How to Control the Molecular Architecture of a Monolayer of Proteins Supported by a Lipid Bilayer

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ABSTRACT In this work, we report the spontaneous formation of a new structure composed of two lipid layers surrounding a dense monolayer of soluble proteins (lysozyme). We extend a process, initially discovered with nonionic surfactants to phospholipids (DMPC and DOPC). The motor of the protein insertion process is the difference between the protein chemical potential in the solution and in the freshly formed Newton black film (NBF). This process is completely controlled by adjusting the protein chemical potential in the solution. By means of x-ray reflectivity, we follow the evolution of the freestanding sandwich structure until a stable equilibrium state is reached. Depending on the lipid concentration with respect to the protein concentration, we observe two different behaviors of the film leading to the formation of such unique structure: at the highest lipid concentration, the usual protein diffusion into the NBF, and, at the lowest lipid concentration, the spontaneous formation of a sandwich structure immediately obtained after the drainage. Finally, we show that the insertion process is reversible, because, if the lipid concentration varies in the bulk solution, a "deswelling" of the film can be observed.

INTRODUCTION

A black film is the final stage of the thinning of a film when drawn from a solution of a surfactant. The film is so thin that visible light reflected at each of the interfaces interferes destructively and the nearly complete lack of reflection gives a black appearance. Depending on the surfactant solution composition, two different types of black films can exist: Common black films (CBF) and Newton black films (NBF) (Mysels et al., 1959). CBF have a rather large equilibrium thickness, well described by the colloid stability theory. The NBF thickness is much thinner and its equilibrium is governed by microscopic interactions at short distance (Mysels et al., 1959). A few years ago, using x-ray reflectivity, we determined the structure of the NBF (Bélorgey and Benattar, 1991). Our experimental approach was based on the fact that the x-ray wavelength is of the order of magnitude of the film thickness, and that constructive interference can occur. We have shown that the NBF is a very reproducible system composed of two opposite molecular walls separated by a central core, which is reduced to an ultimate hydration layer of the polar heads and a weak roughness limited to the sole capillary waves (3.2 Å). Thus, black films that are simple, freestanding bilayer systems involving most of the basic physical interactions appear to be very good models for the study of some specific interactions of biological membranes (Sentenac and Benattar, 1998). Few papers have reported attempts to form black films that include proteins, and these only concern microfilms of pure proteins (Musselwhite and Kitchener, 1967; Clark et al., 1990; Marinova et al., 1997; Postel and Abillon, 1998). Where microscopic CBF and NBF have been obtained, their structures were found to be bilayers of denatured proteins, more complex multilayer films, or thick films.

In the present work, we describe how it is possible to obtain a stable freestanding film, confining a single soluble protein layer within a phospholipid bilayer, and we also evidence the relevant parameters to control such a process. It should be pointed out that this new method should allow the investigation of the protein/lipid interaction at a molecular level.

In a recent paper (Benattar et al., 1999), we have reported the basis of a generally applicable protein insertion method leading to the formation of a close-packed protein single layer within a freestanding surfactant bilayer in a controlled manner by adjusting the protein chemical potential in the solution. Using x-ray reflectivity, we observed a time-dependent insertion of bovine serum albumin (BSA) molecules between the two film layers, constrained mainly of hexaethylene glycol monododecyl ether ($C_{12}E_6$) molecules. The nonionic surfactant stabilizes the native form of the protein as observed for fatty acids at the air–water interface (Graham and Phillips, 1979a,b). We proposed a simple model by comparing the protein chemical potential in the solution at the Gibbs interface (air–water) and in the NBF. Before pulling the film, the Gibbs interface is in thermodynamical equilibrium with the bulk reservoir. This sets the concentration of the protein in the Gibbs film as a function of the bulk concentration C_{BSA}. At time $t = 0$, when the NBF is formed, the surface concentration of each of the two Gibbs layers constituting the NBF does not have time to change. Thus the initial protein surface fraction $\Phi_{NBF}(t = 0)$

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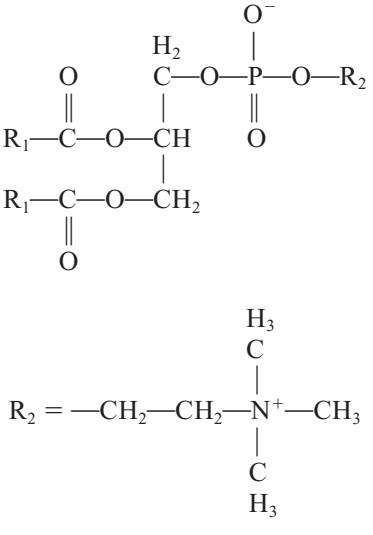
in the film is just $2\Phi_G$ (Φ_G is the protein surface fraction under the Gibbs film). This state is not in equilibrium and there is a protein diffusion toward the NBF (Fig. 1). At long enough times, a surface fraction Φ_{NBF}^{eq} is reached for which the chemical potential of the protein in the NBF equals that in the bulk. If the protein molecules are attracted to the Gibbs interface and if the attraction within the two surfactant walls is weak, then the final concentration in the NBF will be considerably larger than the initial concentration after the NBF formation. Under the experimental conditions of this previous investigation, we estimate that, at equilibrium, the NBF surface fraction should be 0.6 and that the diffusion coefficient is $D \approx$ a few 10^{-7} cm^2/s. This is enough to allow protein–protein interactions in the NBF. Thus, to enable the formation of this new sandwich structure made of two walls of surfactant and a protein single layer, the protein must be soluble, and it must have only slight interactions with the surfactant, which cannot denaturate it.

To extend the preliminary results obtained with the nonionic surfactant $C_{12}E_6$ and the soluble protein BSA, we used completely different systems involving only molecules of biological interest. We replaced BSA by a smaller and positively charged protein, lysozyme, and the surfactant is replaced by noncharged phospholipids.

MATERIALS AND METHODS

Two kinds of phospholipids were used to form freestanding bilayers, but most of the experiments were performed with L-α-phosphatidylcholine dimyristoyl (DMPC) and some complementary experiments with L-α-phosphatidylcholine dioleoyl (DOPC). Their general chemical formula is



For DMPC, R_1 is $CH_3(CH_2)_{12}$; and for DOPC, R_1 is $CH_3(CH_2)_7CH = CH(CH_2)_7$. The compounds were purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification. Both phospholipids have uncharged zwitterionic phosphatidylcholine head—groups (PC) and hydrophobic CH_2—tails with different lengths: DM = 14:0 and DO = 18:1(9). DOPC is an unsaturated phospholipid with double bound C═C at each hydrophobic tail. In the present work, we will mainly discuss the experimental results concerning DMPC; the other results concerning DOPC are very similar and thus will not be detailed. Liposomal dispersions of DMPC and DOPC in water were prepared by sonication procedure for 10 min and filtration with 0.22-μm filters. This preparation technique (Bangham Method) gives suspensions of small unilamellar vesicles (Szola and Papahadjopoulos, 1980).

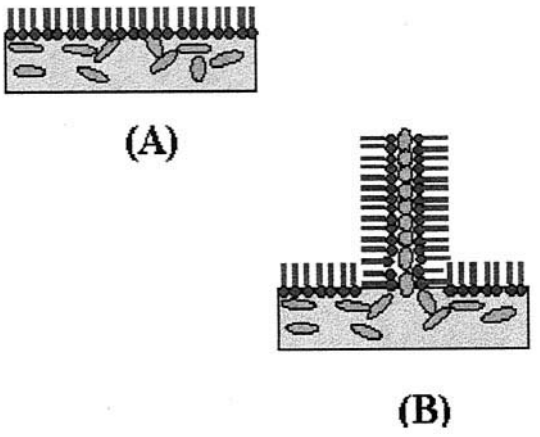


FIGURE 1 Two different equilibrium situations. (*A*) Organization of the air–solution interface before pulling the film. (*B*) When the NBF reaches equilibrium, the proteins form a dense and stable monolayer.

The lysozyme, which is a globular soluble protein, is obtained from chicken egg white and its isoelectric point is pH 11.4. It has a hard ellipsoidal structure whose dimensions are $30 \times 30 \times 45$ Å^3 and it is very stable against denaturation. For the experimental conditions (pH = 5.5 and T = 28°C without addition of salt), the lysozyme is highly positively charged (Sundaram and Stebe, 1997; Sundaram et al., 1998). It was purchased from Sigma and used without further purification.

The lysozyme was added to the liposomal solutions of the lipids. All experiments were performed at T = 28°C, which is well above the phase-transition temperatures of the phospholipids (T_c = 23.5°C for DMPC and T_c = 20°C for DOPC). All the solutions were prepared with deionized water (18.2 MΩ, milli-Q system), sonicated, and then filtered (0.22 μm). The respective surfactant and protein ranges of possible concentrations were found empirically after testing the film stability. The DMPC (and DOPC) solutions were analyzed by chromatography and showed to contain 1% of hydrolysis impurities instead of 0.5% in the solution before sonication.

Usually, in the absence of proteins, the lipid concentration for which large stable films can be obtained is C_{DMPC} = 0.5 mg/ml (Yamanaka et al., 1982; Cuvillier et al., 2000). We also used C_{DMPC} = 0.12 and 0.08 mg/ml, which does not allow the formation of stable NBF without protein. Only one DOPC concentration (C_{DOPC} = 0.08 mg/ml) is presented here. The films are drawn vertically from the solutions by lifting a metallic frame at a constant rate (Fig. 2). The experiment requires a long frame (4 cm) to allow grazing incidence, thus leading to a large film area (2 cm^2).

X-ray reflectivity method

The reflectivity experiment concerns the measurement of the ratio $R(\theta) = I(\theta)/I_0$ at various incidence angles θ. I_0 is the intensity of the incident beam, and $I(\theta)$ that reflected by the film (Fig. 2). The experiments were performed using a high-resolution reflectometer for vertical surfaces (OptiX from Nonius, The Netherlands). A copper tube is used as an x-ray source (λ = 1.5405 Å) and a small vertical slit (100 μm) ensures a low divergence (0.15 mrad). A horizontal slit (1.25 mm) limits the height of the illuminated area of the film. A reflectivity profile (Fig. 3) provides access to the

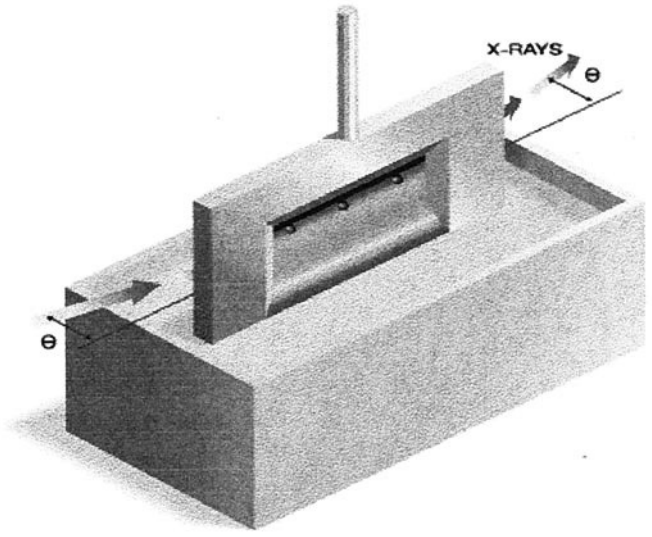


FIGURE 2 Schematic of the set-up installed at the center of the reflectometer. A metallic frame is immersed in the solution and then drawn up to form the film. During the drainage, the film shows interference colors and the NBF appears on the top of the frame.

electron density profile along the film normal. The film, being considered as a succession of homogeneous slabs, for each slab thickness, density and interfacial roughness can be derived from the experimental profile through the use of an optical formalism, taking into account the multiple reflections (Born and Wolf, 1984). The main advantage of freestanding films arises from their high electron density gradients at the interfaces. The reflectivity

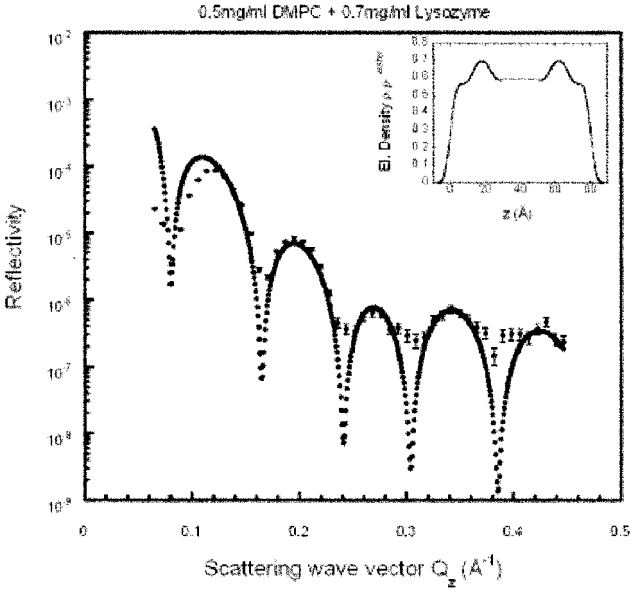


FIGURE 3 Typical x-ray reflectivity curve of a NBF (0.5 mg/ml DMPC with 0.7 mg/ml lysozyme at $t = 23$ h) the positions of the Kiessig fringes indicate the presence of proteins within the central core. The corresponding fit (*plain circles*) using the profile is represented in inset. The electron density profile (normalized by that of the water) is accounted for a five-layer model.

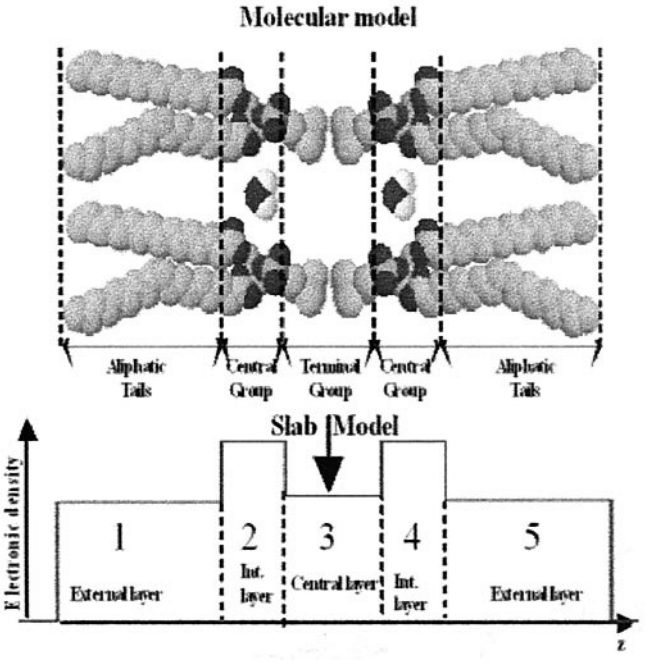


FIGURE 4 Electron density model of a phospholipid bilayer. Three regions of the bilayer can be separated: the aliphatic tails R_1, the central group, and the terminal part of the molecules R_2. They form a five-layer model, which, after taking into account the roughness, can be used to fit the reflectivity curve.

profiles display very strong "Kiessig fringes" that originate from the interference of the beams reflected on each side of the NBF (Bélorgey and Benattar, 1991; Benattar et al., 1999), and these provide an accurate determination of the overall film thickness (Fig. 3).

Data analysis

The films of phospholipids are described using a symmetrical five-box model (Fig. 4) as reported in detail in Cuvillier et al. (2000). This model combines several assumptions that are described here. We assume that the phospholipid molecule is in a quasi-extended state and could be divided into three regions of distinct electron densities. The first zone (*1*) corresponds to the hydrophobic tails of the lipids. Because the tails contain only C and H atoms, the electron density is relatively low. The second zone (*2*) accounts for the central part of the lipid, i.e., around the phosphate group. Due to the presence of several heavier atoms (mainly P and O), it has a higher electron density. Finally, the central box (*3*) accounts for the head group of the lipids and for any liquid water zone, as well as the presence of proteins, in some cases, of mixed films. The electron density is lower than in slabs (*2*) and (*4*) for the pure PC films. The density discontinuities between the different boxes were smoothened using an interfacial roughness. This roughness accounts both for the thermally excited collective motions and for the local disorder of the lipid molecules.

RESULTS

Our results show that, at a given protein concentration, there is a strong influence of the lipid concentration on the structure of the mixed films and on their behavior. Thus, our results will be separated into two parts according to the lipid

TABLE 1 Structural parameters of pure DMPC, DMPC with lysozyme, pure DOPC, and DOPC with lysozyme, resulting from a fit of the experimental profiles using a five-layer model

	h_1 (Å)	h_2 (Å)	h_3 (Å)	$\delta_1 \times 10^6$	$\delta_2 \times 10^6$	$\Delta\delta_3 \times 10^6$	σ (Å)	h_{total} (Å)
0.5 mg/ml DMPC, pure	14.58	11.4	6.6	2.6	3.8	2.7	3.4	58
0.5 mg/ml DMPC and 0.7 mg/ml lysozyme	13.13	10.0	33.83	2.0	2.41	2.0	3.0	80.09
0.08 mg/ml DMPC and 0.7 mg/ml lysozyme	14.58	10.0	31.28	2.0	2.75	2.3	3.0	80.44
0.5 mg/ml DOPC, pure	14.2	11.8	6.6	2.9	3.53	2.25	3.6	59
0.08 mg/ml DOPC and 3 mg/ml lysozyme	13.78	10.06	33.66	2.0	2.64	2.29	3.0	81.24

h_1, δ_1, h_2, δ_2, and h_3, δ_3 are the thickness and the real part of the refractive index of matter for x-rays, of the tails, the heads, and the central core, respectively. σ is the roughness of the film.

concentration. In addition, we will examine the effect of the time left to obtain the saturation of the Gibbs monolayer just before pulling the frame for the film formation.

High concentration of lipids

At a high lipid concentration (0.5 mg/ml DMPC), it is possible to obtain NBF made of the pure surfactant in absence of protein. The structure of such film is described in details in Cuvillier et al. (2000). The mixed films are drawn from a solution prepared as described above with the following concentrations: 0.5 mg/ml DMPC and 0.7 mg/ml lysozyme. The solution is left 3 h to equilibrate in the cell before drawing the films. This delay was necessary to saturate the space around the film with vapors and to obtain stable black films. One reflectivity curve recorded at equilibrium is reported in Fig. 3. It was fitted using a five-layer model for the film structure like that used for pure DMPC. The fit of the experimental curve is not fully satisfying because of some inhomogeneities of the thickness of this complex film structure.

The most important procedure for our purpose was to follow the time evolution of the total thickness of the film and the extra thickness (i.e., the difference between the thickness of the central part of the mixed film with that of the pure DMPC film, which corresponds to the mean value of the protein layer). The central core thickness is increased due to the presence of lysozyme. We have reported in Table 1 the structural parameters corresponding to the fit of the reflectivity profile of Fig. 3 (recorded after 23 h) They are compared to the corresponding parameters of DMPC film. One can notice that the roughness value (3.0 Å) remains as low as usually observed for the pure DMPC film surfaces, and that the insertion process does not significantly increase its value.

The whole set of experimental reflectivity profiles recorded at different times is reported in Fig. 5. It displays a clear shift of the Kiessig fringes toward smaller angles, which evidences the swelling of the initial film due to the protein insertion. This important result allows us to extend the swelling process previously observed in the particular system C12E6-BSA, to another different system of phospholipids and soluble proteins.

In Fig. 6, we have reported the time dependence of the central-core thickness due to the lysozyme insertion within the DMPC NBF. This dependence of the central-core thickness is obtained by fitting the whole set of curves using a five-layer model. The equilibration time (i.e., time required for the formation of a complete protein single layer inserted in the surfactant bilayer by diffusion) for BSA in the previous experiment (Benattar et al., 1999) was 48 h, whereas, in the present case with lysozyme, this time is reduced to only 11 h. The equilibration time for lysozyme is roughly four times smaller than for BSA. A very simple explanation of this value could be given by considering in the difference of the contact area of the BSA and lysozyme with the surfactant walls (for expected similar value of the interaction between the proteins and the surfactants). For BSA, which has an ellipsoidal shape of dimensions 140.9 by 41.6 Å, the contact area is ~4 times higher than that of lysozyme (whose dimensions are 30 by 45 Å) and thus the BSA

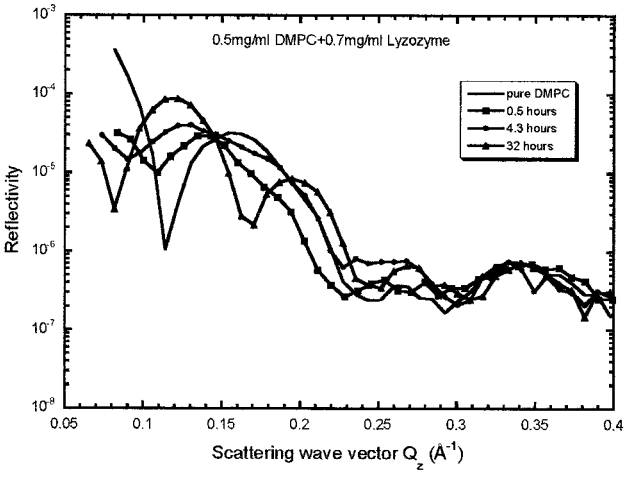


FIGURE 5 Set of experimental reflectivity curves recorded at different times on a NBF (0.5 mg/ml DMPC with 0.7 mg/ml lysozyme). A time function shift of the fringes toward smaller angles is observed and shows the initial film swells due to the protein insertion process.

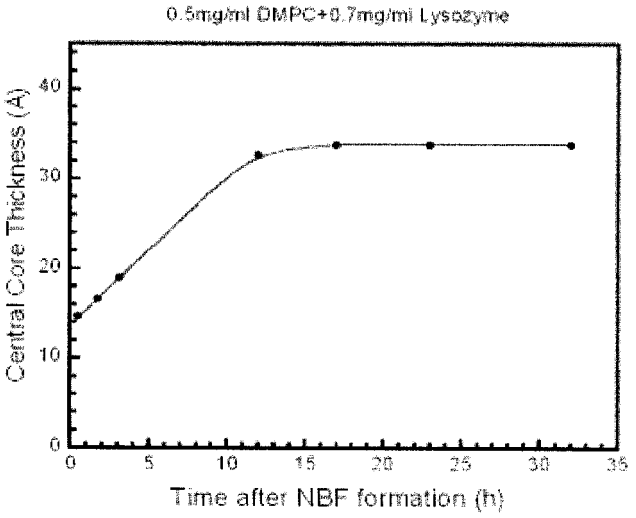


FIGURE 6 Time dependence of the central core thickness due to the lysozyme insertion within the NBF obtained by fitting the whole set of experimental reflectivity profiles (0.5 mg/ml DMPC with 0.7 mg/ml lysozyme). Time 0 is chosen just after the drainage and the NBF formation. At equilibrium, there is a plateau, which means that the protein insertion process is finished.

diffusion rate should be four times slower than for lysozyme.

To understand the film architecture, we tried to relate the state of the Gibbs monolayer with the state of the black film by the investigation of time evolution of the surface tension. Many investigations of protein monolayers reveal the complex behavior of the protein adsorbed at the Gibbs interface (Graham and Phillips, 1979a,b; Uraizee and Narsimhan, 1991; Narsimhan and Uraizee, 1992; Norde and Favier, 1992; Anand and Damodaran, 1995; Murray, 1997). The corresponding surface tension isotherms in the case of high lipid concentration are presented in Fig. 7. The adsorption

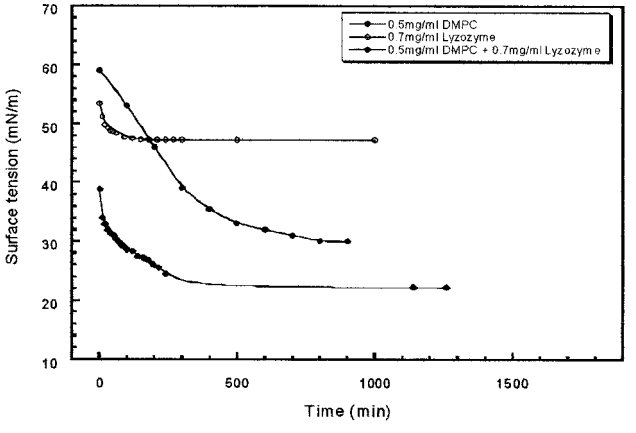


FIGURE 7 Time dependence of the surface tension of pure lysozyme, pure DMPC, and the mixture (0.5 mg/ml DMPC with 0.7 mg/ml lysozyme).

of pure DMPC is a very slow process that requires more than 10 h to reach the surface saturation (Toca-Herrera et al., 1998). The presence of lysozyme in the solution increases the rate of the adsorption kinetic (about 2 h). The values of the surface tension corresponding to the saturation are lower than that obtained for pure DMPC. This could be explained by the balance between the protein adsorption and the lipid adsorption. From Fig. 7., one can observe that the adsorption of a proteins is much faster than lipid adsorption. Thus, for the mixture at the beginning, there is mainly adsorption of proteins, but these proteins are progressively replaced by the smaller lipid molecules. The same process has been described in several papers about mixed lipid–protein monolayers (Nishikido et al., 1982; Goddard and Ananthapadmanabhan, 1993; Netz et al., 1996; Nag et al., 1996). Nevertheless, the value of the surface tension indicates the presence of a small quantity of protein molecules at the Gibbs interface. Such an organization at the surface before the film formation is in agreement with the model of the protein diffusion in the center of the black film after its formation, as reported above (Benattar et al., 1999).

Low concentration of lipids

Some studies about the lipid–protein interactions in the bulk show that the proteins adsorb on the vesicle surface (Matzumura and Dimitrova 1996; Dimitrova and Matzumura, 1997; Dimitrova et al., 1997, 1998). To increase the number of free-protein molecules capable to adsorb at the air–water interface and to obtain a better control of our mixed films, we reduced the lipid concentration. The minimum concentration that allows the formation of stable mixed films at the given protein concentration was found to be ~0.08 mg/ml for DMPC. It should be pointed out that, at this concentration, stable films cannot be obtained without adding the lysozyme. This means that, at such a low phospholipid concentration during the film formation, there is no reservoir of single-lipid molecules that are able to adsorb at the film surfaces and to stabilize the film. A mixed film was drawn from a solution leaving it to equilibrate 3 h. The films were very viscous, and their drainage was much slower than in the previous case at high lipid concentration.

Reflectivity curves have been recorded at different times after the complete drainage of water (Fig. 8). All the curves displayed fringes whose minimum stayed at identical positions. The fringes evolved with time and their intensity increased, leading to a better fringe contrast. This is clearly indicative of a molecular reorganization involving only a slight and irregular change of the total thickness of this "sandwich film." It was not possible to follow the time evolution of this film structure due to the inhomogeneity of the film after its formation (i.e., only one fringe is visible). Three hours later, the second order of the Kiessig fringes appears, indicating an improvement of the film homogeneity. The last reflectivity profile is quantitatively

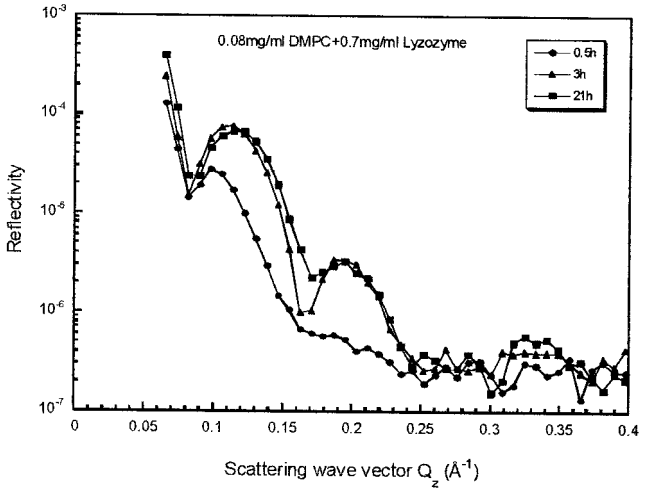


FIGURE 8 Set of three different reflectivity curves recorded at different times for a film made at a low lipid concentration (0.08 mg/ml DMPC with 0.7 mg/ml lysozyme). The solution is left 3 h to equilibrate before the film is drawn. There is a strong increase of the fringe intensity, which shows a molecular reorganization of this sandwich film without any change of the thickness. In a first step, a second order of the Kiessig fringe appears, and, at equilibrium, the fourth-order fringe is visible (the third order remains zero).

similar to that obtained at equilibrium for a higher concentration of lipids, after the swelling process. This means that the film structure should be very close to that described previously—mainly composed of two DMPC layers with a single layer of proteins between them. If most of the proteins are located in the center of the film, it might be possible that a small amount remain located within the surfactant layers. The surface tension isotherms of pure DMPC, lysozyme, and mixture are compared in Fig. 9. It is

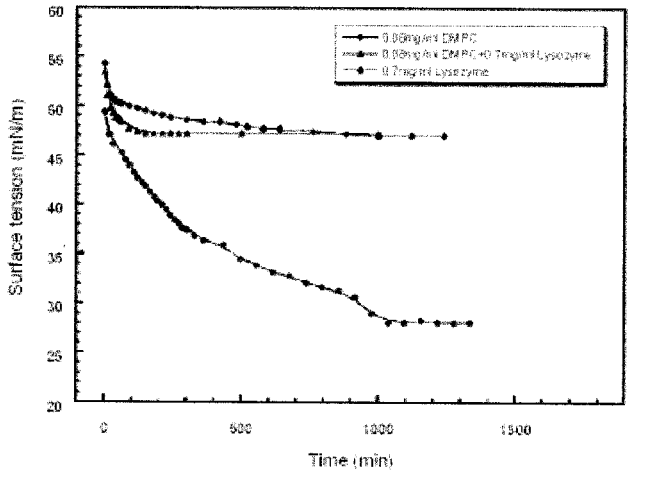


FIGURE 9 Surface tension isotherms at low DMPC concentration (0.08 mg/ml). The surface tension of the mixture (DMPC-Lysozyme) behaves like that of the pure lysozyme.

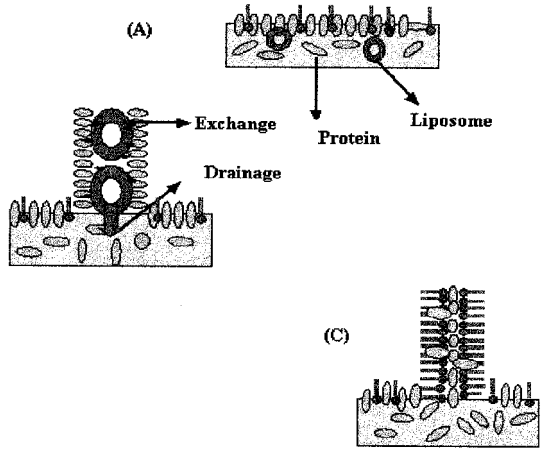


FIGURE 10 Schematic drawing of a possible exchange process of lipids and proteins during the drainage before the NBF formation.

striking to note that the equilibrium value of the surface tension is the same for the pure lysozyme and the mixture. Nevertheless, this equilibrium is reached more rapidly for the mixture (3 h) than for the pure lysozyme (15 h). This adsorption time coincides with the time necessary to draw and obtain stable black films. As we will show further in the text, the initial Gibbs monolayer is mainly composed of proteins, including a small quantity of lipids. If we now compare the reflectivity profile recorded at equilibrium in the two cases, protein insertion by diffusion and spontaneous formation of a mixed film, we can see that the final thickness is the same, as is also possibly the structure. The formation of a sandwich structure that mainly consists of two walls of lipids inserting proteins, thus requires exchanges between lipids and proteins during the drainage to form the external surfactant wall (Fig. 10).

Surprisingly, it appears that, when one increases the protein concentration (in the range from 0.1 to 3 mg/ml), there is no effect on the film thickness. The final thickness remains constant (81 Å) and equal to the thickness of the pure DMPC film with an additional thickness of 30 Å corresponding to the dimension of a lysozyme molecule. It should be pointed out that a similar experiment was made using another lipid (0.08 mg/ml DOPC) but the protein concentration necessary to obtain the same structure was about four times that required for DMPC (3 mg/ml lysozyme). This is possibly due to the unsaturation of DOPC, which leads to a lower surface coverage than for DMPC at the same concentration, that is, a higher area per molecule and thus more free sites for the protein adsorption.

Effect of the equilibration time on the mixed film formation

As mentioned above, from the surface-tension measurements, we observe that the formation of the Gibbs mono-

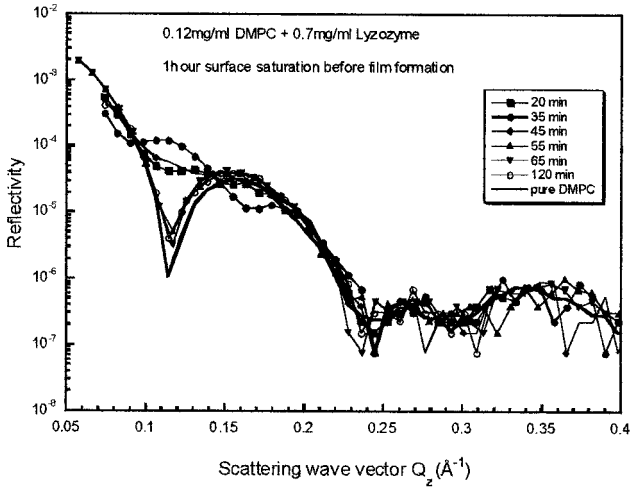


FIGURE 11 Observation of a swelling followed by a deswelling process (0.12 mg/ml DMPC with 0.7 mg/ml lysozyme) occurring when the film is drawn when the surface of the solution is not fully saturated (after 1 h) before the film formation. The chemical potential of the protein varies in the bulk solution (by adsorption of the protein in the liposomes), and this induces a diffusion of the proteins of the film back to the bulk solution.

layer preliminary to the black film formation, requires a rather long time to reach equilibrium (~3 h). To check the behavior of the system when this equilibrium is not reached before the film is drawn (the solution is left only 1 h before the film formation), we recorded a series of reflectivity profiles following the black film just after its drainage (t = 0). This set of curves is reported in Fig. 11. During a first stage (35 min), one can observe a swelling process characterized by a shift of the fringes toward the small Q_z and reaching a total thickness of 81 Å, and, during the next 20 min, a deswelling process characterized by a shift of the fringes toward large Q_z. The reflectivity profile converges to that of the pure DMPC corresponding to a total thickness of 58 Å. A possible explanation could be in a change of the chemical potential with time in the bulk. At the beginning, when the equilibrium is not reached, there is a greater amount of free proteins in the solution, which coincides with the required concentration to initiate a swelling process (i.e., the diffusion of the proteins in the NBF). Indeed, because the equilibrium is not reached at the Gibbs interface, it is also not reached in the bulk. It is possible that some of the free proteins are trapped by the liposomes and the concentration of the free proteins decreases, inducing a change of the chemical potential and a decrease of the protein surface fraction within the NBF.

CONCLUSION

In the present paper, we clearly extend the process leading to the formation of a stable, dense, and robust monolayer of soluble proteins within a freely supported surfactant bilayer to new systems made of phospholipids. We clearly demonstrate that the final architecture at equilibrium is always a sandwich film that is controlled by tuning the chemical potentials (via the respective concentrations) of the lipids and the protein. We evidenced two different behaviors depending on the lipid concentration. At high lipid concentration, we observed a swelling process, whereas, at a lower concentration, we obtained spontaneously a mixed structure, with only a molecular reorganization. If the two processes are clearly identified, the detailed structure and the process of reorganization requires further investigations (like surface diffraction at the interfaces). The difference between the two cases was also confirmed by surface-tension experiments. It should be pointed out that this protein insertion under controlled conditions can be used to form model systems of biological interest for fundamental investigations of interaction between specific lipids and proteins. This new method could also allow the study at a molecular scale of some applied problems like foam stabilization.

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