Insertion Process of a Protein Single Layer within a Newton Black Film

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We report a generally applicable protein insertion process leading to the formation of a close packed protein single layer within a freestanding surfactant bilayer. Very high packing fractions can be obtained in a controlled manner, simply by adjusting the protein chemical potential in the solution. We apply this method to the confinement of a model protein in a Newton black film (NBF) of a non-anionic surfactant. Using x-ray reflectivity, we observed a time dependent insertion of the proteins within the NBF and then a stable equilibrium state. [S0031-9007(99)09430-2]

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Black films are very simple freestanding bilayer systems involving most of the basic physical interactions existing in more complex structures such as biological membranes. The black films made of pure surfactants have been extensively studied in the past, with the primary emphasis concerning the nature and range of molecular forces, the thinning, and the factors affecting their stability [1–5]. Few papers have reported attempts to form mixed black films including proteins. Nevertheless, where microscopic common black films (CBF's) and Newton black films (NBF's) have been obtained, their structures were either bilayers of denatured proteins, more complex multilayer films, or thick films [6–9]. Here, we describe how to obtain a stable freestanding film, confining a single protein layer within a surfactant bilayer.

The black films are the final stages of the thinning of films drawn from solutions of surfactants. They are 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 salt concentration of the surfactant solution, two different black films can exist: the common black films and the Newton black films [3]. CBF's have a rather large equilibrium thickness well described by the colloid stability theory [10]. The NBF thickness is much thinner and its equilibrium is governed by microscopic interactions at short distance [11,12]. A few years ago, using x-ray reflectivity, we determined the NBF structure [13]. Our experimental approach was based on the fact that the x-ray wavelength is of the order of magnitude of the film thickness and constructive interferences can occur. We have shown that the NBF is a very reproducible system composed of two opposite molecular walls whose central core is reduced to an ultimate hydration layer of the polar heads and the weak roughness limited to the sole capillary waves (3.2 Å) [14,15].

The non-ionic surfactant C12E6 (hexaethylene glycol monododecyl ether) is known to have interactions with bovine serum albumin (BSA) without a denaturing effect [16]. C12E6 and BSA were purchased from Sigma. All the solutions were prepared with deionized water (18.2 MΩ, milli-Q system), agitated, and then filtered. The respective surfactant and protein ranges of possible concentrations were found empirically after testing the film stability. Usually, in the absence of BSA, the surfactant concentration for which large stable films can be obtained is $C_{\mathrm{C12E6}} \geq 0.5$ mg/ml ~ 15 cmc (critical micelle concentration). Only in the presence of BSA, we could decrease the concentration down to 2 cmc ($C_{\mathrm{C12E6}} = 0.075$ mg/ml). This last concentration was used for all the experiments. The pH of the solutions is stable roughly after one hour and was found to be 7.4 ± 0.1, that is, above that of the BSA isoelectric point (4.8). At this pH, the BSA is globular with an ellipsoidal shape of dimensions 140.9 and 41.6 Å [16,17]. In the experiments reported here, the protein concentration is $C_{\mathrm{BSA}} = 4$ mg/l; the temperature is regulated at 21 ± 0.1 °C. The films are drawn vertically from the C12E6-BSA solutions by lifting a metallic frame at a slow constant rate [18] (Fig. 1). The experiment requires a long frame (4 cm) to allow grazing incidence, thus leading to a large film area (2 cm^2). A reflectivity experiment concerns the measurement of the ratio $R(\theta) = I(\theta)/I_0$ at various incidence angles θ, between the intensity I_0 of the incident beam and that $I(\theta)$, reflected by the film. The experiments were performed using a high-resolution

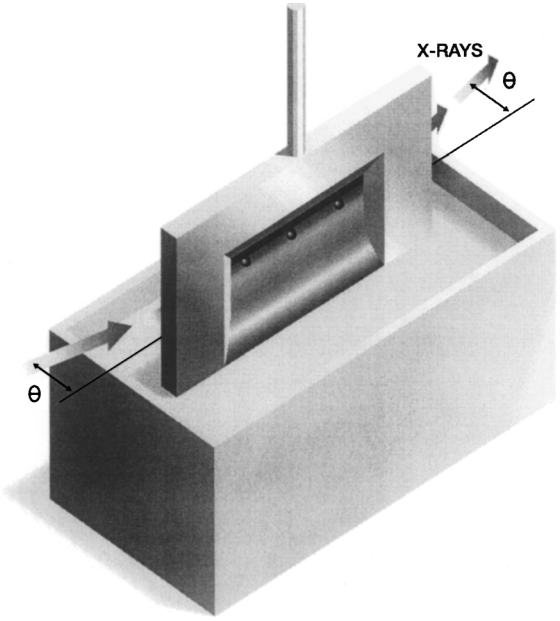

FIG. 1. Schematic of the setup installed at the center of the reflectometer. A metallic frame is immersed in the solution and then drawn up to form the film. During the drainage, the film shows interference colors and the NBF appears on the top of the frame.

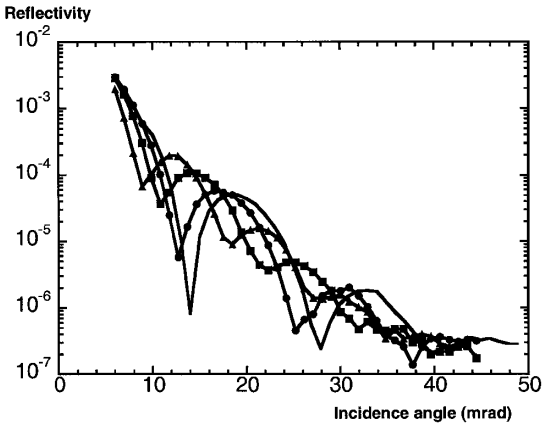

FIG. 2. Set of experimental reflectivity profiles recorded at different times, exhibiting interference Kiessig fringes. A time function shift of these fringes towards smaller angles is observed; it evidences the swelling of the initial film due to the protein insertion. The different profiles correspond to recordings carried out on a pure C12E6-NBF (solid line) and on a BSA-C12E6 NBF ($C_{C12E6} = 0.075$ mg/ml and $C_{BSA} = 4$ mg/ml), after 1 h (circles), after 10 h (squares), and after 18 h (triangles). The mean central core thicknesses between the surfactant walls are, respectively, 0, 3, 15, and 30 Å. It should be noted that, before reaching the equilibrium, the fringe contrast becomes rather low; this is due to the fact that, during the protein insertion process, the NBF is not homogeneous. The profiles are fitted using the weighted average of the area with (103 Å) and without (63 Å) proteins.

reflectometer for vertical surfaces (OptiX from Microcontrole). A copper tube is used as an x-ray source ($\lambda = 1.5405$ 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 provides access to the electron density profile along the film normal. The film being considered as a succession of homogeneous chemical 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 [19,20]. The main advantage of freestanding films arises from their high electron density gradients at the interfaces. The reflectivity profiles display very strong "Kiessig fringes" that originate from the interference of the beams reflected on each side of the NBF [21] which enable an accurate determination of the overall film thickness.

The reflectivity curve, recorded immediately after its formation, is slightly different from that of the pure surfactant; it is indicative of only a small increase of the thickness ($\sim$3 Å). Nevertheless, this confirms that the protein interacts with the surfactant. Immediately after its formation, the NBF displayed a remarkable time dependent "swelling," characterized by a continuous shift of the Kiessig fringes (Fig. 2). The time dependence of the overall film thickness has been obtained after a fit of the experimental data [12,13]. We call extra thickness the difference between the overall thickness of the NBF and that of the pure surfactant (63 $\pm$ 0.5 Å); it represents the matter swelling the NBF. Its time dependence is shown in Fig. 3. After 45 h, there is a plateau, which indicates the end of evolution. The system then reaches an equilibrium state where the extra thickness is 40 Å. This value remains constant (30 h) until the film bursts. Between 0 and 45 h, the curve exhibits a slow and continuous increase. It should be noticed that, for $C_{BSA} \geq 4$ mg/ml, the film rapidly bursts, probably due to a greater amount of BSA moving to the NBF. Experiments carried out at different BSA concentrations in the bulk solution (between 2 and 4 mg/ml) show increasing equilibrium thicknesses; thus the amount of BSA migrating to the NBF depends on the initial concentration. The results suggest that the additional matter swelling the film results from the protein insertion and not from the adsorption of water. The increase of the overall film thickness is indeed much smaller than that due to a transition to a CBF [3] by the formation of an aqueous core (in general, $>$100 Å). The film remains a NBF with an overall thickness smaller than that characteristic of a CBF. The swelling is, therefore, due solely to the protein insertion. It should be pointed out that, when no BSA is present, a pure C12E6-NBF does not present any thickness change during its lifetime (several days). The last problem is to locate the protein with respect to the surfactant and to interpret the maximum extra thickness value ($\sim$40 Å). This value may correspond either to roughly twice the size of a totally unfolded BSA molecule (β strands) or

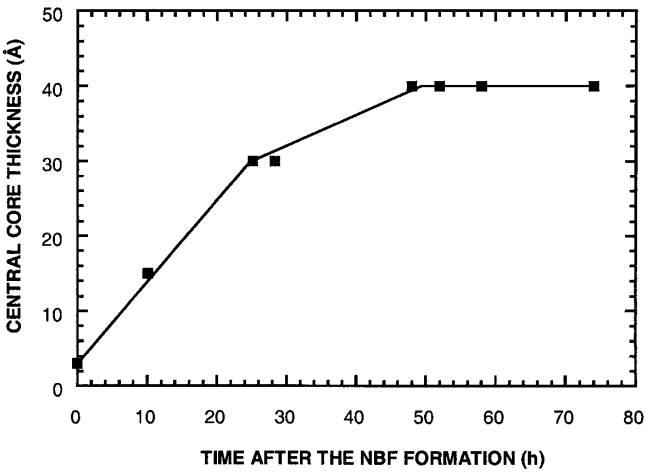

FIG. 3. Time dependence of the central core thickness due to the BSA insertion within the NBF obtained by fitting the whole set of experimental reflectivity profiles ($C_{C12E6} = 0.075$ mg/ml and $C_{BSA} = 4$ mg/ml). Time zero is chosen just after the drainage and the NBF formation. During the insertion of individual proteins, the measured "extra thickness" corresponds to a weighted average between areas without proteins and areas including proteins; its value varies between 0 and 41 Å (BSA thickness). At equilibrium, there is a plateau, which means that the protein insertion process is finished.

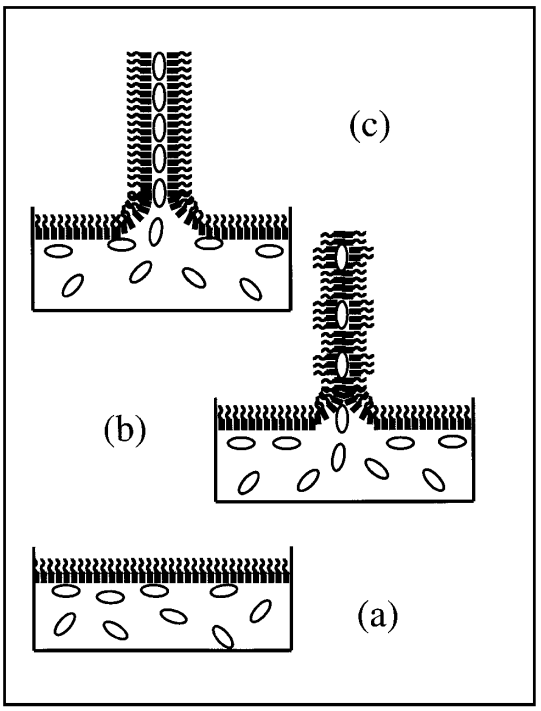

FIG. 4. Three different stages of the protein insertion process: (a) air/solution interface before pulling the film; (b) just after the drainage, a few proteins are trapped in the NBF and the film is locally distorted; (c) at equilibrium, the proteins form a dense and stable monolayer.

to the width of a native molecule (41 Å) situated in the central core of the NBF. To obtain an answer, we first formed a stable pure C12E6-NBF and then injected in the reservoir with a syringe a stock solution of pure BSA. To make this experiment possible, we increased C_{C12E6} to 0.5 mg/ml and, subsequently, we also increased C_{BSA} to 6.6 mg/ml (final concentrations), so reducing the film stability. We made a series of reflectivity profiles at regular time intervals and we again observed the swelling process. This is a direct experimental proof that the protein ascends within the NBF. It is thus clear that, at equilibrium, the new system is a "sandwich NBF" whose central core is a single layer of protein molecules. During the experiments, the BSA molecules are never in contact with the air-water interface: In the solution they are protected by the Langmuir film and, in the NBF, by the two surfactant walls. The C12E6 molecules could stabilize the native form of the protein as the fatty acids do at the air-water interface [17].

Our observations may be incorporated into a very simple model, by comparing the BSA chemical potential in the solution, at the Langmuir (air-water) interface and in the NBF. Before pulling the NBF, the Langmuir interface is in thermodynamical equilibrium with the bulk reservoir [Fig. 4(a)]. This sets the concentration of BSA in the Langmuir film as a function of C_{BSA}. At time $t = 0$, when the NBF is formed [Fig. 4(b)], the surface concentration of each of the two Langmuir films constituting the NBF does not have time to change. Thus the initial BSA surface fraction $\Phi_{NBF}(t = 0)$ in the film is just $2\Phi_L$ (Φ_L is the BSA surface fraction in the Langmuir film). This state is obviously not in equilibrium and, at long enough times, a surface fraction Φ_{NBF}^{eq} is reached for which the chemical potential of the BSA molecules in the NBF equals that in the bulk. *A priori*, the final concentration might be either larger or smaller than the initial value. We show in the following that, if the BSA molecules are attracted to the Langmuir 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 after the NBF formation. Let us call μ_{NBF}, μ_L, and μ_b the chemical potentials, respectively, of BSA in the NBF, in the Langmuir film, and in the bulk. In the dilute regime,

$$\mu_b = \mu_b^0 + kT \ln\Phi_b , \tag{1}$$

$$\mu_L = \mu_L^0 + kT \ln\Phi_L , \tag{2}$$

$$\mu_{NBF} = \mu_{NBF}^0 + kT \ln\Phi_{NBF} , \tag{3}$$

Φ_b is the volume fraction of BSA in the bulk, and μ_b^0, μ_L^0, and μ_{NBF}^0 are essentially the interaction free energy of a BSA molecule with its surroundings. The number of C12E6 micelles in the solution is, under our conditions, about 100 times smaller than the number of BSA free molecules. As a result, the expression (1) of the bulk chemical potential is obtained reasonably accurately if Φ_b

is taken as the nominal volume fraction (i.e., if one ignores the proportion of BSA adsorbed on the micelles; note that this approximation is also consistent with a "small" Φ_L). If the native BSA surface is essentially homogeneous and the NBF considered as being made of two Langmuir walls, then $\mu_b^0 - \mu_{\mathrm{NBF}}^0 \approx 2(\mu_b^0 - \mu_L^0) - w$, where w is the energy required to pull apart the two surfactant layers. None of these numbers are known, and, if the protein as a whole has two different sides, this estimate could be grossly wrong. Yet, if one neglects w [i.e., the disjoining pressure in this NBF is weak ($\approx$100 Pa) [4]] and takes as a trial value $\mu_b^0 - \mu_L^0 \approx 2.5$ kT (which is a rather small attraction to the Langmuir interface), thus the observations can be easily explained. Indeed, for $\Phi_b = 4 \times 10^{-3}$ ($C_{\mathrm{BSA}} = 4$ mg/ml), we find $\Phi_L \approx 5 \times 10^{-2}$ and $\Phi_{\mathrm{NBF}} \approx 0.6$. In other words, although the attraction by the Langmuir interface is small, that of the NBF is big. Actually a surface fraction of 0.6 is such that BSA-BSA interactions in the NBF should be taken into account [Fig. 4(c)]. According to these orders of magnitude, the estimate of the initial thickness should be the following (where d, d_L, and d_{BSA} are, respectively, the thicknesses of the NBF, of the Langmuir film, and the width of BSA): $d(t = 0) \approx 2(d_L + \Phi_L d_{\mathrm{BSA}})$; thus, $d(t = 0) - 2d_L \approx 4$ Å, which is approximately the value we found experimentally (3 Å). For surface fractions exceeding the natural close packed fraction, short-range repulsive steric forces become preponderant. The film is under internal pressure and its stability should drop abruptly. This is what is observed for $C_{\mathrm{BSA}} \geq 4$ mg/ml. The dynamics of BSA migration into the NBF should be essentially diffusive. Indeed the BSA flux is controlled by the chemical potential gradients and the characteristic time for equilibrium should be given by $\tau \approx L^2/D$, in which L is roughly half of the NBF height (0.5 cm) and D is the BSA diffusion constant in the NBF. From $\tau \approx 50$ h, we estimate $D \approx$ a few $\times 10^{-7}$ cm^2 sec^{-1}. It is important to realize that most of the arguments are not specific to BSA and should be valid for a wide class of amphiphilic proteins. It should be pointed out that successful experiments using another protein (lysozyme) and phospholipids are in progress. This protein insertion process under controlled conditions is very promising since it could be used to form new systems of biological interest for fundamental investigations of specific lipid-protein interactions or foams stabilized by proteins. It could open a new way for a two-dimensional crystallization over very large areas within free-standing films.

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[1] M. N. Jones, K. J. Mysels, and P. C. Scholten, Trans. Faraday Soc. **42**, 42 (1966).
[2] K. J. Mysels and M. N. Jones, Discuss. Faraday Soc. **42**, 42 (1966).
[3] K. J. Mysels, K. Shinoda, and S. Frankel, *Soap Films* (Pergamon, New York, 1959).
[4] T. Kolarov, R. Cohen, and D. Exerowa, Colloids Surf. **42**, 49 (1989).
[5] S. Clunie, J. F. Goodman, and B. T. Ingram, *Thin Liquid Films, in Surface and Colloid Science*, edited by E. Matijevic (Interscience, New York, 1971), Vol. 3, pp. 167–239.
[6] P. R. Musselwhite and J. A. Kitchener, J. Colloid Interface Sci. **24**, 80 (1967).
[7] D. Platikanov, G. P. Yampol'skaya, N. Rangelova, Zh. K. Ahgarska, L. E. Bobrova, and V. N. Izmaila, Kolloid. Z. **43**, 753 (1981).
[8] D. C. Clark, M. Coke, R. Mackie, C. Pinder, and D. R. Wilson, J. Colloid Interface Sci. **138-1**, 207 (1990).
[9] G. K. Marinova, T. D. Gurkov, O. D. Velev, I. B. Ivanov, B. Campbell, and R. P. Borwankar, Colloid Surf. A, Physicochem. Eng. Aspects **155**, 123 (1997).
[10] E. J. W. Verwey and J. T. G. Overbeeck, *Theory of the Stability of Lyophobic Colloids* (Elsevier, Amsterdam, 1949).
[11] J. Israelachvili, *Intermolecular and Surface Forces* (Academic, San Diego, 1985).
[12] D. Sentenac and J. J. Benattar, Phys. Rev. Lett. **81**, 160 (1998).
[13] O. Bélorgey and J. J. Benattar, Phys. Rev. Lett. **66**, 313 (1991).
[14] Z. Gamba, J. Hautman, J. C. Shelley, and M. Klein, Langmuir **8**, 3155 (1992).
[15] J. J. Benattar, A. Schalchli, and O. Belorgey, J. Phys. I (France) **2**, 955 (1992).
[16] N. Nishikido, T. Takahara, H. Kobayashi, and M. Tanaka, Bull. Chem. Soc. Jpn. **55**, 3085 (1982).
[17] D. E. Graham and M. C. Phillips, J. Colloid Interface Sci. **70-3**, 403 (1979).
[18] P. Guenoun, A. Schlachli, D. Sentenac, and J. J. Benattar, Phys. Rev. Lett. **74**, 3628 (1995).
[19] J. Penfold and R. K. Thomas, J. Phys. Condens. Matter **2**, 1369 (1990).
[20] M. Born and E. Wolf, *Principles of Optics* (Pergamon, London, 1984), 6th ed., pp. 51–60.
[21] J. J. Benattar, A. Schalchli, and D. Sentenac, Prog. Colloid Polym. Sci. **105**, 113 (1997).