Study of Protein Adsorption by Dynamic Surface Tension Measurements: Diffusive Regime

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We present new data on the adsorption of bovine serum albumin (BSA) at the free water interface using an improved setup of the pendant drop method. We show that it is possible to describe quite accurately the surface pressure−time behavior until the surface pressure is about half of its equilibrium value by only taking into account the diffusion of the proteins from the bulk solution to the surface and the thermal convection inside the measurement cell, without invoking the thermodynamics of the adsorption. We believe that this is a first and necessary step for a simpler description of the adsorption dynamics of proteins.

1. Introduction

Protein adsorption is a widely encountered phenomenon in colloidal science and is an important issue in food science when proteins are used for their emulsifying properties. Proteins are made of a set of amino acids condensed in a polypeptide chain; this chain adopts a specific folding (called secondary and tertiary structure) according to the interactions of its pendant groups. An interesting problem is the study of the possible unfolding (or *denaturation*) of the polypeptide chain at the interface. Indeed, since the pendant groups of the backbone have different affinities for water, we can imagine that, at the air−water interface for instance, the hydrophobic groups of the proteins tend to escape the bulk to spread at the interface and destroy the original folding. This process can been evidenced by the loss of the biological activity of the protein[1] or the direct observation of a modification of the polypeptide conformation.[2] Our aim is to study this surface-induced denaturation through an interfacial effect and namely the surface pressure (or equivalently the surface tension).

The interfacial activity of proteins is usually studied from the $\Pi = f(\Gamma)$ isotherms, where Π and Γ, respectively, are the surface pressure and surface concentration. Due to the large molecular weight of proteins (compared to surfactants), it can take several hours for the proteins to reach the equilibrium state described by $\Pi = f(\Gamma)$. Figure 1 represents a typical plot of surface pressure versus time $\Pi(t)$ of a midrange-concentration BSA solution (experimental conditions are given in the next section). The initial steps are governed by the diffusion of the proteins from the bulk to the interface while the last stage should be ruled by the details of the incorporation of the proteins inside the surface layer. This late stage should thus contain relevant information on the denaturation process, that could be extracted but only if the initial diffusive part of adsorption is sucessfully modeled. Previous studies[3−6] have proposed models of the dynamics of adorption that could only take into account distincts parts of the $\Pi(t)$ curve. Our very aim is to describe $\Pi(t)$ by using only the parameters describing the bulk diffusion and the possible denaturation.

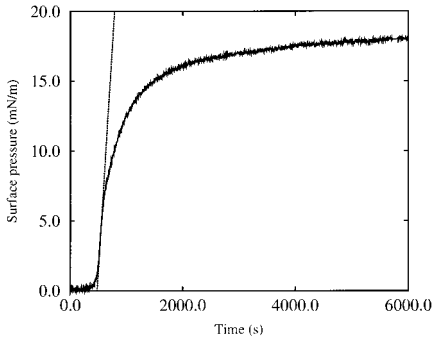

Figure 1. Typical adsorption curve for BSA ($c_0 = 5 \times 10^{-4}$%).

In this paper, we present an accurate description of the inital stage of adsorption.

2. Experimental Section

2.1. Materials. Bovine serum albumin (BSA, purity >97%, essentially fatty acid free grade) was purchased from Sigma and used as received. Solutions were prepared with Milli-Q water; pH was about 5.5, very close to the BSA isoelectric point.[7] All glassware and Teflon components of the setup were cleaned before use with a piranha solution (1:3 H_2O_2, 2:3 H_2SO_4) and then rinsed with ample amounts of distilled water. We used a new spectroscopic polystyrene cell for each experiment, and all experiments were performed at room temperature (20 ± 2) °C.

2.2. Experimental Setup. We have built an automatic pendant drop tensiometer similar to the one described by Hansen and Rødsrud.[8] An air bubble is created with a 1-mL Hamilton syringe at the tip of a Teflon tube guided by a U-shaped glass capillary tube. The Teflon tube has the advantage of being easily cut with sharp edges and provides an enhanced bubble stability. The image of the bubble is captured by a CCD video camera (Micam VHR 1000) mounted on a binocular microscope (Olympus

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SZ11) and connected to a PC frame grabber board (Data Translation DT55). A bubble has a radius of about 1 mm while a pixel of the CCD-sensitive device corresponds to 7 μm. The main features of the data processing are as follows: (i) the contour points are found in a row by local thresholding, allowing subpixel resolution; (ii) the contour search is switched from rows to columns once the contour tangent slope exceeds ±45°; (iii) the bubble symmetry axis is determined by folding the half contours and a mean-square minimization; (iv) the equatorial diameter D_E and the diameter D_S at a distance D_E from the apex are measured on the experimental contour, defining the parameter $S = D_S/D_E$; (v) a first estimation of the surface tension γ is obtained from $\gamma = \rho g R_0^2/\beta$, where R_0 and β, respectively the radius of curvature at the bubble apex and the shape factor, are calculated with polynomial expressions of S;[8] (vi) the estimation is improved by comparison of the experimental bubble profile with the eight closest (on a β, R_0 grid) theoretical profiles, calculated by a standard Runge−Kutta method. The experimental setup allows real-time measurements with a time resolution of 1 s and a noise of about 0.1 mN/m.

3. Theory of the Transport from the Bulk to the Interface

Without any stirring of the solution, the transport to the interface is expected to be entirely governed by diffusion, but the possible relevance of free convection was also put forward.[4,9,10] Natural free convection is always present in the cell, due to thermal inhomogeneities and turns out to homogenize concentrations on length scales greater than a characteristic length δ. In the most general case, this length δ is dependent on the physical properties of the fluid (density, viscosity, coefficient of volume expansion), the cell (size and geometry), and the diffusion coefficient D of the protein. To take into account both effects, we have developed a model of diffusion in a layer of width δ. The transport within this layer is governed by diffusion while free convection ensures that the protein concentration at the distance δ from the interface is always c_0, where c_0 is the bulk concentration. Assuming that, during the first stages of the adsorption, the interface behaves as an infinite well, the protein bulk concentration close to the interface, $c(x = 0, t)$ is taken as zero.[11] The transport problem is then ruled by the following equation:

$$\frac{\partial c}{\partial t} = D\frac{\partial^2 c}{\partial x^2} \tag{1}$$

together with the conditions

$$c(x,0) = c_0 \tag{2}$$

$$c(0,t) = 0 \tag{3}$$

$$c(\delta,t) = c_0 \tag{4}$$

The flux $J(t)$ toward the interface, defined by

$$J(t) = D\frac{\partial c}{\partial x}|_{x=0} \tag{5}$$

is obtained in the Laplace space from eqs 1−4

$$\tilde{J}(p) = c_0\sqrt{\frac{D}{p}}\frac{1}{\tanh(q\delta)} \quad \text{with} \quad q = \sqrt{\frac{p}{D}} \tag{6}$$

Finally, the surface concentration Γ is given, assuming that $\Gamma(t=0)$ is zero, by a time integration of J:

$$\Gamma(u) = \frac{c_0\delta}{\pi^2}\left(u^2 + 2\sum_{n=1}^{\infty}\frac{1 - e^{-n^2u^2}}{n^2}\right) \tag{7}$$

where u is defined by

$$u = \frac{\pi}{\delta}\sqrt{Dt} \tag{8}$$

At small times the usual result for a simple diffusion model in an infinite medium is recovered:

$$\Gamma(t) = 2c_0\sqrt{\frac{Dt}{\pi}} \tag{9}$$

whereas at large times the simple stationary layer[12] model is found:

$$\frac{d\Gamma}{dt} = \frac{D}{\delta}c_0 \tag{10}$$

4. Results and Discussion

4.1. The Lag Time. The lag time, during which the surface pressure is about zero, seems to be characteristic of protein adsorption curves. It is found experimentally as the intersection between the time axis and the straight line following the beginning of the pressure increase. The surface concentration at the end of this initial period can be evaluated in the same way from surface−pressure isotherms,[3,10] which gives for BSA at pH 5.5 $\Gamma_{lat} \simeq 1$ mg/m^2. The surface coverage corresponding to this concentration is about 25%—assuming BSA is a 116 × 27 × 27 Å^3 ellipsoid[13]—and the pressure given by a perfect gas law is 0.04 mN/m. We then see this plateau as corresponding to the gaseous part of the protein isotherm, the part for which the expected pressure appears to be of the same order as the noise of measurement. It is then consistent to consider that, at least during this period, there is no energy barrier to adsorption, which means that the dynamics is dominated by the transport in the bulk. Mac Ritchie and Alexander[4] for example have already checked that lag times were consistent with a transport by diffusion to the interface down to about 10^{-3}% (w/w), but they found below this concentration a drift between calculated and measured values, presumably due to the presence of free convection. We have carrefully studied the BSA concentration region going from 10^{-2}% to 10^{-5}% (w/w) in order to complete the experimental measurements in the diffusion-controlled regime and to verify the influence of free convection. We present in Figure 2 the experimental results and the prediction of our bulk-transport model, in which the only free parameter is the width of the diffusion layer imposed by free convection. The lag time exhibits in the diffusion limit (high concentrations) a well-defined c^{-2} behavior, according to eq 9, and it changes in the convection limit (low concentrations) to a c^{-1} behavior

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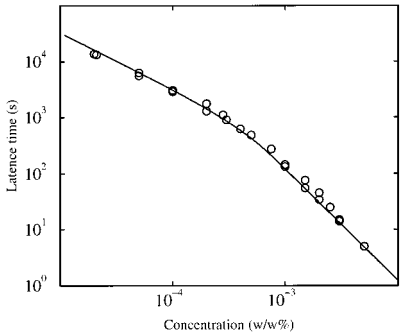

Figure 2. Evolution of the lag time with the bulk concentration of BSA: (○) experimental points, (—) model prediction (Γ_{lat} = 1 mg/m^2, δ = 0.22 mm).

(eq 10). The crossover between those two regimes occurs when proteins from distances greater than δ are required to reach Γ_{lat}; that is, $Dt_{lat} \geq \delta^2$. Values found for the lag time are well in agreement with those previously reported.[3,4] The best fitted value for the width δ of the diffusion layer is $\delta \simeq 0.2$ mm. Using results taken from heat transport in fluids and adapted to the transport of molecules by free convection,[14] the mass-transfer coefficient k is given by

$$\frac{kL}{D} = 0.07Gr^{0.33}Sc^{0.407} \qquad (11)$$

where the Grashof and Schmidt numbers are respectively

$$Gr = \frac{g\rho^2\alpha L^3}{\eta^2}\Delta T \qquad Sc = \frac{\eta}{\rho D} \qquad (12)$$

In eq 12, L is the typical size of the cell, g is the acceleration of gravity, ρ, η and α are the volumic mass, the viscosity, and the thermal expansivity of the fluid, and finally ΔT is the typical temperature inhomogeneity responsible for the convection. As $k = D/\delta$ and using L = 2 cm, $D = 6.7 \times 10^{-11}$ m^2/s,[15] $\Delta T \simeq 0.2$ K, and for ρ, η, and α the values of water,[16] we obtain the estimation δ = 0.4 mm, in good agreement with the measured value. We have also tried to check the accuracy of our description through the viscosity dependency of the lag time. For that purpose, we chose to add glycerol to the solution, as it lowers the surface tension of pure water only by 3 mN/m at a concentration of 50% (w/w)[16] and should therefore leave the surface behavior of proteins almost unaffected. Various relative viscosities of the solution were then obtained by changing the glycerol concentration: the resulting density was taken from ref 16 and the relative viscosity was measured for each experiment using a long and small capillary. The measured induction time is plotted with respect to the relative viscosity of the solution in Figure 3, for a given c_0 of 10^{-3}%. For this concentration well in the diffusion asymptotic regime, the relationship predicted by our model is simply

$$t_{lat} = \frac{\pi}{D}\left(\frac{\Gamma_{lat}}{2c_0}\right)^2 \eta_{relat} \qquad (13)$$

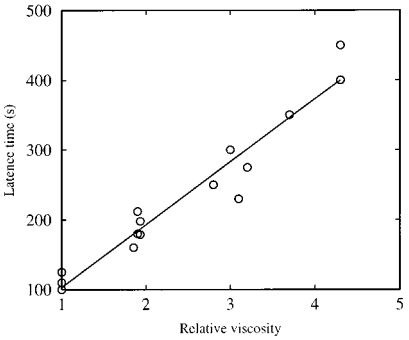

Figure 3. Evolution of the lag time with the relative viscosity with BSA at 10^{-3}% (○) experimental points; (—) linear regression.

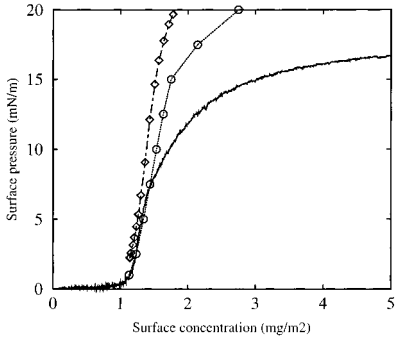

Figure 4. Comparison of the different $\Pi(\Gamma)$ relations: (○) experimental isotherm as taken from Hansen and Myrvold, (◇) measurements with bubble retraction method; (—) calculated relation, assuming only diffusion and convection.

The data exhibit the expected linear behavior, and the estimation of Γ_{lat} provided by the slope is 0.9 mg/m^2, which agrees very well with the expected 1 mg/m^2.

4.2. Post-Lag-Time Dynamics. Previous studies[3,4,17] had limited the region governed by bulk-transport dynamics to the only induction period (ending at the lag time). We have investigated the insight of this hypothesis through a comparison between experimental and calculated surface−pressure relationships and through the influence of protein concentration and fluid viscosity on the evolution of surface pressure $\Pi(t)$.

4.2.1. Surface Pressure as a Function of Γ. It is possible with our experimental setup to measure pressure as a function of surface concentration by monitoring Π while changing the volume (and hence the area) of the bubble. Practically, the bubble is kept immobile until it reaches the end of the induction period, for which surface concentration is known. The bubble is then retracted and the new surface concentration is deduced from the previous one through the area compression factor (which can be varied up to about 2). To ensure that the total amount of protein on the surface is constant during the procedure, retraction (or expansion) has to be done quickly (typically within 15 s) and the measured pressure is thus not the equilibrium pressure.

Combining the experimental $\Pi(t)$ and the theoretical expression (eq 7) for $\Gamma(t)$, it is possible to calculate what would be Π as a function of Γ if the dynamics was always dominated by transport in the bulk. $\Pi(\Gamma)$ relations, resulting from the above calculation and from the bubble retraction experiments, are plotted in Figure 4, in addition to the HSA equilibrium isotherm at pH 5.8 taken from Hansen and Myrvold.[3] To facilitate comparison, the

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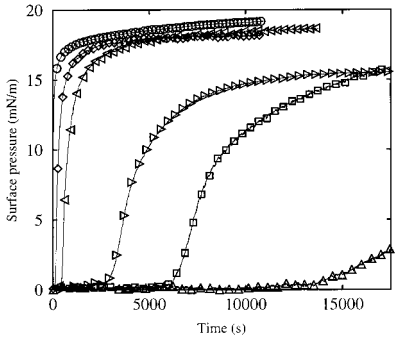

Figure 5. Evolution of $\Pi(t)$ with the concentration. (○) 5×10^{-3}%; (◇) 10^{-3}%; (left-pointing triangle) 5×10^{-4}%; (right-pointing triangle) 10^{-4}%; (□) 5×10^{-5}%; (△) 2×10^{-5}%.

isotherm has been translated by -0.15 mg/m^2 to let the pressure rises coincide. The different curves agrees very well up to about 7 mN/m; then, the calculated $\Pi(\Gamma)$ leads to lower pressures (for a given surface concentration) than measured $\Pi(\Gamma)$, indicating that the model overestimates the flux of proteins to the surface. This can be understood as follows: when the interface is covered enough, the time for proteins to incorporate the surface is larger than the time needed to diffuse from the bulk to the surface and diffusion cannot describe the dynamics of adsorption anymore.

4.2.2. Rescaling Procedure. The accuracy of the model up to about 7 mN/m was shown for a particular concentration ($c_0 = 5 \times 10^{-4}$%) and a particular viscosity ($\eta = \eta_w$, with η_w the viscosity of water). It is obviously of importance to verify that this remains true for the whole concentration range presently studied. Assuming that each pressure corresponds, independently of the bulk concentration, to a given protein surface concentration, the time was rescaled as follows. We choose as reference a certain couple of bulk concentration and viscosity values (c_{ref} and η_{ref}), and for each set of concentration and viscosity (c_0, η), we change the time scale $t \rightarrow t'$, with t' defined by the relation

$$\Gamma(c_0,\eta,t) = \Gamma(c_{ref},\eta_{ref},t') \tag{14}$$

where Γ obeys eq 7. In the limit $\delta \rightarrow \infty$, or $t \rightarrow 0$ corresponding to a negligible free convection, the transport is purely diffusive and Γ is given by eq 9. In this case the rescaling is simply

$$t' = \frac{\eta_{ref}}{\eta}\left(\frac{c_0}{c_{ref}}\right)^2 t \tag{15}$$

The matching parts in the rescaled curves should indicate the region where the time evolution of the surface concentration is well described by the bulk-transport model. The concentration and viscosity of the reference experiment are $c_{ref} = 5 \times 10^{-4}$% and $\eta_{ref} = \eta_w$.

4.2.3. Rescaled Data with Respect to Concentration. We present in Figure 5 data corresponding to concentrations from 5×10^{-3}% to 2×10^{-5}% and $\eta = \eta_w$ before any rescaling: the strong dependency in concentration can be seen through both the lag time and the further dynamics. The same curves are drawn in Figure 6 after the rescaling procedure. The correspondence between the curves is striking, indicating that the bulk-transport model is relevant. However the matching remains after the expected limit of 7 mN/m, which is a little puzzling in a region apparently governed by adsorption dynamics. In fact, adsorption dynamics can be written in the general

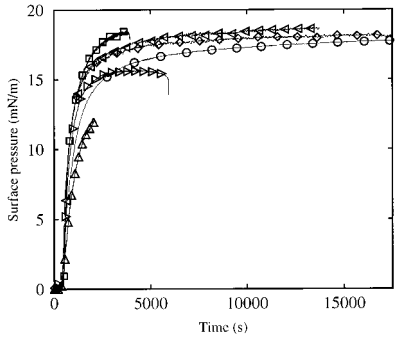

Figure 6. Rescaled curves $\Pi(t)$ for different concentrations of protein: (○) 5×10^{-3}%; (◇) 10^{-3}%; (left-pointing triangle) 10^{-4}%; (right-pointing triangle) 10^{-4}%; (□) 5×10^{-5}%; (△) 2×10^{-5}%.

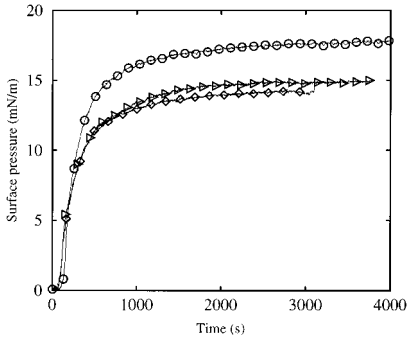

Figure 7. Rescaled curves $\Pi(t)$ for different relative viscosities ($\eta_{ref} = \eta_w$): (○) $\eta_{rel} = 1$; (◇) $\eta_{rel} = 2$; (right-pointing triangle) $\eta_{rel} = 3$.

form

$$\frac{d\Gamma}{dt} = c_0 f(\Gamma,\Pi) \tag{16}$$

When eq 16 is compared to the asymptotic behavior of our model corresponding to large time (eq 10), the concentration dependency appears the same. The complete matching is not in contradiction with results from the $\Pi(\Gamma)$ relationship, it simply verifies, for large pressure, the concentration dependency assumed in eq 16. Contrary to the results from Graham and Phillips[10] or Gonzalez and Mac Ritchie[18] no dramatic decrease in the equilibrium pressure is observed on decreasing the bulk concentration (at least for $c_0 > 2 \times 10^{-5}$%). At very low concentrations, the loss of proteins because of the adsorption on the cell walls is no more negligible and we have pre-equilibrated our cells with the solution before measurement to avoid protein material loss and artifactual equilibrium pressure decrease.

4.2.4. Rescaled Data with Respect to Viscosity. We now present the viscosity dependence of $\Pi(t)$ at fixed concentration $c_0 = 10^{-3}$%. As the adsorption dynamics model (eq 16) does not depend on viscosity, we expect that the rescaling procedure should not be valid above 10 mN/m. In Figure 7 are presented the rescaled curves for relative viscosity from 1 to 3. The rescaling is indeed satisfactory until about 10 mN/m. However, most experiments appear noisier and the reproducibility of the experiments is poorer in the presence of glycerol (typically from 1 to 3 mN/m). This is attributed to possible interactions between BSA and glycerol. This view is supported by the fact that the dynamics is highly affected for large glycerol concentrations (above 45% (w/w)).

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5. Conclusion

We have presented new measurements of the dynamics of adsorption of proteins at the air−water interface. The use of a Teflon tube in our setup for measuring bubble shapes and the careful experimental procedure (incubation of the cells, determination of the surface pressure every second) have allowed us to obtain improved adsorption curves. Taking into account the thermal convection inside the measurement cell has permitted us to extend the description of the dynamics until the pressure reaches about half of its equilibrium value and then beyond what is usually described in the literature.

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