, as it showed a much greater temperature dependence, yielding D values 2–3 times higher at 50°C.

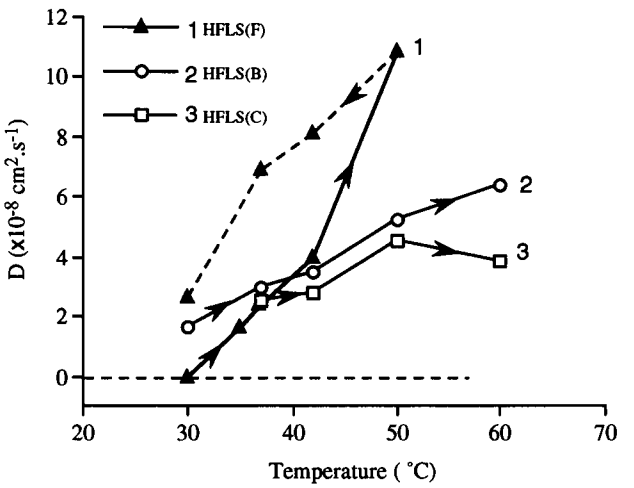

FIGURE 6 Temperature dependence of the diffusion coefficient (D) of surface-adsorbed 5-*N*-(octadecanoyl)aminofluorescein in black foam films stabilized by different hydrophobic fractions of lung surfactant (HFLS). Solution conditions: 1.0 mg/ml HFLS in 0.14 M NaCl. Curve 1, common black film (CBF) of fraction HFLS(F); curve 2, CBF of fraction HFLS(B); curve 3, CBF of fraction HFLS(C). For extraction conditions see Materials and Methods. Capillary pressure, P_c = 0.3 cm H_2O. Radius of the film r = 2×10^{-2} cm. ——, heating; – – –, cooling.

The explanation of the differences in surface diffusion properties of these samples, forming CBFs of equivalent thickness, must be based on their differing compositions. In our view two factors are key. First, our previous systematic study of mixtures of lipids that were present in the HFLC fractions showed that the ratio of negatively charged lipid to zwitterionic lipid significantly influences surface diffusion properties (Lalchev et al., 1994, 1995b). Our findings revealed that D increased markedly with increasing content of negatively charged lipid. Indeed, it was only in the presence of negatively charged lipid that surface diffusion in FFs was observed in the gel state. The percentage of negatively charged lipid varied between the HFLS fractions (Table 2), with HFLS(F), (B), and (C) containing 20%, 12%, and 17%, respectively. So the differences in the surface diffusion properties within the fractions could be due to the different proportions of negatively charged lipid within them. Second, in contrast to HFLS(C), HFLS(F) and (B) samples are known to contain 1–2 wt% of the hydrophobic lung surfac-

tant proteins SP-B and SP-C. Our hypothesis is that the different diffusion behaviors observed in CBFs of the HFLS samples were not due to the slight film thickness changes (Table 1) and can be explained by contributions from charged lipids and hydrophobic SP-B,C.

The temperature dependence of D in CBFs of HFLS(F) and HFLS(B) shows (Fig. 6) good agreement with the established effect of negatively charged lipids on D (Lalchev et al. 1994, 1995b), because the HFLS(F) sample contains 20% negatively charged lipids and shows a much greater temperature dependence than the HFLS(B) sample, which contains 12% negatively charged lipids. Invoking the effect of negatively charged lipids, we would predict that the D-T curve for the HFLS(C) sample, consisting of 17% charged lipid, should lie between that of HFLS(F) and (B). However, the results show that the D-T curve for HFLS(C) is very similar to that of HFLS(B) up to 50°C. On the other hand, the first measurable diffusion for HFLS(C) is at 37°C, which is significantly higher than that of the (B) sample (30°C). The latter effect could be connected with the absence of hydrophobic lung surfactant proteins in the former sample. It is therefore compelling to propose that the presence of SP-B and -C in lung surfactant could facilitate the surface diffusion in FFs at low temperatures by a mechanism of lowering the phase transition temperature of the lung surfactant complex. Such a hypothesis would be consistent with the proven effect of hydrophobic surfactant apoproteins enhancing the rate of spreading and adsorption of phospholipid molecules (mainly DPPC) at the air/liquid interface (e.g., Hall et al., 1992).

The heating-cooling curve obtained from a single temperature cycle of HFLS(F) is also shown in Fig. 6, and it can be seen that the cooling curve lies above the heating curve. Identical hysteresis behavior was observed in our systematic studies of synthetic phospholipids including DMPC, DPPC, and DPPA (Lalchev et al., 1995b). In contrast, the heating-cooling behavior of HFLS(B) and (C) samples differed with immediate abolition of surface diffusion at initiation of the cooling phase, where the temperature was reduced from 60°C to 50°C and finally resulted in complete surface immobilization. This behavior was consistent with the appearance of surface heterogeneity in these FFs, which was confirmed by epi-illumination microscopy, revealing that many aggregates had appeared in the film surfaces. Within the limits of our study, the latter effect could only be connected with the lower content of charged lipids in HFLS(B) and (C) compared to HFLS(F), but a more detailed study is required.

Molecular mobility in foam films composed of DPPC, HFLS, and reconstituted lung surfactant samples

FFs stabilized by HFLS in the absence and presence of SP-A were studied to elucidate the role of this hydrophillic lung surfactant-specific protein. The temperature dependence of D in CBFs stabilized by the major phospholipid component of HFLS(F), DPPC (*curve 3*); the same with added SP-A (*curve 4*) or albumin (symbol X), HFLS (F) alone (*curve 1*); and reconstituted lung surfactant, composed of SP-A added to HFLS(F) (*curve 2*), is shown in Fig. 7. D in the DPPC films remains at the immobile level up to 42°C, and higher temperatures (up to 75°C) only induce small increases in D. The first measurable diffusion in these films was detected at 43°C, slightly above the DPPC phase transition temperature. The addition of albumin (1:5 w/w) decreased the value of D by ~40% (symbol X). The addition of SP-A to DPPC dispersion (1:3 w/w) resulted in immobilization at 45°C and a decrease in D by ~50% at 65°C (curve 4). In contrast, in HFLS(F) FFs, D is much higher. The higher D associated with the HFLS(F) films compared to those of DPPC could be explained by a number of effects: 1) the presence of other phospholipids in addition to DPPC in HFLS(F), some of which are charged or have shorter acyl chain lengths and/or head group size, unsaturated chains, etc.; 2) a shift in the phase transition temperature of HFLS(F) sample to a lower temperature (due to the presence of the above-mentioned phospholipid species) with resultant fluidization of the film surfaces. The films of mixtures of HFLS(F) and SP-A are characterized by D values lower than that of HFLS(F) alone but higher than that of DPPC (*curve 2* in Fig. 7). This effect could be attributed to the association of the large, lipid-binding SP-A molecules with adsorbed lipid at the interface, resulting in reduced lateral diffusion. It is important to note that FFs formed

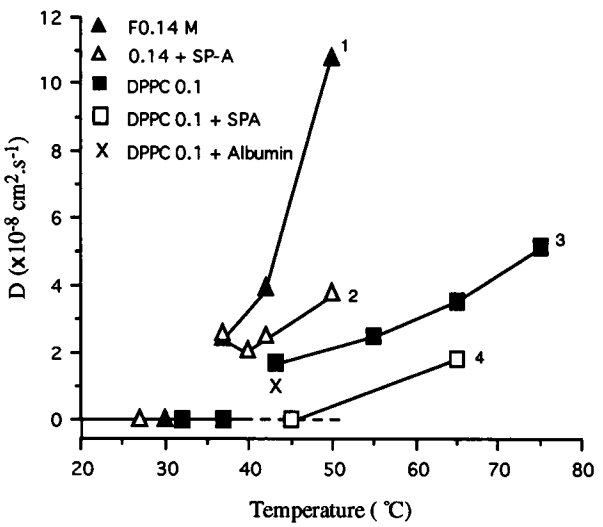

FIGURE 7 Temperature dependence of the diffusion coefficient (D) of surface-adsorbed 5-*N*-(octadecanoyl)aminofluorescein in black foam films stabilized by hydrophobic fraction of lung surfactant alone (curve 1) and in the presence of SP-A (curve 2). Curve 1, 0.8 mg/ml HFLS(F) in 0.14 M NaCl solution. Curve 2, curve 1 plus SP-A (3:1 w/w). Curve 3, as the main component of HFLS, DPPC in 0.125 M NaCl solution. A single experiment was done with albumin added (×) to DPPC (1:5 w/w) in 0.125 M NaCl solution. Curve 4, as curve 3 plus SP-A (3:1 w/w). All samples formed CBFs. Capillary pressure, P_c = 0.3 cm H_2O. Radius of the film $r = 2 \times 10^{-2}$ cm.

from mixtures of HFLS and SP-A gave D values similar to those observed with LSAM-1 and LSAM-2, from which HFLS and SP-A were extracted (compare curve 2 in Fig. 7 with the curves for the CBFs in Figs. 4 and 5). We conclude therefore that the surface diffusion characteristics of LSAM are governed by HFLS and SP-A, and that LSAM can be reconstituted in terms of its surface diffusion properties by mixing these components.

In summary, the effects of the specific hydrophillic SP-A and hydrophobic fraction of lung surfactant, consisting of surfactant lipids and hydrophobic proteins SP-B, C, on the lateral diffusion in pure DPPC FFs are different. The hydrophilic protein SP-A reduces D in both HFLS(F) and DPPC stabilized films. In contrast, the constituents in chloroform-soluble HFLS samples lead to an enhancement of D compared to that of pure DPPC foam films; this may be due to the presence of charged lipids in them. Indeed, the possible effects of both hydrophobic SP-B, C on surface diffusion and hydrophylic SP-A (at physiological concentrations), must be investigated in detail by using simple phospholipid mixtures or pure phospholipids with further separated samples of SP-A, SP-B and SP-C, which will be the subject of our further studies.

CONCLUDING REMARKS

Because FFs and structures of the foam film type are found in vivo and likely contribute to the anticollapse forces in the alveoli, their physical properties, particularly surface diffusion properties, have been investigated. Our focus in this study has been the mobility of the lung surfactant molecules in the plane of different foam films, which must play an essential role in two processes in vivo—the alveolar stabilization, due to the molecular interactions normal to the plane of the film monolayers, and the regulation of surface tension during respiration. Because FFs of different thicknesses could be realized in the lung, we studied FFs of thickness h from ~90 to 20 nm. Because D is dependent on h, we first proved that at physiological C_{el} all films studied were CBFs with equivalent thickness. Furthermore, for the CBF model we have estimated the contributions of the different components and fractions of lung surfactant to the surface diffusion.

Our results show that the surface diffusion coefficient in FFs stabilized by lung surfactant sample (LSAM-2 or its fraction HFLS(F)) is much higher than that in FFs stabilized by the principal lipid component, DPPC. This effect appears to be due to contributions from minor charged lipid components with lower transition temperatures and not to the effect of film thickness. In contrast, the hydrophilic protein SP-A and non-lung surfactant proteins reduced the magnitude of D. The role of charged surfactant phospholipids in enhancing the lateral diffusion in FFs stabilized by HFLS was clearly established. This confirmed the same effect of the charged synthetic phospholipids as previously shown (Lalchev et al., 1994). However, it was impossible in this study to separate the effect of charged lipids from that of hydrophobic SP-B, C on the lateral diffusion, especially their possible potential to decrease the temperature where measurable diffusion was first observed.

We emphasize that the configuration, thickness, size, capillary pressures, etc. of the in vitro formed foam films, stabilized by lung surfactant samples and/or their fractions, support their use as realistic structural analogs of the surface films that exist in vivo in the lung, which opens up new opportunities for the investigation of lung surfactant. The foam film model offers new opportunities compared to other models currently used (e.g., Boyle and Mautone, 1982; Enhorning, 1977; Hawgood and Clements, 1990; Schurch et al., 1989) for the simulation of the properties and functions of alveolar surface films. In contrast to the above models (films), the formation of stable black foam films involves not only the lateral molecular interactions of the surfactant constituents, but also the interactions normal to the film surfaces, which more realistically reflect the molecular interactions in in vivo surface films. In addition, because the orientation of the phospholipid molecules in black foam films is opposite that in BLMs (black lipid membranes) and these films have similar thicknesses (especially NBFs) as BLMs, the NBF system allows the dependence of the molecular dynamics (e.g., Beck and Peters, 1985) on molecular orientation to be studied and comparison with other membrane systems (e.g., lipid monolayers, liposomes, BLMs, etc.) to be made.

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