

# Monolayers of Globular Proteins on the Air/Water Interface: Applicability of the Volmer Equation of State

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We performed simultaneous measurements of the instantaneous values of the surface pressure versus time,  $\Pi(t)$  (by the Wilhelmy plate method), and the adsorption versus time,  $\Gamma(t)$  (by ellipsometry), for aqueous solutions of a globular protein ( $\beta$ -lactoglobulin, BLG). The resulting dependence  $\Pi(\Gamma)$  was found to be well described by the Volmer equation of state (when  $\Gamma \leq 1.6 \text{ mg/m}^2$ , a value corresponding to an almost complete monolayer), for all times and bulk concentrations. The excluded area per molecule,  $\alpha$ , turned out to be twice as large as the maximum cross-sectional area of the molecule,  $\omega$ , in accordance with the theoretical considerations. We processed in the same way available literature data for various proteins (BLG,  $\alpha$ -lactalbumin, bovine serum albumin), both for equilibrium and for nonequilibrium adsorbed layers, as well as for spread layers. In all cases, the experimental dependencies  $\Pi(\Gamma)$  were fitted well by the Volmer equation; the excluded area either was almost exactly twice the maximum cross-sectional area (for spherical molecules) or could be interpreted in a similar way (for nonspherical molecules), by means of qualitatively equivalent reasoning. These results have led us to the following conclusions for the studied globular proteins: (i) The surface state depends only on the instantaneous adsorption,  $\Gamma$ , regardless of how it was reached. (ii) The Volmer equation is obeyed for surface coverages close to or lower than the monomolecular adsorption. (iii) No denaturation occurs during the adsorption process.

## 1. Introduction

The adsorption of globular proteins on liquid interfaces is a widely studied phenomenon, due to its importance for the stabilization of food dispersions (dressings and sauces, mayonnaise, ice cream, etc.). The complexity of the protein adsorption comes from the fact that the molecules on the surface usually undergo substantial conformational changes, unfolding and partial denaturation.<sup>1</sup> The latter changes are driven by the free energy gain when hydrophobic moieties from the molecule enter into the hydrophobic phase (oil or air). The extent of the configurational rearrangement is often dependent on the layer density:<sup>2</sup> at lower surface concentrations the globular protein molecules tend to unfold more, and in tightly packed layers the scope for reorganization is rather limited.<sup>3</sup> Typically, these processes of partial denaturation are slow (with characteristic times of the order of several hours<sup>4</sup>). They affect the macroscopic properties of the layer, such as the surface pressure. On the other hand, for relatively short interfacial aging (e.g., up to about an hour in the case of lysozyme<sup>4</sup>) the layer state can be understood with the premise that the protein molecules have not succeeded to unfold. This of course depends on the particular protein, the bulk concentration, and so forth; in the present work

we analyze several cases in which the layer behavior is not affected by surface denaturation.

Information about the physical state of the protein molecules on the fluid interface can be obtained from measurements of the surface pressure,  $\Pi$  (the lowering of the interfacial tension due to the layer), and the adsorbed protein amount per unit area,  $\Gamma$ . Different types of  $\Pi(\Gamma)$  relation, that is, surface equations of state, have been proposed and tested in the literature. In refs 5–8, an equation of the Szyszkowski–Langmuir type was adopted,  $\Pi \sim \ln(1 - \omega\Gamma)$ , where  $\omega$  has the meaning of (average) partial molecular area. Statistical thermodynamical considerations<sup>9</sup> confirm that the parameter  $\omega$  can be interpreted as the area physically occupied by one molecule. The authors of refs 5–8 assumed that protein molecules on the interface, in equilibrium with the bulk solution, could exist in a number of states with different molar areas. In that model, the main factor determining the state of the adsorbed molecules was the value of  $\Pi$ : at low surface pressure the molecular states with larger size were strongly favored over those with small size. The latter fact was interpreted as deeper denaturation.<sup>8</sup> Two-dimensional aggregation of the protein in the surface layer was considered in ref 5. The influence of the electrostatic interactions between charged protein molecules on the air/water (A/W) boundary was investigated in refs 5–8, in the frames of the Gouy–Chapman theory. The multiple-

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state model incorporating electrostatics was compared with experimental data for the dependence of the surface pressure,  $\Pi$ , on the bulk protein concentration,  $c_b$ , for bovine serum albumin (BSA),<sup>5</sup>  $\beta$ -casein,<sup>6,7</sup> and human serum albumin (HSA).<sup>6–8</sup> Fits (with three or four adjustable parameters) showed good agreement between theory and experiment, for  $\Pi$  up to 15–25 mN/m (refs 5–8).

Serrien et al.<sup>10</sup> introduced a model with a first-order surface chemical reaction between native and unfolded forms of the protein molecule (with only the native form being directly exchangeable with the bulk solution). Reorientation was also included as a possible change. For both BSA and casein, two reaction mechanisms were found to be operative on the interface, with characteristic times of ca. 120 and 3000 s for BSA and ca. 12 and 300 s for casein, respectively.<sup>10</sup> Another model surface equation of state for globular proteins on the A/W boundary<sup>2</sup> took into account the statistics of the adsorbed segments (at different degrees of unfolding), the mixing enthalpy within the interfacial layer (i.e., the segment–solvent interactions), and the electric double layer free energy. Good four-parameter fits with that model were reported for the experimental  $\Pi(\Gamma)$  relations for BSA and lysozyme,<sup>2</sup> including the region of constant  $\Pi$  at high  $\Gamma$ .

In a recent work, Meinders and colleagues<sup>11</sup> used another equation of state:

$$\Pi = \frac{kT\Gamma}{(1 - \omega\Gamma)^2} \quad (1)$$

Although this equation was originally derived for a layer of hard disks,<sup>12</sup> Meinders and colleagues<sup>11</sup> applied it to viscoelastic disks by assuming that  $\omega$  depends on the surface coverage. Thus, the authors of ref 11 developed a four-parameter model. They interpreted the  $\Pi(\Gamma)$  data for spread layers of  $\beta$ -lactoglobulin (BLG),  $\alpha$ -lactalbumin, BSA, and  $\beta$ -casein in an interval for  $\Gamma$  up to rather high values (4–5 mg/m<sup>2</sup>).

In contrast to those complicated cases, in which denaturation of the protein had been developing with time, some systems containing globular proteins were found to exhibit simpler behavior under certain conditions. (Such conditions include not very long interfacial aging, so that significant unfolding would not take place.) Sufficiently diluted layers should obey the 2D ideal gas law, or laws assuming that the protein molecules are nonpenetrable objects occupying a constant surface.<sup>13</sup> Indeed, at low values of  $\Pi$  and  $\Gamma$  the spread BLG layers investigated in ref 11 had constant molecular area,  $\omega$ . As another example, in refs 13 and 14 the experimental  $\Pi(\Gamma)$  dependence for spread BLG layers on concentrated salt, at surface pressures below  $\sim 0.8$  mN/m, was described very well by the equation of Volmer:

$$\Pi\left(\frac{1}{\Gamma} - \alpha\right) = kT \quad (2)$$

This equation of state, valid for hard disks,<sup>9,15</sup> has only one parameter,  $\alpha$ , which is often called “excluded area per

molecule”;  $kT$  is the thermal energy, and the adsorption  $\Gamma$  is measured in number of molecules per cm<sup>2</sup>. The value of  $\alpha$  reported in ref 13 (with the data taken from ref 14) is 74.3 nm<sup>2</sup> for BLG; this is far greater compared to what follows from other data (see section 4.1 below). The probable reason for this discrepancy is the difference in the composition of the aqueous subphase: in ref 14 it was 35 wt % (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. The validity of eq 2 was confirmed also for spread egg albumin on concentrated salt (35 wt % (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>), at  $\Pi \leq 0.4$  mN/m (ref 16).

In the present work, we have studied layers of BLG formed by adsorption on the air/water interface (as opposed to the spread layers in the papers cited above), measuring  $\Pi$  and  $\Gamma$  independently, as functions of time. It is demonstrated that the instantaneous values of the surface pressure,  $\Pi(t)$ , and the adsorption,  $\Gamma(t)$ , obey eq 2 when the adsorption is below (or close to) that for a complete monolayer, irrespective of the time and the bulk concentration in the solution. We discuss also literature data for BLG from different sources. These data (for equilibrium adsorbed or spread layers, or for adsorbed layers under dynamic conditions) turn out to comply with eq 2, and the value of the parameter  $\alpha$  coincides with that obtained from our results (see section 4.1 below):  $\alpha = 19$ – $20$  nm<sup>2</sup>. The  $\Pi(\Gamma)$  relations for diluted layers of  $\alpha$ -lactalbumin and BSA, measured by other authors, are also found to be in good agreement with eq 2 and give reasonable values for  $\alpha$  (section 4.2). Therefore, it can be concluded that all investigated proteins do not undergo denaturation and the molecules do not interact appreciably when the layers on the air/water surface are relatively diluted (with coverage lower than or close to a monolayer).

## 2. Experimental Section

**2.1. The Setup.** The adsorbed protein amount was measured by ellipsometry, using a “rotating analyzer” apparatus (with a fixed angle of incidence of 50°).<sup>17,18</sup> The scheme of the ellipsometer is shown in Figure 1a. The two ellipsometric parameters  $\Psi$  and  $\Delta$ , which characterize the protein layer, were determined as functions of time. An automatic Wilhelmy balance with computer data acquisition was used to measure the surface pressure. Both data sets (surface pressure and ellipsometric angles) were simultaneously recorded every second.

**2.2. The Measuring Cell.** The experiments were performed in the measuring cell shown in Figure 1b. A glass semicylinder (with diameter 6 cm and length 12 cm) was filled up with aqueous solution. A barrier made from poly(tetrafluoroethylene) (PTFE, Teflon), protruding less than 1 mm inside the solution, allowed us to clean up the surface and remove the adsorbed layer. At the end of the cylinder, a Wilhelmy plate was mounted. The ellipsometric beam spot was situated more than 2 cm away from the measuring plate and from the cylinder walls, to avoid any change of the angle of incidence due to surface curvature. The cell was covered with a glass plate (except in the region of the laser beam and the Wilhelmy plate), to protect the surface from contamination and airflow.

**2.3. Materials and Experimental Procedure.** Protein solutions at three different bulk concentrations  $c_b$ , 0.01, 0.005, and 0.0005 wt %, containing 0.1 g/L sodium azide (NaN<sub>3</sub>, antibacterial agent), were prepared with deionized water from a Milli-Q system (Millipore);  $\beta$ -lactoglobulin from bovine milk (mixture of A and B variants, catalog no. L-0130) was purchased from Sigma. The pH was adjusted to 5.2–5.4 by HCl. The isoelectric point of BLG is at pH  $\approx 5.2$ , so in our system the protein molecules were essentially uncharged. All solutions were used within 2 h after their preparation.

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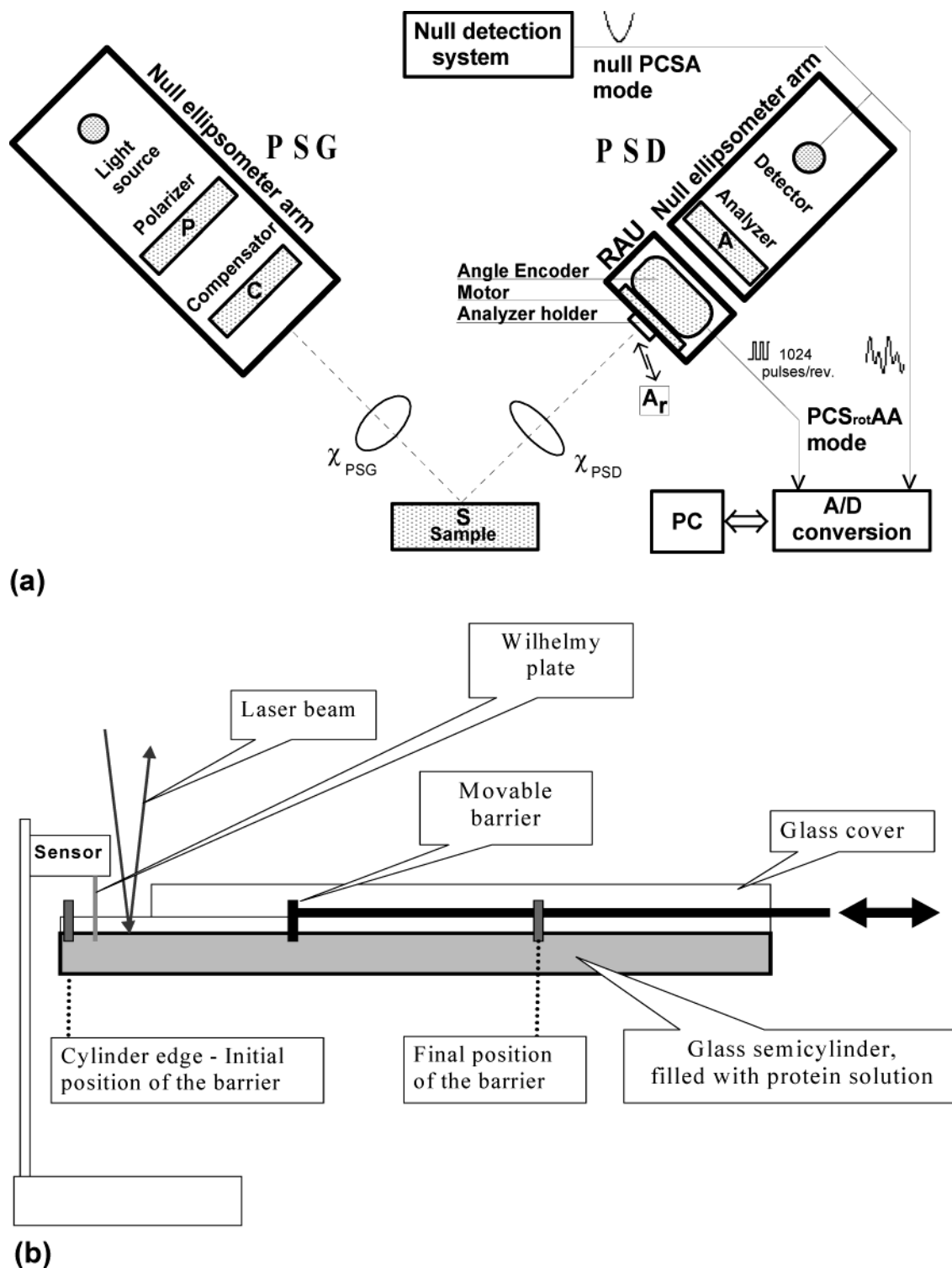
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**Figure 1.** Scheme of the experimental setup. (a) The ellipsometer, equipped with rotating analyzer unit, which allows data acquisition at every second. (b) The experimental cell, in which ellipsometry and surface tension measurements are performed simultaneously. There is a barrier for cleaning the air/water boundary.

The experimental cell and the glassware were dipped in fresh sulfochromic acid (solution of potassium dichromate in sulfuric acid) for 10 min; then they were put in distilled water for 20 min, rinsed with deionized water (from Millipore), after that soaked in Millipore water for 20 min, and subsequently rinsed with Millipore water. The cell was then dried and placed on the sample holder. We tested the cleanness of the setup walls and the pure water used for the solutions in the following way: The vessel was filled up with Millipore water and left for several hours to allow emerging of any surface-active contamination. Meanwhile, the water surface was monitored ellipsometrically. Afterward, the surface was highly compressed by the

barrier, and if no change in the ellipsometric signal occurred, we assumed that the system was clean. The pure water was then sucked out and replaced by protein solution. After that, the surface of the protein solution was cleaned by sweeping with the barrier, and immediately the tensiometer plate was put in contact with the interface. From that moment on, the measuring devices were simultaneously operating. The data coming from both apparatuses (ellipsometer and tensiometer) were recorded and stored every second for 30–40 min after the surface cleaning. Next, the surface of the protein solution was cleaned again by the barrier and a new experiment (called a “run”) was performed.

### 3. Data Processing

In ellipsometry, one determines the change of the polarization state of the incident light after its interaction with the sample. This change is described by two parameters, the ellipsometric angles  $\psi$  and  $\Delta$  (or equivalently, by the complex ellipsometric ratio  $\rho$ ):<sup>17</sup>

$$\rho = e^{i\Delta} \tan \psi \quad (3)$$

The two measured quantities,  $\psi$  and  $\Delta$ , are connected with the optical parameters of the sample by the relation

$$\rho = R_p/R_s \quad (4)$$

where  $R_p$  and  $R_s$  are the generalized Fresnel coefficients for p- (parallel) and s- (perpendicular) polarization, respectively. For a given model of the optical system,  $R_p$  and  $R_s$  are usually known functions of the refractive indices and the layer(s) thickness. Thus, eqs 3 and 4 can be used to find two unknown system parameters from a single ellipsometric measurement (i.e., from a couple  $\psi$ ,  $\Delta$ ). In the case of the system air/(surface protein layer)/bulk solution, these two quantities are the thickness,  $d$ , and the refractive index,  $n$ , of the layer.

It is well-known<sup>17</sup> that for very thin layers (in comparison to the used wavelength) only one of the measured ellipsometric angles (viz.,  $\Delta$ ) is sensitive to the change of the layer thickness and/or refractive index. The other angle,  $\psi$ , changes only very slightly from its value for a clean surface. This means that we have only one useful measured quantity, and consequently, only *one* physical parameter of the layer can be found. On the other hand, the optical model of the system presumes that the interfacial layer is a homogeneous volume phase with finite thickness and plane-parallel surfaces (air/layer and layer/water); for such a layer the unknown optical parameters are *two*: the thickness and the refractive index. This problem is usually overcome by using additional information (e.g.,  $dn/dc$  data, where  $c$  represents the concentration of the solute per unit volume of the layer phase).

Since the protein layers are very thin (thickness/wavelength  $\equiv d/\lambda \ll 1$ ), we can use the following expression for the change of  $\Delta$  (which is valid up to the first-order term in  $d/\lambda$ , cf. refs 17, 19, 20):

$$\delta\Delta = \Delta - \bar{\Delta} =$$

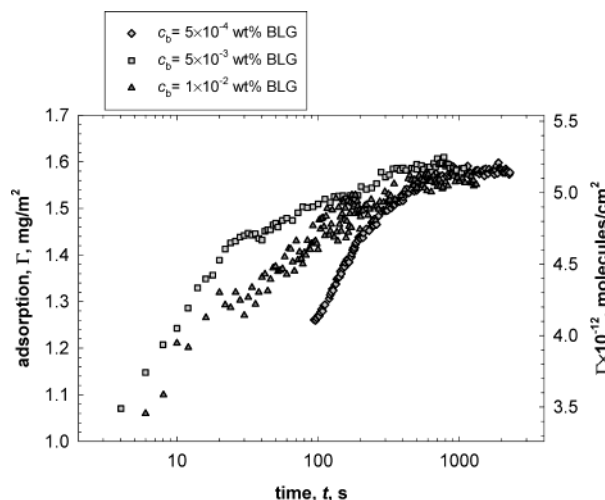
$$\frac{4\pi}{\lambda} \frac{n_0 \sin \varphi \tan \varphi}{[n_2^2 - n_0^2][1 - (n_0/n_2)^2 \tan^2 \varphi]} F_x = A F_x \quad (5a)$$

where

$$F_x = d_{1x} \left( n_{1x}^2 + \frac{n_0^2 n_2^2}{n_{1x}^2} - n_0^2 - n_2^2 \right) \quad (5b)$$

Here  $\delta\Delta$  is the difference in  $\Delta$  between the interface carrying a layer and the bare interface (to which  $\bar{\Delta}$  refers),  $\varphi$  is the incidence angle,  $n_0$ ,  $n_{1x}$ , and  $n_2$  are the refractive indexes of the medium (upper phase), the layer, and the substrate, respectively, and  $d_{1x}$  is the thickness of the layer.

The two unknowns,  $n_{1x}$  and  $d_{1x}$ , cannot be separately determined solely from eqs 5, but the so-called “ $dn/dc$



**Figure 2.** Ellipsometric results for the time dependence of the amount of BLG adsorbed at an air/water interface, at three bulk concentrations  $c_b$ .

approximation” can be used to provide one additional relation:

$$n_{1x} = n_2 + \frac{dn}{dc} c_{1x} \quad (6)$$

where  $dn/dc = 0.180 \text{ cm}^3/\text{g}$  is a typical value for proteins,<sup>21,22</sup>  $n_2 = 1.332$  is the refractive index of water, and  $c_{1x}$  is the protein concentration in the layer (in units of  $\text{g}/\text{cm}^3$ ). Further, the surface coverage,  $\Gamma_{1x}$ , is related to the concentration  $c_{1x}$ :

$$\Gamma_{1x} = c_{1x} d_{1x} \quad (7)$$

Equations 5–7 lead to a relationship between the surface coverage (adsorption),  $\Gamma_{1x}$ , and the ellipsometric angle  $\Delta$ :

$$\Gamma_{1x} = k(\Delta - \bar{\Delta}) \quad (8)$$

with

$$k = \frac{n_{1x}^2}{A(dn/dc)[n_{1x}^2 - n_0^2](n_{1x} + n_2)} \quad (8a)$$

Although the coefficient  $k$  in eq 8 depends on the layer refractive index  $n_{1x}$ , this dependence is relatively weak if  $n_{1x}$  does not change considerably. (The entire expected range of the refractive index  $n_{1x}$  during the layer buildup is  $1.332 < n_{1x} < 1.5$ .) Therefore, using constant  $k$  as an approximation would give sufficient accuracy for  $\Gamma_{1x}$  in thin layers. The coefficient  $k$  was calculated according to eq 8a, for the particular system and angle of incidence under consideration (the result was  $k \approx 0.6 \text{ mg m}^{-2} \text{ deg}^{-1}$ ).

## 4. Results and Discussion

### 4.1. Equation of State from $\Pi$ – $\Gamma$ Data for BLG.

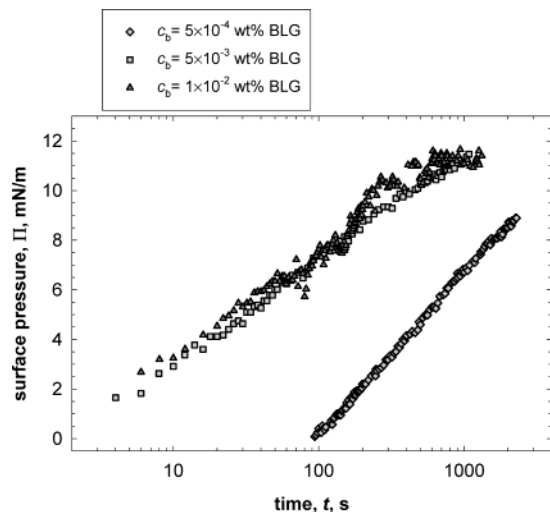
The ellipsometrically measured time dependence of the adsorbed amount of BLG, at the three studied bulk concentrations,  $c_b$ , is presented in Figure 2. One notices that the adsorption process is initially rather fast; in the first seconds after starting the experiment  $\Gamma$  already

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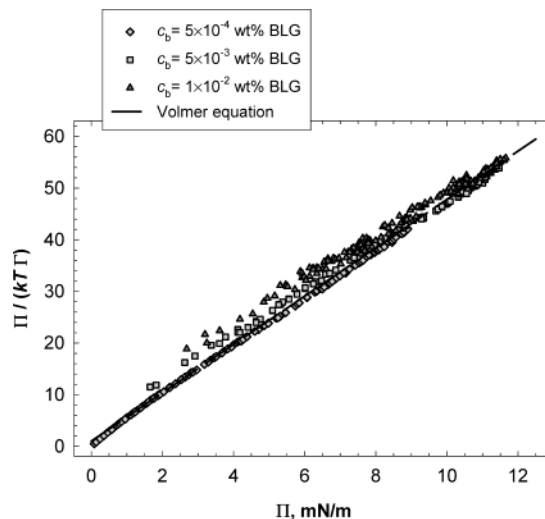
**Figure 3.** Results for the time dependence of the surface pressure of adsorbed BLG layers ( $\Pi = \sigma_0 - \sigma$ , i.e., the decrease of the surface tension,  $\sigma$ , with respect to that at the bare air/water interface).

exceeds  $1 \text{ mg/m}^2$ . The very beginning (the first several seconds) of the adsorption kinetics is actually lost in our measurement. We could not start exactly from time zero because initially the interface was perturbed by the cleaning barrier (Figure 1b), as well as by the insertion of the Wilhelmy plate. Therefore, the three curves in Figure 2 may have different (and unknown) shifts along the abscissa, with magnitudes of the order of seconds. In our interpretation below, however, the time will be excluded to yield the  $\Pi(\Gamma)$  relation, so these time shifts will hardly affect the results for the equation of state which follow from the  $\Pi(\Gamma)$  data.

From Figure 2, one sees that after the initial fast adsorption the process slows down and in a few (around 5) minutes  $\Gamma$  reaches plateau values; it levels off at about  $1.55\text{--}1.60 \text{ mg/m}^2$ . This plateau adsorption is close to what was found in ref 23 by means of neutron reflectivity:  $\Gamma = 1.64 \text{ mg/m}^2$  was measured for BLG monolayers on the air/water boundary, at  $10^{-3}$  and  $10^{-2}$  wt % protein in the bulk.<sup>23</sup>

The time dependence of the surface pressure,  $\Pi(t)$ , measured simultaneously with  $\Gamma(t)$ , is shown in Figure 3. We observe that  $\Pi$  is a linear function of the logarithm of time, at all studied concentrations of BLG. A similar trend of  $\Pi \sim \ln(t)$  was reported by Beverung et al.,<sup>24</sup> for different globular proteins at the interface between heptane and water. It is not our purpose in the present work to explain the peculiar dependence  $\Pi \sim \ln(t)$ . We mention only that a barrier adsorption mechanism is likely to hold, with  $d\Pi \sim e^{-\text{const}\cdot\Pi} dt$  (the latter relation gives  $\Pi \sim \ln(t)$  directly). The exponent suggests existence of a barrier, probably connected with the jumping of molecules from the subsurface onto the interface (see, e.g., ref 25).

Another specific feature of the curves in Figure 3 is the presence of lag time. Especially at a low bulk concentration ( $5 \times 10^{-4}$  wt %), the surface pressure is seen to remain zero while the adsorption has increased to reach values above  $1 \text{ mg/m}^2$ . This phenomenon with globular proteins is known in the literature. Ybert and di Meglio<sup>26</sup> discussed the case of BSA on the water/air interface. It has been



**Figure 4.** Plot of the data from Figures 2 and 3 according to the Volmer equation of state, eq 9. The adsorption,  $\Gamma$ , is taken in units of molecules/cm<sup>2</sup>.

suggested that during the lag time the adsorbed layer is diluted and obeys the two-dimensional ideal gas law. The latter predicts very small changes of the surface tension (below  $0.1 \text{ mN/m}$ ), which are not measurable.<sup>26</sup> Thus, although there are adsorbed molecules on the interface, due to the lack of appreciable interactions the surface pressure remains vanishingly small.

By eliminating the time, we combined our results from Figures 2 and 3, for  $\Gamma(t)$  and  $\Pi(t)$  at different  $c_b$ , to determine the surface equation of state, that is, the relation  $\Pi(\Gamma)$ . We tried to fit those data using the Langmuir isotherm with a single constant parameter,  $\omega$ :  $\Pi = -(kT/\omega) \ln(1 - \omega\Gamma)$ ; however, the equation in this form turned out to be inadequate. The attempt to utilize eq 1 was also unsuccessful. The most appropriate equation for the purpose of  $\Pi(\Gamma)$  fit was the Volmer isotherm, eq 2. We present it as

$$\frac{\Pi}{\Gamma kT} = 1 + \frac{\Pi\alpha}{kT} \quad (9)$$

and draw the corresponding plot in Figure 4. What is remarkable here is that the points of  $\Pi$  and  $\Gamma$  at *different times and bulk protein concentrations* all lie on the same straight line. This fact proves that the physical state of the surface layer is entirely determined by the instantaneous value of the adsorption,  $\Gamma$ . There are no prehistory or aging effects; at each moment of time the surface pressure corresponds to the instantaneous  $\Gamma$ . Thus, for the duration of the experiment the protein molecules residing on the interface do not undergo any noticeable configurational rearrangement and denaturation. The excluded area per molecule,  $\alpha$ , determined from the line in Figure 4, is  $19.3 \text{ nm}^2$ . The latter value will be discussed below in view of the molecular size of BLG.

The fact that eq 2 is the only suitable one-parameter equation is indicative for the physical behavior of the protein molecules in the adsorption layer. The Volmer eq 2 corresponds to nonlocalized adsorption of hard disks which do not interact with any long-range forces.<sup>9,15,27</sup> Equation 2 can be regarded as a particular case of the two-dimensional van der Waals equation of state, with the long-range interaction parameter equal to zero.<sup>15</sup> Hence, one may infer that the adsorbed molecules of BLG

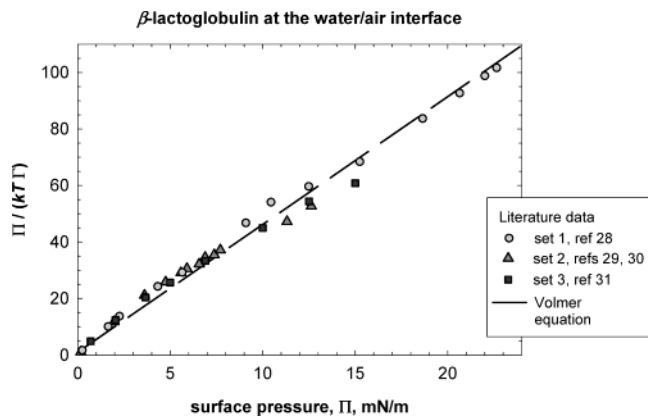
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**Figure 5.** Literature data for BLG at the air/water interface, plotted according to eq 9. Set 1: Equilibrium isotherms for  $\Pi(\alpha_b)$  and  $\Gamma(\alpha_b)$ , from ref 28. Set 2: Dynamic  $\Pi(t)$  and  $\Gamma(t)$  data at  $\alpha_b \sim 10^{-4}$  wt % BLG, from refs 29 and 30. Set 3: Equilibrium  $\Pi(\Gamma)$  isotherm for spread layers of BLG (at pH = 5.6), from ref 31.

behave as hard disks, if the surface coverage,  $\Gamma$ , is not greater than the value corresponding to the monolayer state (i.e.,  $1.64 \text{ mg/m}^2$ , ref 23). We have ellipsometric data showing that at longer times, up to 10 h, nothing happens in the system with  $\alpha_b = 0.01$  wt % BLG;  $\Gamma$  remains practically constant (the layer has reached saturation). In contrast, at higher bulk concentrations one observes complicated phenomena connected with denaturation and/or multilayer formation.

We compare now our findings about the applicability of the Volmer equation with other authors' data for  $\beta$ -lactoglobulin. A graph with such data, plotted according to eq 9, is displayed in Figure 5. We have selected the following three sets of experimental results (sets i and ii pertain to adsorbed layers from bulk aqueous solutions with concentration  $\alpha_b$ , and set iii to spread layers): (i) equilibrium isotherms for  $\Pi(\alpha_b)$  and  $\Gamma(\alpha_b)$ , measured in separate experiments (by axisymmetric drop shape analysis and by ellipsometry, respectively), from ref 28; (ii) dynamic  $\Pi(t)$  and  $\Gamma(t)$  data at  $\alpha_b \sim 10^{-4}$  wt % BLG, obtained simultaneously in a Langmuir trough (by means of a Wilhelmy plate and a radiotracer technique with  $^{14}\text{C}$ , respectively), from refs 29 and 30; (iii) equilibrium  $\Pi(\Gamma)$  isotherm for spread layers of BLG (at pH = 5.6), compressed by a barrier in a trough, from ref 31.

Figure 5 demonstrates that all types of  $\Pi$ - $\Gamma$  data, for equilibrium or dynamic adsorption, or with spread layers, satisfy one and the same surface equation of state: that of Volmer (eqs 2 and 9), with the same value of the adjustable parameter,  $\alpha$ . The latter is determined from the slope of the line in Figure 5. Let us denote  $1/\alpha$  with  $\Gamma_\infty$ ; in the framework of the model eq 2, this is the maximum possible adsorption, at which  $\Pi$  would diverge. The literature data collected in Figure 5 yield

$$\alpha = 18.6 \text{ nm}^2$$

$$\Gamma_\infty = 1/\alpha = 5.38 \times 10^{12} \text{ cm}^{-2} = 1.65 \text{ mg/m}^2 \quad (10a)$$

The results from our measurements (Figure 4) give similar

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values of the relevant parameters:

$$\alpha = 19.3 \text{ nm}^2$$

$$\Gamma_\infty = 1/\alpha = 5.19 \times 10^{12} \text{ cm}^{-2} = 1.59 \text{ mg/m}^2 \quad (10b)$$

We have analyzed also the  $\Pi(\Gamma)$  data of Meinders et al.<sup>11</sup> for spread BLG layers at  $\Gamma < 1.5 \text{ mg/m}^2$ : they comply with eq 9, with  $\alpha \approx 22 \text{ nm}^2$  (which is close to  $\alpha$  in eqs 10). Thus, there is a good agreement between the different types of data with BLG, measured independently with different methods and by different authors. The obtained values of  $\Gamma_\infty$  are close to the monolayer adsorption,  $1.64 \text{ mg/m}^2$  (ref 23). We may conclude that irrespective of the concrete procedure for preparation of the BLG layer on the air/water interface, the protein molecules behave as noninteracting hard disks if  $\Gamma \leq \sim 1.65 \text{ mg/m}^2$ .

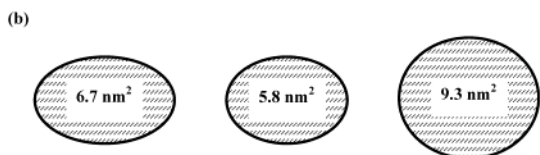
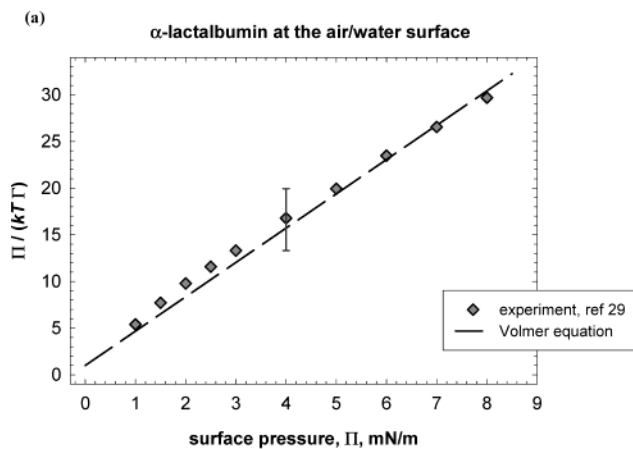
An attempt to rationalize the physical significance of the constant  $\alpha$  can be made in view of the meaning of the parameters in the van der Waals and Volmer equations of state. If the layer is diluted, so that virial expansion of  $\Pi$  with respect to the surface density,  $\Gamma$ , is possible, then the statistical derivation of the van der Waals equation suggests that  $\alpha$  is the excluded area per molecule.<sup>9,15,27</sup> The two-dimensional equation of state for a layer of molecules with finite size is  $\Pi(a - a_{\text{exc}}) = kT$ , where  $a = 1/\Gamma$  is the area per molecule, and the excluded area,  $a_{\text{exc}}$ , is the area around each molecule which is inaccessible for other molecules (taken as a statistical average). If  $d$  is the diameter of a molecule (a hard disk), then  $d$  is the minimum possible center-to-center distance between approaching molecules, see Figure 8a. The inaccessible area (shaded in Figure 8a) is equal to  $\pi d^2$ , and the excluded area per one molecule would be  $a_{\text{exc}} = \pi d^2/2$ . The multiplier (1/2) takes into account the fact that the excluded area effect is due to the mutual interaction between the molecules, that is, each act of exclusion always involves two molecules, A and B. So, only half of the inaccessible area can be ascribed to a given molecule. A comprehensive discussion and proof of these considerations can be found in ref 9 (the chapter for the van der Waals equation of state), as well as in ref 27 and in section 6.3 of ref 15.

Now, the comparison of the "real gas" equation of state,  $\Pi(a - a_{\text{exc}}) = kT$ , with eq 2 yields  $\alpha = a_{\text{exc}} = \pi d^2/2 = 2\omega$ , where  $\omega = \pi d^2/4$  is the disk area, that is, the area physically occupied by one molecule. In this approach, only binary interactions between the particles are accounted for (let us recall that the layer is assumed to be diluted). In a concentrated system of hard spheres, the excluded volume becomes an extremely complicated function of the density (due to overlapping). The problem was explored theoretically in ref 32.

The BLG molecule in a bulk aqueous solution is a sphere with diameter  $3.58 \text{ nm}$ ,<sup>29</sup> which has a cross-sectional area of  $10 \text{ nm}^2$ . If the protein molecules do not undergo significant configurational changes on the water/air boundary, then we would have  $\omega \approx 10 \text{ nm}^2$  and  $\alpha = 2\omega \approx 20 \text{ nm}^2$ , which compares well with the experimental value of  $\alpha \approx 19\text{--}20 \text{ nm}^2$ . The fact that the surface dimension coincides with the bulk dimension of the molecule suggests that substantial denaturation (accompanied with changes in the molecular shape) is not very likely to take place in the undersaturated layers considered above.

The relation  $\alpha = 2\omega$ , with  $\omega$  being the disk area, follows also from eq 1 for diluted layers: at small  $\omega\Gamma$  the denominator of eq 1 becomes  $1 - 2\omega\Gamma$ , so eq 1 transforms into the Volmer eq 2, with  $2\omega$  corresponding to the constant

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**Figure 6.** (a) Literature data for adsorbed  $\alpha$ -lactalbumin on the air/water interface (dynamic  $\Pi(t)$ ,  $\Gamma(t)$  values, at  $c_b = 0.5 \times 10^{-4}$ ,  $1 \times 10^{-4}$ , and  $2 \times 10^{-4}$  wt % protein), taken from ref 29 and plotted according to eq 9. (b) Plane projections of different orientations of the  $\alpha$ -lactalbumin molecule. The three cases correspond to dimensions of  $2.3 \times 3.7$  nm,  $2.3 \times 3.2$  nm, and  $3.2 \times 3.7$  nm, respectively.

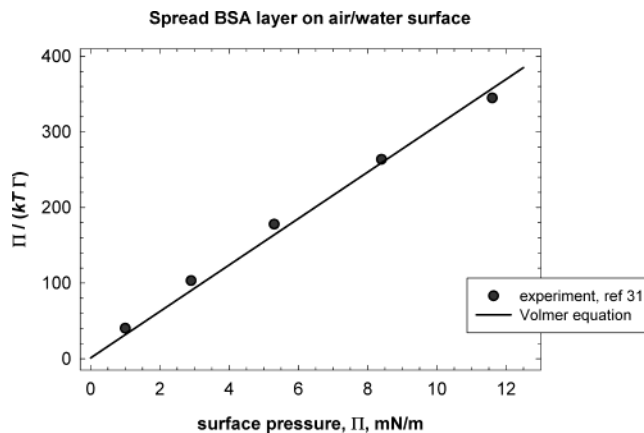
$\alpha$ . However, as emphasized very clearly by Landau and Lifshitz,<sup>27</sup> the van der Waals equation should be regarded as an interpolation formula whose validity should be judged from the comparison with the experiment. It should not be considered to hold for *low* densities only (in gaseous systems). Indeed, in our case the Volmer equation is found to be valid also when the surface layer of BLG is not very diluted.

**4.2. Other Globular Proteins.** The findings with BLG stimulated us to check if diluted layers (with surface coverage up to a monolayer) of other globular proteins would also follow eq 2. Figure 6a shows literature data for adsorbed  $\alpha$ -lactalbumin on the air/water interface, taken from ref 29. Dynamic  $\Pi(t)$  and  $\Gamma(t)$  values (measured by Wilhelmy plate and by radioactivity of  $^{14}\text{C}$ -labeled protein, in a Langmuir trough), at  $c_b = 0.5 \times 10^{-4}$ ,  $1 \times 10^{-4}$ , and  $2 \times 10^{-4}$  wt % protein, are used to extract the  $\Pi(\Gamma)$  relation. Obviously, the Volmer equation is satisfied, and the slope of the straight line in Figure 6a gives

$$\alpha = 14.9 \text{ nm}^2$$

$$\Gamma_\infty = 1/\alpha = 6.72 \times 10^{12} \text{ cm}^{-2} = 1.56 \text{ mg/m}^2 \quad (11)$$

The size of the  $\alpha$ -lactalbumin molecule in a bulk aqueous solution is  $2.3 \times 3.7 \times 3.2$  nm (ref 29). The respective cross-sectional areas, corresponding to different orientations of the ellipsoid, are 6.7, 5.8, and 9.3 nm<sup>2</sup>. For the sake of clarity, the three possible orientations on a surface, with the respective cross-sections, are depicted in Figure 6b. The areas are estimated according to the formula for an ellipse ( $\pi \times 2.3 \times 3.7/4 = 6.7$  nm<sup>2</sup>, etc.). The mean value of the three areas is 7.3 nm<sup>2</sup>. If we suppose that the protein molecule behaves at the surface as a disk with effective area  $\omega \approx 7.3$  nm<sup>2</sup>, corresponding to the three possible states in Figure 6b as an average, then we will obtain for the constant  $\alpha = 2\omega$  a value of 14.6 nm<sup>2</sup>. The



**Figure 7.** Literature data for spread monolayers of BSA on the air/water interface at pH = 5.6 (equilibrium  $\Pi$ – $\Gamma$  values), taken from ref 31 and plotted according to eq 9.

latter estimate is quite close to the experimental value of  $\alpha$  from eq 11,  $\alpha = 14.9$  nm<sup>2</sup>. As an alternative to this interpretation, there is one more possibility to explain the experimental results. Indeed, if the protein molecule is oriented with the side  $2.3 \times 3.7$  nm sitting on the interface (the first case in Figure 6b), then the calculated value of the parameter  $\alpha = 2\omega$  would be  $2 \times 6.7 = 13.4$  nm<sup>2</sup>, which is again close to the experimental value of  $\alpha$  from eq 11,  $\alpha = 14.9$  nm<sup>2</sup>. The other two molecular orientations then seem less probable. In any case, we can conclude that the relation  $\alpha = 2\omega$  is confirmed, similarly to what was found with BLG.

The structure of the  $\alpha$ -lactalbumin molecule has been considered as less stable in solution compared to BLG and more capable of denaturation.<sup>33</sup> Nonetheless, the above results suggest that surface denaturation of lactalbumin (accompanied with substantial molecular shape changes) does not take place, at least in the interval of concentrations and times where the data from Figure 6a have been measured.

BSA is another globular protein whose properties on liquid interfaces have been studied extensively in the literature. We verify the applicability of the Volmer equation (eqs 2 and 9) to equilibrium  $\Pi$ – $\Gamma$  data for spread monolayers of BSA at pH = 5.6 in a trough, taken from ref 31. In Figure 7, we see again a good agreement with the Volmer formula; the adjustable parameter  $\alpha$  is

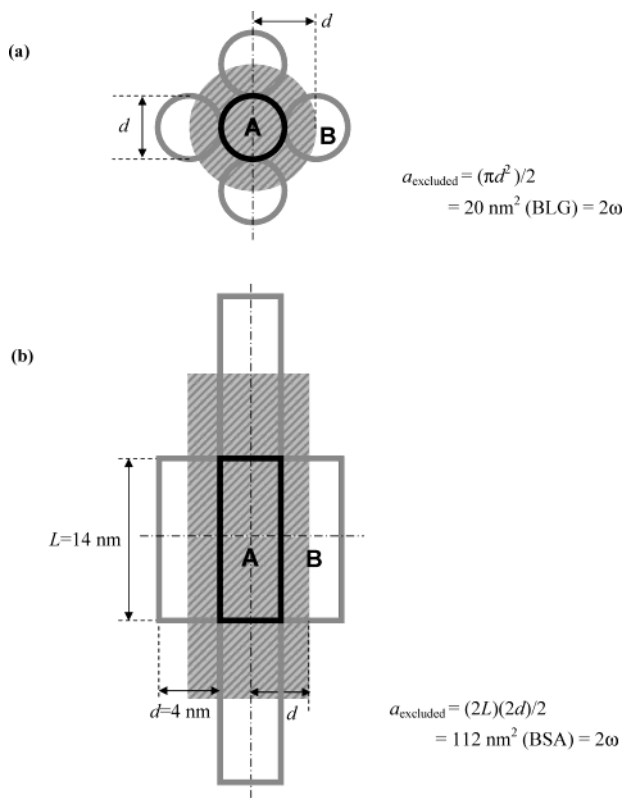
$$\alpha = 124.2 \text{ nm}^2$$

$$\Gamma_\infty = 1/\alpha = 8.05 \times 10^{11} \text{ cm}^{-2} = 0.895 \text{ mg/m}^2 \quad (12)$$

The BSA molecule in a bulk aqueous solution has approximately cylindrical shape, with dimensions  $4 \times 4 \times 14$  nm (ref 34). It has been proven that in relatively diluted layers BSA lies in a side-on position at the liquid boundary.<sup>34</sup> The latter conclusion was based on measurements of the layer thickness by neutron reflection.<sup>34</sup> Taking the molecular size in the bulk state, cited above, we infer that the corresponding cross-sectional area at the surface would be 56 nm<sup>2</sup>. The value of  $\alpha$  from eq 12 is not far from (slightly larger than) twice the latter area ( $2\omega = 112$  nm<sup>2</sup>, if  $\omega \approx 56$  nm<sup>2</sup>); that is, the relation  $\alpha \approx 2\omega$  may turn out to be fulfilled.

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(34) Lu, J. R.; Su, T. J.; Thomas, R. K. *J. Colloid Interface Sci.* **1999**, *213*, 426.



**Figure 8.** Sketches illustrating the excluded area of a molecule: (a) circular shape; (b) rectangular shape. The central molecule, A, occupies an area  $\omega = \pi d^2/4$  (a) or  $\omega = Ld$  (b) on a plane interface. A second molecule, B, comes to a close approach. With empty circles (a) or rectangles (b) we show different possible positions of the second molecule, B, around the central molecule A. The inaccessible area for the center point of B is hatched in gray; the excluded area per molecule is one-half of that area (due to statistical averaging).

Of course, we are aware that the nonspherical shape of the (probably rotating) molecules could infringe the interpretation of the constant  $\alpha$  as being equal to  $2\omega$ , as discussed in section 4.1 above and illustrated in Figure 8a for the case of disks. To clarify this point, we estimate the excluded area for rectangular-shaped molecules, following the reasoning for disks. Figure 8b shows collision of rectangular particles on a surface (with one particular possibility for mutual orientation). The molecule B can take different positions around the central molecule A, and the shaded area in Figure 8b is inaccessible for the center point of B. The corresponding excluded area per molecule is one-half of the inaccessible area, due to statistical averaging (in just the same way as it was in the case of disks, see section 4.1 above). Each of the particles occupies an area  $\omega = Ld$ ; for BSA  $L = 14$  nm and  $d = 4$  nm (the length and the cross-sectional diameter of the cylindrical molecule, respectively);  $\omega = 56$  nm<sup>2</sup>. Thus, the inaccessible area around the central particle A in Figure 8b will be  $(2L)(2d) = 224$  nm<sup>2</sup>, so the excluded area per one molecule is  $a_{\text{exc}} = 2Ld = 112$  nm<sup>2</sup>. There is one more

extreme possibility for mutual orientation of the two molecules A and B upon collision (not shown as a picture), whose contribution to the excluded area can be calculated in a similar manner,  $a_{\text{exc}} = 162$  nm<sup>2</sup>. If all configurations have equal probability, then the average excluded area would amount to 137 nm<sup>2</sup> (that is,  $2.45\omega$ , instead of  $2\omega$  for disks). Comparing with eq 12, we observe that the excluded area obtained from the experimental data,  $\alpha = 2.22\omega$  (viz.,  $124.2$  nm<sup>2</sup> =  $2.22 \times 56$  nm<sup>2</sup>), is in reasonably good agreement with the above assessment. As far as the latter is implemented with  $\omega$  corresponding to the molecular size in the bulk state, we can say that BSA under these conditions is not likely to unfold and denature at the air/water interface.

## 5. Conclusions

In this work, we present experimental results for simultaneously measured surface pressure ( $\Pi$ ) and adsorbed amount ( $\Gamma$ ) of  $\beta$ -lactoglobulin on the air/water interface. From the time-dependent  $\Pi(t)$  and  $\Gamma(t)$  at different bulk concentrations, we eliminate the time and extract the  $\Pi(\Gamma)$  relation, which is well described by the Volmer equation of state. The layer obeys one and the same equation of state irrespective of the surface age and the concentration in the bulk, for values of  $\Gamma$  up to those for a complete monolayer ( $\sim 1.64$  mg/m<sup>2</sup>). The latter fact points to the absence of processes of denaturation and unfolding, aging and prehistory effects; the surface pressure corresponding to a given  $\Gamma$  establishes instantaneously. The Volmer equation suggests that the layer behaves as a collection of noninteracting hard disks.

Our results are compared with literature data for BLG, for equilibrium and dynamic adsorption, and with data for spread layers. In all cases, despite the differences in the way of layer preparation, the Volmer equation is satisfied. There is one adjustable parameter, the excluded area per molecule,  $\alpha$ , whose value is determined from the data fits. The result for  $\alpha$  which follows from our measurements ( $\alpha \approx 19.3$  nm<sup>2</sup>) is in good agreement with the outcome from the other authors' data. If the protein molecule does not undergo substantial unfolding and denaturation upon adsorption, then the area occupied by it on the A/W surface,  $\omega$ , would be equal to about 10 nm<sup>2</sup> (the cross-sectional area in the bulk solution state, which is a sphere). Thus, we observe  $\alpha \approx 2\omega$ , in accordance with the statistical interpretation of the constant parameter,  $\alpha$ , in the Volmer equation.

We discuss other two globular proteins:  $\alpha$ -lactalbumin and BSA. Their layers on the air/water interface, under certain conditions (low coverage and relatively short surface age), also obey the Volmer equation of state. Reasonable values for the excluded molecular area,  $\alpha$ , are obtained from the fits of  $\Pi(\Gamma)$  data taken from literature sources.

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