

Interactions in oil/water/oil films stabilized by β -lactoglobulin; role of the surface charge

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Dedicated to Professor Ivan B. Ivanov (LCPE, University of Sofia) on the occasion of his 70th birthday.

Abstract

In this work we investigate emulsion films (oil/water/oil), stabilized by β -lactoglobulin (BLG). Isotherms of disjoining pressure versus the film thickness are measured experimentally, at different pH (4.0, 5.2, and 6.5), and ionic strength. The data are fitted successfully with the classic theory of DLVO (assuming superposition of electrostatic repulsion and van der Waals attraction). One adjustable parameter, the surface potential, is determined from the best fit; the results are used to calculate the surface charge density in the films. At the pH of 5.2 (which is the isoelectric point, pI , in the bulk solution), the interface is charged. Possible reason is the conformational change, which the protein undergoes upon adsorption. At bulk pH of 4.0, the BLG-laden oil/water interface is close to isoelectric state (the surface charge density is very low). Under these conditions, there is evidence for long-range steric repulsion, possibly due to favored aggregation at the interface. In some cases, after eventual collapse of the repulsion, we observe formation of spots of very thin Newton Black Films (NBF). Addition of inorganic salt, NaCl, leads to increase of surface charge (up to a certain limit). This effect is derived both from results with thin liquid films, and from zeta-potentials of emulsion drops. At the “natural” pH of 6.5, with 150 mM ionic strength, the extent of charging of adsorbed BLG is considerably lower than that in the bulk aqueous solution. Thus, also at pH 6.5, the charge state of the protein molecules residing on the oil/water interface is significantly influenced by the conformational transformations that accompany the adsorption. The emulsion films with BLG are less stable than the foam films, and rupture without overcoming a barrier (maximum) in the disjoining pressure isotherm. The latter fact implies that certain weakness of the interfacial layer is brought about by the contact with the oil phase (hydrophobization).

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1. Introduction

Thin liquid films of the type oil/water/oil are important for the stability of O/W emulsions, because such films form between drops which are pressed against each other either by some external force (for example, buoyancy), or due to restricted volume. The conditions for film formation, and its consequences, are discussed in [1]. Emulsion- and foam-type films are convenient as a model system that allows quantitative determination of the surface forces (or the “disjoining pressure”, Π), as a function of the thickness, h . Molecular interactions of different origin, van

der Waals, electrostatic, steric, structural, hydration, protrusion, etc. [2] contribute to Π and influence to a large extent the film behavior (thinning and stability). One of the preferred experimental methods for precise measurement of Π versus h involves film formation in a porous glass cell (also called Mysels cell, or Exerowa-Scheludko cell); it is suitable both for foam-type films [3] and for emulsion films [4]. Depending on the pore size and the interfacial tension, one can reach capillary and disjoining pressures as high as thousands of Pascals [4].

β -Lactoglobulin (BLG) is the major whey protein in the milk; its stabilizing role in films and emulsions is a topic of practical interest in view of the numerous food applications. Air/water/air films with BLG were investigated in [5]. The measured surface force at larger thicknesses ($h > \sim 24$ nm) was well represented as a superposition of van der Waals attraction and electrostatic

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repulsion, while in thinner foam films a steep steric repulsion arose [5]. In an earlier work, Clark [6] reported equilibrium thicknesses of about 25 nm for foam films and ~ 35 nm for emulsion films with 0.02 wt.% BLG. Some other results with thin aqueous films containing proteins (not specifically disjoining pressure data, $\Pi(h)$) are reviewed by van Aken et al. [7]. Isotherms $\Pi(h)$ for foam films in the presence of β -casein and BSA are reported in [8].

Our purpose in this work is to carry out a systematic study of the surface forces in emulsion films stabilized by BLG. We measured disjoining pressure isotherms, $\Pi(h)$, at three different values of pH (4.0, 5.2, which is the isoelectric point in the bulk solution, and 6.5, the “natural” pH when protein is just dissolved in water). The concentration of inorganic salt, NaCl, was also varied.

Let us estimate, as an order of magnitude, the typical pressures exerted on the drops by buoyancy, in an O/W emulsion with oil volume fraction, φ , of approximately 0.7, and mass density difference $\Delta\rho \approx 0.1 \text{ g/cm}^3$ between the two phases. An emulsion column of 10 cm height (H) will develop pressure $\Delta\rho g\varphi H \approx 70 \text{ Pa}$ (the gravity acceleration, g , is 981.55 cm/s^2). The disjoining pressures, Π , measured by us, are of this order (see Section 4 below). Hence, such data would bear relevance, for example, to stabilization of concentrated emulsions during shelf storage.

We focus on the following particular tasks related to the $\Pi(h)$ data obtained for films with 0.02 wt.% BLG:

- To reveal what interactions are important in the films. For this purpose, we fit the disjoining pressure curves with the known theory of electrostatic and van der Waals forces. In most cases the agreement is good; the surface potential and the surface charge in the film are deduced from one adjustable parameter in the fits.
- To analyze how the electric charge state of the adsorbed protein at the oil/water interfaces is influenced by the pH and the salt; to explore if it is different from the charge state in the bulk solution. Specifically, we have to clarify whether the surface isoelectric point is the same as the pI in the aqueous phase (the latter is equal to 5.2).
- To compare the behavior and stability of emulsion and foam films, and to see whether their rupture is associated with passing over a barrier (maximum) in the curve $\Pi(h)$.

Determination of surface charge through fitting of force versus distance data (obtained by means of surface force apparatus) was accomplished by Sivasankar et al. [9], who investigated the pH-dependent electrostatic properties of a single binding face of streptavidin. Thin film balance (porous plate method) was used in [10], where the surface charge was found by fitting of disjoining pressure in films stabilized by alkyl glucosides. We basically follow the data management procedures implemented by those authors.

The question about the charge state of adsorbed protein, and how it differs from the protein in bulk solution, bears significance for many biomolecular interactions. Such are, for instance, the directed ligand associations, kinetic binding rates, bio-fouling,

etc. [9]. All these phenomena are sensitive to electrostatic interactions, since the energy is affected; they underlie applications related to molecular recognition and detection, sensor design, etc. The methods employed here may be helpful for determination of protein's surface charge under various conditions (pH, electrolytes, mixed adsorption layers), and in presence of specific ligands.

2. Materials and methods

2.1. Materials

The used protein was β -lactoglobulin from bovine milk, product of Sigma, lyophilized, Cat. No. L-0130, Lot No. 20K7043, mixture of A and B variants. Aqueous solutions with concentration of 0.02 wt.% were made. The “natural” pH of the solution, without additives for controlling it, was measured to be 6.5. We needed also solutions with pH lower than the natural pH; those were prepared by addition of small portions of diluted solution of HCl by means of a micropipette. Thus, the required pH was not surpassed. The ionic strength that corresponds to the set pH is taken into account as a contribution to the total ionic strength (Section 3.1).

All solutions were prepared with deionized water, purified by a Milli-Q system (Millipore). In some cases we added inorganic electrolyte, NaCl (Merck), in the aqueous phase, with concentrations 0.001, 0.002, 0.005, and 0.010 M. All solutions were used immediately after preparation. The pH was measured both before and after the experiments, and exhibited insignificant variation.

The oil phase was always soybean oil (from a local supplier). It was purified by passing through a glass column filled with alumina adsorbent (Florisol F101), as described by Gaonkar [11].

2.2. Methods

2.2.1. Liquid films in a porous glass cell

Equilibrium forces in thin liquid films are gauged by the disjoining pressure, Π , which is a function of the distance of separation, or film thickness, h . At equilibrium, and in the flat portion of the film, the disjoining pressure, Π , is equal to the capillary pressure, P_c . The latter is a measurable quantity, and represents the difference between the pressure in the outer phase that surrounds the film (in our case, oil), and the bulk liquid pressure in the Plateau border, i.e., in the aqueous phase from which the film is made.

The force law (the disjoining pressure isotherm, $\Pi(h)$) was measured with a thin-film balance, using a modified version of the porous-plate Mysels cell [4]. The experimental cell is shown schematically in Fig. 1. The film is formed by sucking out the inner liquid phase (aqueous solution). The cell is directly connected to a pressure transducer (Omega PC 136G01), the reference pressure being the atmospheric one. This configuration provides the opportunity to investigate both emulsion-type and foam-type films. The measured value of the pressure, P_m , should be corrected for the hydrostatic pressure difference between the plane of the film and the level of the measuring membrane of the

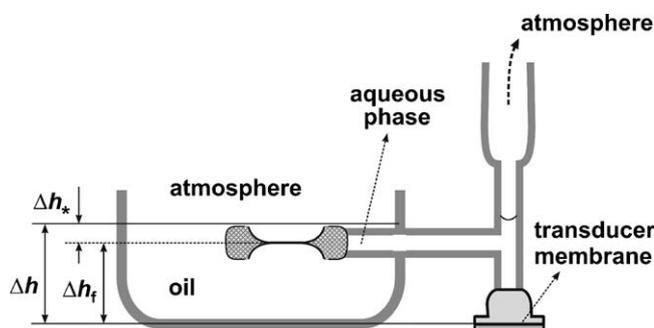


Fig. 1. Schematic presentation of the experimental porous-plate (Mysels) cell, suitable for emulsion and foam films up to high capillary pressures.

transducer (see Fig. 1). That difference is obtained by calibration in the following way.

The porous cell is first loaded with the aqueous solution, and the beaker, which contains the porous cell is filled up with oil (Fig. 1); no film is made, and the aqueous layer in the cell is thick. In mechanical equilibrium, the measured “calibration” pressure is:

$$P_{\text{cal}} = -\rho_a g \Delta h + \rho_o g \Delta h_* + \rho_w g \Delta h_f \quad (1)$$

where ρ_a , ρ_o and ρ_w are the mass densities of air, oil, and water, respectively, and the heights Δh , Δh_* and Δh_f are designated in Fig. 1. Then, in the presence of a thin emulsion film in the cell, the measured running pressure accounted by the transducer is:

$$P_m = -\rho_a g \Delta h + \rho_o g \Delta h_* - P_c + \rho_w g \Delta h_f \quad (2)$$

therefore, from Eqs. (1) and (2) we see that the capillary pressure in question is simply

$$P_c = P_{\text{cal}} - P_m \quad (3)$$

In the case of foam films (air/water/air), the outer phase is gaseous. Both the calibration and the experiments have been performed in a saturated atmosphere in order to prevent the evaporation of water from the film and the porous cell. Some amount of the aqueous solution (a few ml) is put in the beaker, which contains the porous cell (Fig. 1), with the liquid level being below the edge of the cell. A glass plate is used as a lid to cover the vessel. The pressure calibration is carried out by using the same procedure as above; P_{cal} is found with a thick aqueous layer. The air pressure inside the beaker is equal to the atmospheric pressure. After a thin film is formed, Eq. (3) holds.

Special care should be taken when one fixes the transducer to the cell. The liquid column inside the connecting glass tube should be free from any air bubbles. Otherwise, the measured value of the pressure will be incorrect.

The maximum and minimum capillary pressures obtainable with the porous-plate technique are determined by the geometric characteristics of the porous holder. In our experiments, we used a cell in which the diameter of the hole that enclosed the film was equal to 1.0 mm; the nominal pore size was about 16 μm . The maximum capillary pressure P_c that could be reached was limited by entry of oil/water menisci into the pores of the Mysels cell. The emulsion films in all studied systems with β -lactoglobulin ruptured at pressures lower than that limit.

The cell was attached on the table of a Jenavert microscope (Carl Zeiss Jena), employing a special home-made device, which provided a possibility to adjust the horizontality of the cell by means of two screws. The latter adjustment was necessary in order to ensure the plane of the film to coincide with the focal plane of the objective. This is a condition for reliable determination of the film thickness. The microscope was equipped with a long focal distance objective (Zeiss, 20 \times) and with a monochromatic light source. The experiments were visualized by means of a highly sensitive video camera Sony SSC-C370P, and a VCR Panasonic AG-7335. The film thickness (effective water thickness) was determined via standard interferometry [12,13]. The intensity of the reflected light is connected with the film thickness, h , by the expression:

$$h = \frac{\lambda}{2\pi n} \left(l\pi \pm \arcsin \sqrt{\frac{I - I_{\text{min}}}{I_{\text{max}} - I_{\text{min}}}} \right) \quad (4)$$

where I_{max} and I_{min} denote the maximal and minimal intensity of the reflected light, $l=0, 1, 2, \dots$ is the order of the interference maximum, λ is the wavelength of the incident light (546 nm in our case), and n is the refractive index of the liquid forming the film. A photo-multiplier tube was employed to determine the intensity of the reflected light with great precision. Thus, the film thickness, h , was calculated with maximum uncertainty of about ± 1 nm (in the range $h \geq \sim 20$ nm). Since we measure directly and independently the disjoining pressure (i.e., P_c) and the film thickness, we are able to evaluate the disjoining pressure versus distance isotherms, $\Pi(h)$. The method allows us to explore only the repulsive branches of the disjoining pressure curves.

Appropriate experimental procedure was contrived so as to attain equilibrium values of Π and h : after the initial film formation, the pressure P_c was increased in small steps (1–2 Pa). After each step, the change of the thickness h was monitored until a constant value was reached (that usually happened for 10–15 min), and the latter value was taken as the equilibrium h . In some runs, the $\Pi(h)$ curve was scanned backwards, by decreasing the pressure P_c in steps of 1–2 Pa, and waiting for the increasing thickness to equilibrate. The observed hysteresis of h (increasing versus decreasing P_c) was minor, the differences in the thickness for a given Π did not exceed ~ 2 nm.

All measurements were performed at the ambient temperature of 23 ± 0.5 °C. For each set of experimental conditions we carried out several independent runs of $\Pi(h)$ measurement. Representative curves are shown in Section 4 below.

2.2.2. ζ -Potentials

We prepared O/W emulsions with 10% volume fraction of soybean oil. The aqueous phase contained BLG at a concentration of 0.02 wt.%; sodium azide (0.1 g/l) was added to prevent bacterial contamination. Systems with different amount of inorganic salt, NaCl (0, 10, 75, 150, and 250 mM) were studied. The dispersions were formulated by intensive stirring of corresponding volumes of the protein solution and the soybean oil, by means of a rotor-stator homogenizer Ultra Turrax T25 (Janke & Kunkel GmbH, IKA-Labortechnik), operating at 13,500 rpm. The duration of stirring was fixed at 5 min for all emulsions. The mean

Sauter diameter of the produced emulsion drops, d_{32} , was about 40 μm ; the size distribution was rather polydisperse. It has been previously verified that after 3 min of homogenization the mean drop size does not change upon further stirring. The dependence of the ζ -potential of the drops on the electrolyte concentration in the aqueous phase was investigated, at the “natural” pH of 6.5. This was done with a fraction of smaller drops in the emulsion (with diameters below $\sim 5 \mu\text{m}$), in order to avoid sedimentation during the measurement of ζ . Such a fraction was obtained by keeping the emulsion at rest for some time after the stirring, so as to ensure that the large drops had separated.

The electrophoretic mobility, U_E , was measured on a Zeta-sizer II C equipment (Malvern Instruments, Ltd., England), following the guidelines from Sections 3.1, 5.1 and 5.2 in the Instruction Manual. The measurements in the low-salt systems, below 100 mM NaCl and without added NaCl, were accomplished in a PC4 cell, after careful adjustment of the position of the scattering volume with respect to the glass wall of the cylindrical capillary containing the sample. This was necessary in order to avoid the electroosmotic effect. The dispersions with salt concentration above 100 mM NaCl could not be measured in the standard PC4 cell for low ionic strength, because of the large electric current. A special thin ($\sim 1 \text{ mm}$) glass capillary, with wall covered by hydrophilic polymer (to prevent electroosmosis), PC3 cell, was used for those experiments. The cell was first rinsed with electrolyte solution, and subsequently, with protein solution. The ζ -potential was determined according to the simple Smoluchowski formula, $U_E = (\varepsilon\zeta/4\pi\eta)$, where ε , η denote the dielectric permittivity and the dynamic viscosity of the medium, respectively.

3. Disjoining pressure according to the DLVO theory

Below we will examine if the experimentally obtained disjoining pressure isotherms, $\Pi(h)$, would comply with the classical theory of DLVO [14,15], in the case of emulsion films stabilized by BLG, at pH values of 4, 5.2, and 6.5. In the DLVO framework, the disjoining pressure is represented as a sum of contributions from van der Waals and electrostatic interactions between the film surfaces. Here follows a short summary of the relevant equations, necessary for data interpretation, and explanation of the fitting procedure.

3.1. Electrostatic interactions

We denote by ϕ the dimensionless electric potential, $\phi = e\Psi/(k_B T)$, where e is the electronic charge, $k_B T$ is the thermal energy, and Ψ is the absolute value of the potential. Integration of the Poisson–Boltzmann equation in a plane-parallel film yields [14,15]:

$$\left(\frac{d\phi}{dx}\right)^2 = 2\kappa^2[\cosh\phi(x) - \cosh\phi_m] \quad (5)$$

in the case of 1:1 electrolyte. Here x is the coordinate perpendicular to the film surfaces, ϕ_m refers to the plane in the middle of the film (where $d\phi/dx=0$ due to the symmetry), and κ rep-

resents the inverse Debye screening length; $\kappa = \kappa_c\sqrt{c_{el}}$, with $\kappa_c = 0.001338 \text{ cm}^{1/2}$ (a constant), and c_{el} being the salt concentration in cm^{-3} .

Let ϕ_0 be the potential at the onset of the diffuse part of the electric double layer, at each one of the two opposing film surfaces. We ascribe $\phi_0 = \phi[x=0] = \phi[x=h]$. The planes where $\phi = \phi_0$ lie very close to the respective physical surfaces [16], so we accept that h is approximately equal to the thickness of the aqueous core of the film. The balance of electric charge in the film is written with account for the condition of total neutrality, and leads to the relation [14]:

$$\left(\frac{d\phi}{dx}\right)_{x=0} = -\frac{1}{2}\kappa_c^2\Gamma_{ch} \quad \left(\frac{d\phi}{dx}\right)_{x=h} = \frac{1}{2}\kappa_c^2\Gamma_{ch} \quad (6)$$

where Γ_{ch} is the surface charge density, expressed as number of elementary charges e (without sign), per unit area. From Eqs. (5) and (6) one derives

$$\Gamma_{ch} = \frac{2}{\kappa_c} \sqrt{2c_{el}(\cosh\phi_0 - \cosh\phi_m)} \quad (7)$$

Eq. (7) will be used below for evaluation of Γ_{ch} .

The electrostatic disjoining pressure, Π_{el} , can be found from a simple formula which connects it with ϕ_m [14,15]:

$$\Pi_{el} = 2c_{el}k_B T[\cosh(\phi_m) - 1] \quad (8)$$

(Eq. (8) suggests that Π_{el} may be identified as the excess osmotic pressure of ions in the middle of the film.) On the other hand, ϕ_m depends on the film thickness h in a straightforward manner. Integration of Eq. (5) from $x=0$ to $x=h/2$ gives:

$$\frac{\kappa h}{2} = \int_{\phi_m}^{\phi_0} \frac{d\phi}{\sqrt{2\cosh\phi - 2\cosh\phi_m}} \quad (9)$$

Eq. (9) can be cast in an equivalent and more convenient form with the use of the Incomplete Elliptic Integral of the First Kind [14,15]:

$$\frac{\kappa h}{2} = K \int_0^{t_0} \frac{dt}{\sqrt{1-t^2}\sqrt{1-K^2t^2}} = KF(t_0, K) \quad (10)$$

where

$$\frac{1}{K^2} = 1 + \left[\sinh\left(\frac{\phi_m}{2}\right)\right]^2, \quad t_0^2 = 1 - \left[\frac{\sinh(\phi_m/2)}{\sinh(\phi_0/2)}\right]^2$$

We adopt a procedure for calculation of Π_{el} versus h that involves one free parameter, the surface potential, ϕ_0 . After specifying a value for ϕ_0 , we use Eq. (9) or (10) to determine ϕ_m for a given film thickness, h . Then, Eq. (8) provides the corresponding value of Π_{el} for this h . The curve $\Pi_{el}(h)$ is obtained under the condition for constant surface potential, that is, ϕ_0 is independent of h . According to Derjaguin [14], this regime ensures minimum loss of free energy due to the electrostatic repulsion, compared to the regimes of constant surface charge or charge regulation. The films which we investigate in this work are thick, in the sense that κh is always larger than ~ 2.5 . In such a case, the quantitative difference between the regimes of constant potential or charge becomes negligibly small [14]. The fact that we assume $\phi_0 = \text{const.}$ leads to a slight dependence of Γ_{ch} upon the

film thickness, h (see Eq. (7), where ϕ_m depends on h). However, for all studied systems this variation of Γ_{ch} is less than a few percent, so it will be disregarded.

3.2. Van der Waals interactions

For the van der Waals force we use a description that takes into account the finite thickness of the protein layers on the two film surfaces [17]. We envisage the system oil/adsorbed protein layer of thickness δ /aqueous core of thickness h /adsorbed protein layer of thickness δ /oil. For such films (of plane-parallel geometry), the van der Waals disjoining pressure, Π_{vdW} , is given by [17]:

$$\Pi_{vdW} = \frac{1}{6\pi} \left[\frac{A_{pr-w-pr}}{h^3} - \frac{2A_{o-pr-w}}{(h+\delta)^3} + \frac{A_{o-pr-o}}{(h+2\delta)^3} \right] \quad (11)$$

The respective compound Hamaker constants, A_{i-j-k} , pertain to the interaction in a system that consists of a layer of phase j sandwiched between two adjacent phases i and k (pr = protein, w = water, o = oil). A combining relation [14,18] is used to express A_{o-pr-w} :

$$A_{o-pr-w} = A_{pr-w-pr} - A_{pr-w-o} \quad (12)$$

Values of the necessary constants, $A_{pr-w-pr}$, A_{o-pr-o} , and A_{pr-w-o} , are taken from [18]:

$$A_{v=0}^{pr-w-pr} = 0.645 k_B T; \quad A_{v>0}^{pr-w-pr} = 2.455 k_B T; \quad (13a)$$

$$A_{v=0}^{o-pr-o} = 0.005 k_B T; \quad A_{v>0}^{o-pr-o} = 2.325 k_B T; \quad (13b)$$

$$A_{v=0}^{pr-w-o} - A_{v=0}^{pr-w-pr} = 0.010 k_B T;$$

$$A_{v>0}^{pr-w-o} - A_{v>0}^{pr-w-pr} = -1.570 k_B T \quad (13c)$$

Polar and dispersion components [17] are designated by $v=0$ and $v>0$, respectively. The effect of electrostatic screening of the polar interactions across aqueous solution of salt is taken into account by using a correction for $A_{v=0}^{pr-w-pr}$, according to the formula [17], [18]:

$$A_{pr-w-pr} = A_{v=0}^{pr-w-pr} (1 + 2\kappa h) \exp(-2\kappa h) + A_{v>0}^{pr-w-pr} \quad (14)$$

The polar components in Eqs. (13b) and (13c) are negligible, so A_{o-pr-o} and $(A_{pr-w-o} - A_{pr-w-pr})$ are set equal to the dispersion components.

In the case of foam films, oil is replaced by air (subscript a); $A_{a-pr-a} = A_{pr-pr} = 23.42 k_B T$ [18] (for interaction of protein layers across vacuum). Application of Eq. (11) requires that A_{a-pr-w} should be known; it is found from combining relations:

$$2 A_{a-pr-w} = A_{pr-pr} - A_{w-w} + A_{pr-w-pr} \quad (15)$$

where $A_{w-w} = 0.71 k_B T (1 + 2\kappa h) \exp(-2\kappa h) + 8.28 k_B T$ for the water phase [17].

The effective thickness of the protein adsorption layer is taken to be $\delta = 1.5$ nm, as suggested by the neutron reflection results reported in [19]. Thus, the van der Waals disjoining pressure, Eq. (11), is calculated without any free/adjustable parameters.

The theoretical isotherm of total disjoining pressure, $\Pi = \Pi_{vdW} + \Pi_{el}$, as a function of h , Eqs. (8) and (11), is fitted to the

experimentally measured data, using one adjustable parameter, the surface potential ϕ_0 (or Ψ_0 in mV).

4. Results and discussion

Fig. 2 displays a series of four $\Pi(h)$ isotherms obtained at pH 5.2 with different concentrations of NaCl. What we see is that in all cases the agreement between the experiment and the DLVO calculation is satisfactory. In the presence of 1, 2, and 5 mM NaCl the plots are linear in the scale $\ln \Pi$ versus h (Fig. 2a–c). Such a behavior is in agreement with the asymptote of the electrostatic disjoining pressure in the limit of weak overlapping of the diffuse layers at the two film surfaces [14,15]:

$$\Pi_{el} \approx 64c_{el} k_B T \left(\tanh \frac{\phi_0}{4} \right)^2 \exp(-\kappa h) \quad (16a)$$

$$\ln \Pi_{el} = \text{const.} - \kappa h \quad (16b)$$

The slopes of the lines in Fig. 2a–c practically coincide with the corresponding values of the inverse Debye screening length, κ , as suggested by Eq. (16b). Indeed, in Fig. 2a, the slope is $9.9 \times 10^5 \text{ cm}^{-1}$, and $\kappa = 10.4 \times 10^5 \text{ cm}^{-1}$; in Fig. 2b, the slope is $1.42 \times 10^6 \text{ cm}^{-1}$, and $\kappa = 1.47 \times 10^6 \text{ cm}^{-1}$; in Fig. 2c, the slope is $2.24 \times 10^6 \text{ cm}^{-1}$, and $\kappa = 2.32 \times 10^6 \text{ cm}^{-1}$. One can conclude that the predominant interaction in these films is the electrostatic repulsion. Contingent influence of steric forces (which are also exponential versus h) should be ruled out. The decay length of the electrostatic repulsion is equal to the Debye screening length, and the latter depends on the salt concentration; so do the slopes of the lines in Fig. 2a–c. At higher salt (Fig. 2d), the van der Waals attraction becomes significant (the plot is curved).

Another important aspect of the results in Fig. 2 is that the protein adsorption layers at pH 5.2 are charged (the concrete values of Ψ_0 will be discussed below). In bulk aqueous solution, the molecules of BLG are electrically neutral at this pH, since the isoelectric point, pI , has been reported to lie at 5.2 [20]. Therefore, it seems likely that the conformational changes, which accompany the adsorption lead to a shift in the isoelectric point. The charged state of the protein on the oil/water boundary is different from that in the bulk.

Fig. 3 shows disjoining pressure curves at the “natural” pH of 6.5 (no acid or base has been added to the solution). The measured data are in compliance with the DLVO theory. The situation changes when the pH is set to 4.0 (see Fig. 4). The isotherms $\Pi(h)$ presented in Fig. 4 exhibit peculiar behavior. The electrostatic and van der Waals interactions (DLVO) are predominant at thicknesses larger than ~ 23 –24 nm. In thinner films a steep repulsion emerges (Fig. 4); its origin is probably from steric interaction. The films with $h = 23$ –24 nm are much thicker than the size of the individual molecule of β -lactoglobulin; the latter is a sphere with diameter of 3.58 nm [21]. A plausible physical mechanism for explanation of this relatively long-range repulsive force is the existence of protein aggregates residing on the two opposing film surfaces. As suggested by Tcholakova et al. [22], chain-like aggregates can extend and dangle in the aqueous phase. Steric repulsion is brought about when such chain-like protein aggregates at each surface experience hindrance from

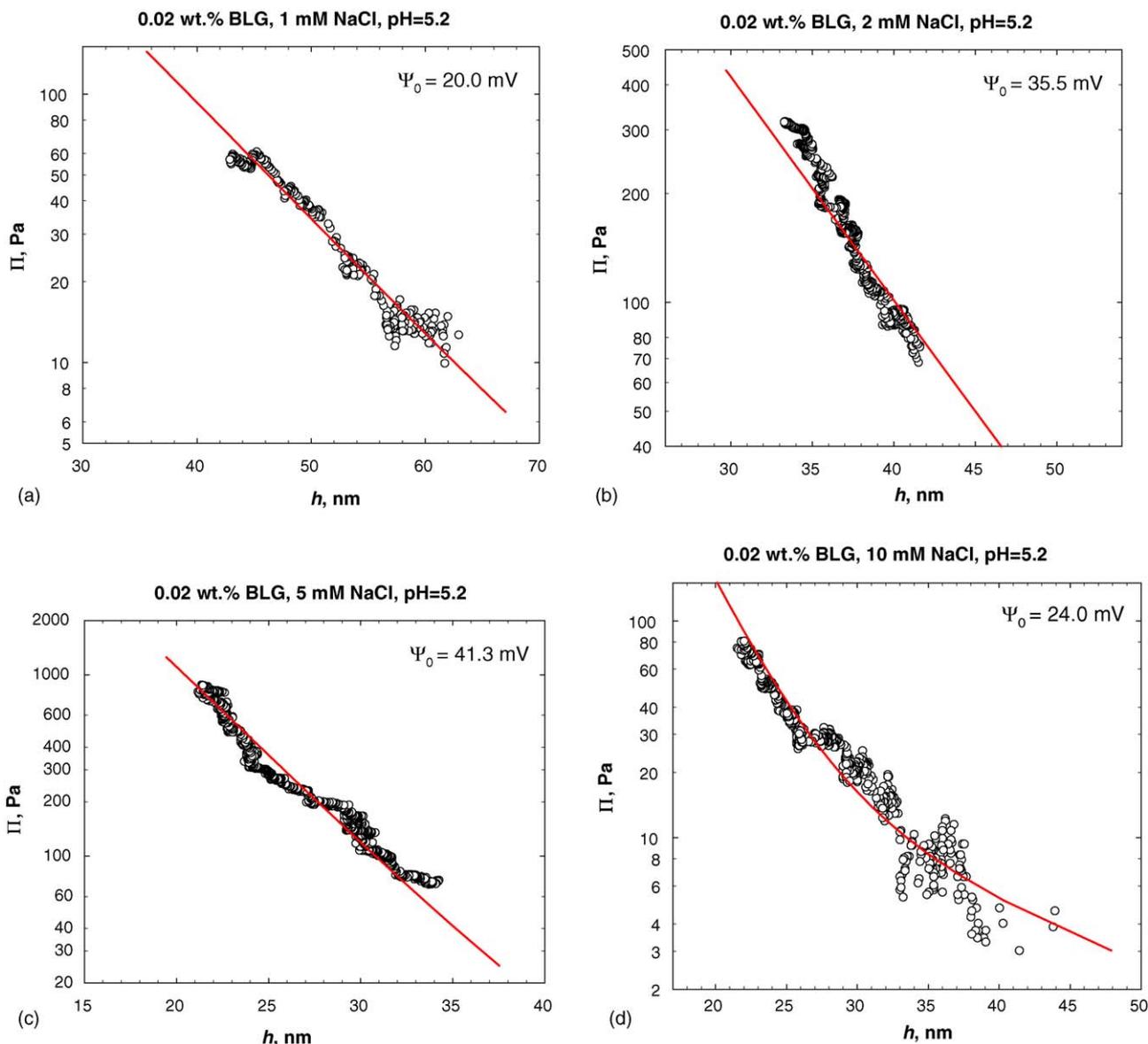


Fig. 2. Disjoining pressure isotherms of emulsion films stabilized by BLG at pH 5.2, with different concentrations of NaCl: (a) 1 mM; (b) 2 mM; (c) 5 mM; (d) 10 mM. The films rupture at the highest measured value of Π . The lines represent the best DLVO fits.

those attached at the other surface. More detailed description of this interaction, with numerical estimates, is provided in [22]. Thus, the aggregation of BLG on the liquid boundary seems to be favored at pH 4.0.

From the above, we can infer that pH 4.0 should be close to the isoelectric pH of the adsorbed protein on the oil/water interface (while in the bulk $pI = 5.2$). Such a hypothesis is supported by direct observation of emulsion films, by means of interference microscopy. It turns out that in some films, at pH 4.0, a thickness transition occurs, with step-wise formation of very thin Newton Black Film (NBF). This is illustrated in Fig. 5b and c. The NBF is essentially a bilayer; shortly after its appearance the film ruptures. The pictures in Fig. 5b and c are taken just before the rupture.

At pH values different from 4.0 we did not register formation of spots of Newton Black Film. Fig. 5 shows films at pH 3.0 and 5.2 (cases a, d). They stay relatively thick, due to the electrostatic

stabilization. On the other hand, one can envisage that at pH 4.0 the surface charge is low (see below), and the aggregation is promoted, so the films are stabilized at $h \sim 23\text{--}24$ nm by steric repulsion; the aggregates which are responsible for this may eventually collapse and/or be expelled from the film, and then NBF forms (Fig. 5b and c); afterwards the film ruptures.

Let us now discuss the charge state of the protein on the film interfaces. For this purpose we will use the surface potential, ϕ_0 , determined from the best fit of experimental $\Pi(h)$ data with the DLVO theory. The charge per unit area, Γ_{ch} , is calculated from ϕ_0 through Eq. (7), and the results are plotted in Fig. 6. As expected, the charge density at pH 4.0 is very low, which substantiates our conjecture that this pH is close to pI on the O/W boundary.

At pH 5.2 and 6.5, Fig. 6 reveals a trend for increase of Γ_{ch} upon addition of salt, NaCl. The effect is well pronounced at lower concentrations of NaCl; at pH 5.2 with 10 mM NaCl, Γ_{ch}

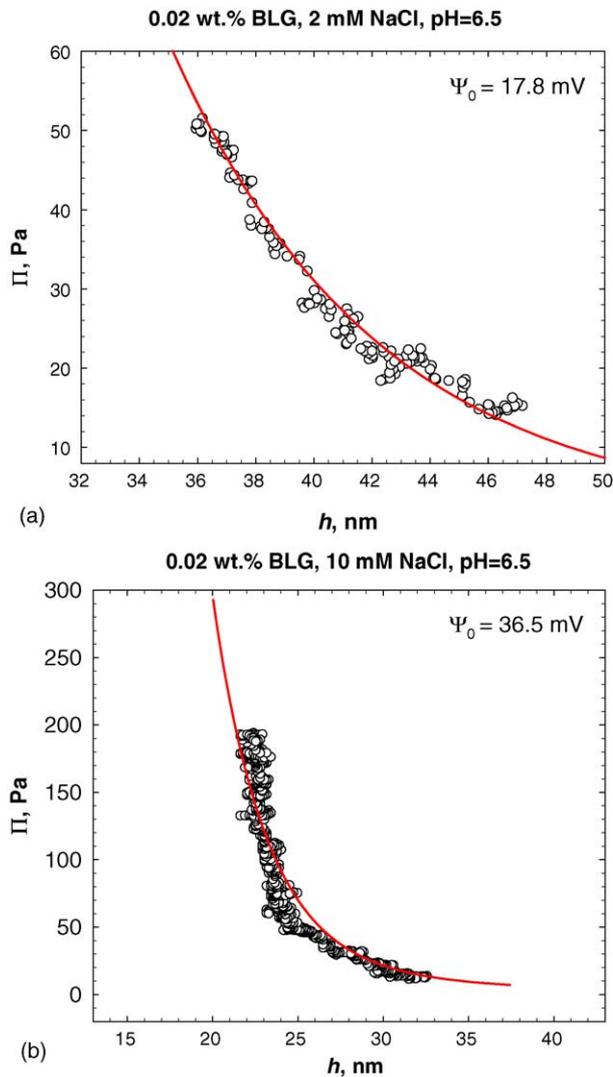


Fig. 3. Disjoining pressure isotherms of emulsion films stabilized by BLG at the natural pH (without adjustment), that is, at pH 6.5; (a) 2 mM NaCl; (b) 10 mM NaCl. The curves represent the best DLVO fits.

becomes slightly lower (Fig. 6). These results indicate a possible influence of the inorganic salt upon the protein configuration and charge state on the interface. We decided to verify the trend in Γ_{ch} by independent measurements of ζ -potentials of emulsion drops, as a function of the NaCl content. The experimental data are listed in Table 1; they are included also in Fig. 7.

Table 1
 ζ -Potentials of soybean oil-in-water emulsion drops, stabilized by 0.02 wt.% β -lactoglobulin

Concentration of added NaCl (mM)	ζ -Potential (mV)
0	-52.0
10	-35.0
75	-19.7
150	-15.0
250	-9.6

The aqueous phase contains 0.1 g/l NaN_3 (an antibacterial agent); the pH is 6.5 ("natural").

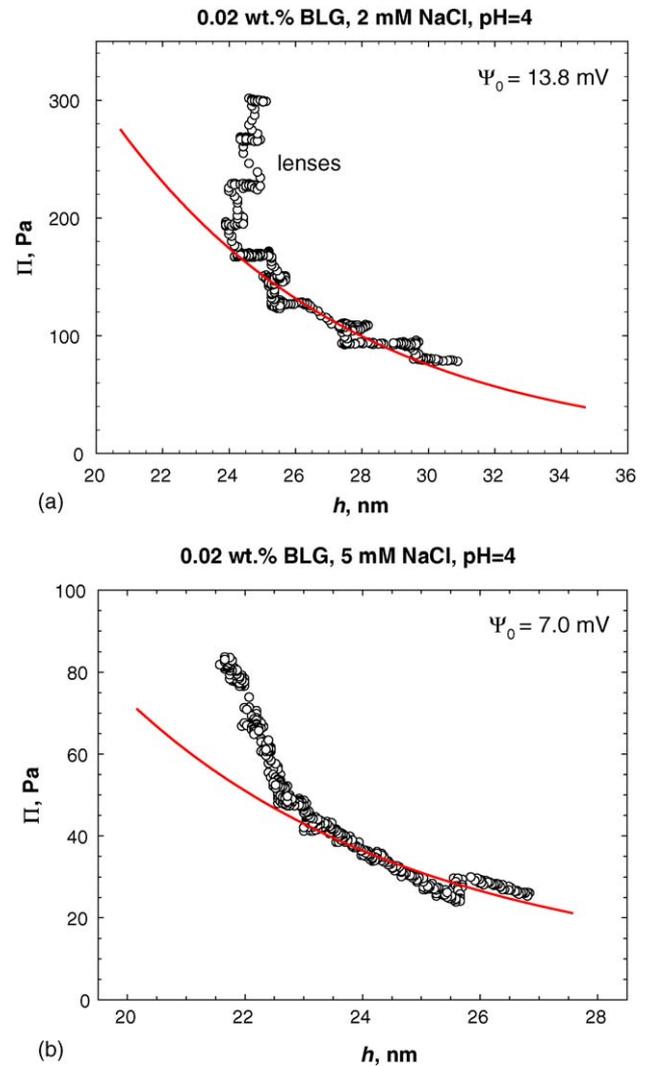


Fig. 4. Disjoining pressure isotherms of emulsion films stabilized by BLG at pH 4.0; (a) 2 mM NaCl; (b) 5 mM NaCl. The curves represent the best DLVO fits.

In order to estimate the surface charge from the ζ potential, we need to know how far from the physical interface lies the shear plane (to which ζ refers). Different cases have been described in the literature. If the shear plane is away to a distance $\sim 1/\kappa$ from the surface [23], this would lead to a significant difference between Ψ_0 and ζ . On the other hand, it was discussed by Tcholakova et al. [22] that with protein (BLG) the shear plane is most likely placed very close to the outer Helmholtz plane (i.e., $\Psi_0 \approx \zeta$). To resolve this question for our systems, it is pertinent to compare values of Ψ_0 , determined from $\Pi(h)$ in films, with the ζ potential of drops. Such a comparison can be made for the system at pH 6.5 (natural), with 10 mM NaCl. The fitting of $\Pi(h)$ isotherms in films gave $\Psi_0 = 46.0$ mV and 36.5 mV (for two experimental curves, corresponding to the two square points in Fig. 6 at 10 mM salt), while $|\zeta| = 35$ mV from Table 1. The values of Ψ_0 , $|\zeta|$ are close; the deviation is consistent with a distance between the shear plane and the outer Helmholtz plane [$\approx \kappa^{-1} \ln(\Psi_0/|\zeta|)$] from zero to no more than ~ 8 Å. For our purposes this is insignificant, so we assume $\Psi_0 \approx \zeta$ and calculate

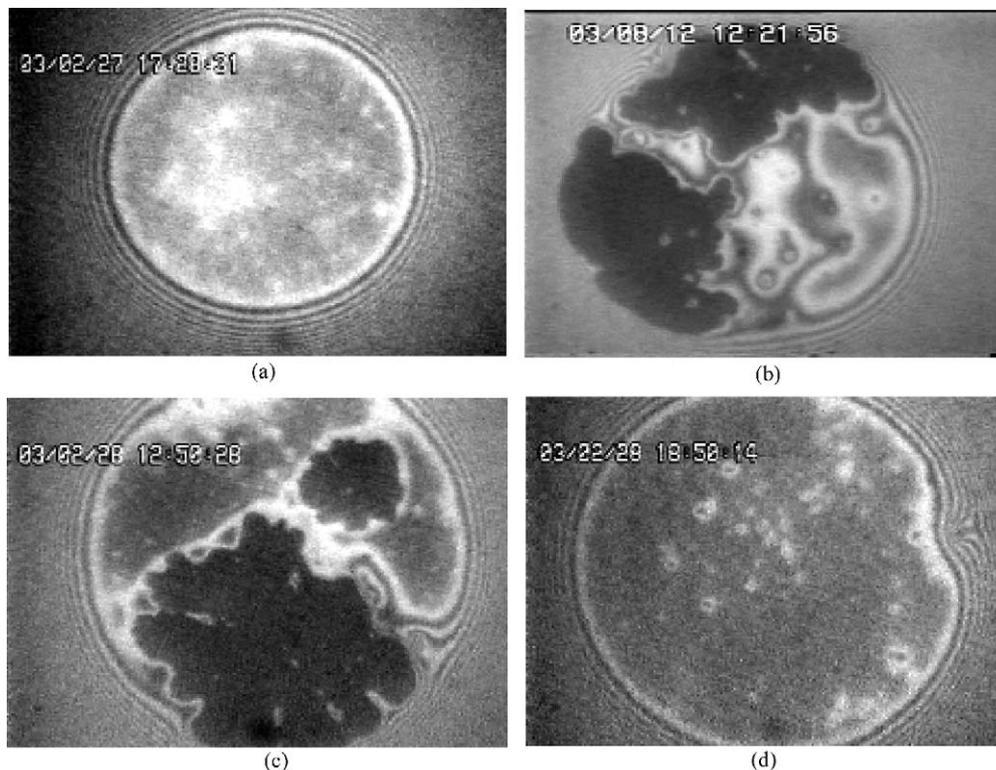


Fig. 5. Interference pictures of emulsion films stabilized by 0.02 wt.% BLG, observed in reflected monochromatic light. (a) pH 3.0; (b), (c) at pH 4.0 (note the very thin and dark portions of Newton Black Film); (d) pH 5.2.

Γ_{ch} from the relation [24]

$$\Gamma_{\text{ch}} = \frac{4}{\kappa_c} \sqrt{c_{\text{el}}} \sinh \frac{\phi_0}{2} \quad (17)$$

Eq. (17) is a corollary from Eq. (7), in which we put $\phi_m = 0$ (single surface, not a film). The results for Γ_{ch} , together with the measured $|\zeta|$, are presented in Fig. 7. One observes the peculiar trend of Γ_{ch} first ascending with the increase of the NaCl content, and next, slightly descending. This behavior is similar to what was found from the film data at pH 5.2, Fig. 6. Evidently, some influence of the salt on the charge state of the adsorbed BLG takes place.

The maximum surface charge density of about 1.4×10^{-11} mol/cm² with 100–150 mM NaCl (Fig. 7) can be discussed in view of the charged state of BLG in the bulk solution, at this pH 6.5. According to [20], at ionic strength of 150 mM KCl and pH 6.5 there are about 5 negative charges per one molecule of BLG in solution. On the other hand, at monolayer coverage of the liquid interface, the adsorption is about 1.6 mg/m² BLG [19], equal to 8.9×10^{-12} mol/cm². With $\Gamma_{\text{ch}} \approx 1.4 \times 10^{-11}$ mol/cm², we deduce that an average of 1.6 charges per molecule are available on the O/W boundary. Therefore, adsorbed molecules of BLG are charged to a considerably lesser extent than those in the bulk solution (under the conditions of “natural” pH in the bulk, and in the presence of 150 mM salt). A plausible mechanism to explain this fact may be the following: Initially, charged molecules from the bulk (pH 6.5) come to the interface and adsorb; the consequent surface electric field attracts counterions (H⁺), and the local pH at the surface decreases; the protein

responds by gradually binding some H⁺; finally, an equilibrium state is established, with the adsorbed protein molecules being less charged than those in the bulk.

With surface potential, Ψ_0 , of about 40 mV at pH 6.5 (see above), the shift of pH (bulk-surface) is $0.68 = \log_{10} [\exp(e\Psi_0/k_B T)]$; the resulting pH at the oil/water boundary, 5.8, would correspond to ~ 3.2 charges per one protein molecule, if

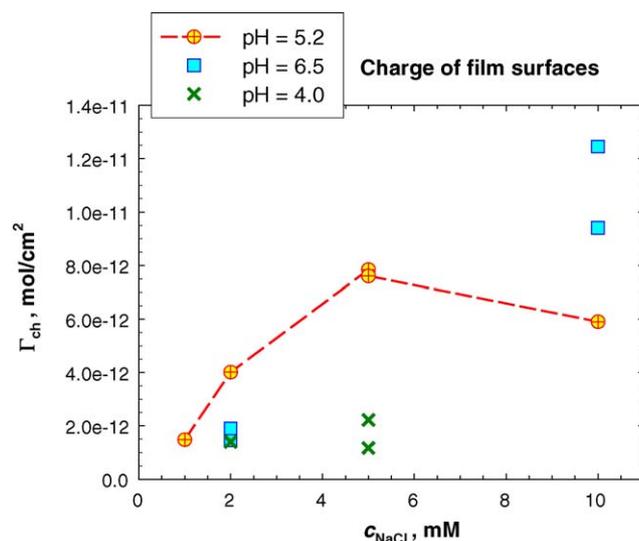


Fig. 6. Electric charge density on the O/W interfaces of emulsion films, calculated from the surface potentials according to Eq. (7). The surface potentials were determined from the best DLVO fits of $\Pi(h)$ data. Each point in Fig. 6 corresponds to one $\Pi(h)$ isotherm (the results from Figs. 2–4 are included).

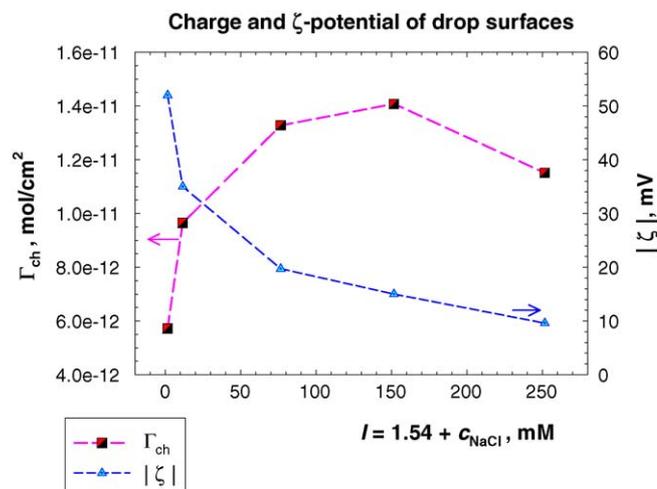


Fig. 7. Electric charge density on the O/W interfaces of emulsion droplets, calculated from the ζ -potentials according to Eq. (17). The measured ζ -potentials are listed in Table 1.

BLG is in dissolved state in the aqueous phase (cf. Fig. 9 in [20]). This is still higher than the actual surface charge density determined from Ψ_0 in films, ~ 1.6 charges per molecule. The disparity can be attributed to different conformation of the protein residing on the interface, in comparison with the bulk solution. It is worthwhile to point out that levels of charging even lower than 1 charge per molecule (Fig. 6) may be responsible for the predominant stabilizing force, Π_{el} , in the emulsion films.

One should be aware that the above mechanism of “charge regulation” cannot be operative at pH 5.2. This is the isoelectric point in the *bulk*, while the adsorption layer of BLG is charged (Fig. 6). So, conformational changes in the molecules upon adsorption are the most likely reason for the differences in the bulk-surface charge state at pH 5.2.

Finally, let us comment on some results with foam films (air/solution/air). As a rule, the disjoining pressure in such films is again described well by the DLVO theory. Fig. 8 shows a sample curve $\Pi(h)$ in a system at pH 5.2 with 2 mM NaCl. The air/water surfaces are charged, similarly to the case of oil/water interfaces at this pH (equal to pI in the bulk). An essential difference between the foam and emulsion films is that the former ones are much more stable than the latter. As illustrated by Fig. 8, the foam films exist at considerably higher pressures than the emulsion films under the same other conditions (compare with Fig. 2b). We did not observe rupture of the film from Fig. 8 (the upward arrow indicates that the pressure may continue to increase, the film remains stable, but measurements had to be stopped because of entry of menisci into the glass cell pores). Similar high stability was observed for other foam films made at pH 5.2 and 6.5, with different salt concentrations.

In contrast, all *emulsion* films from Figs. 2–4 rupture at the point when the highest measured value of Π is reached. Moreover, the instability is not connected with passing over a barrier (maximum) in $\Pi(h)$. One can suppose that weakness of the interfacial layers is caused by the contact of the adsorbed protein with the oil phase, which leads to hydrophobization.

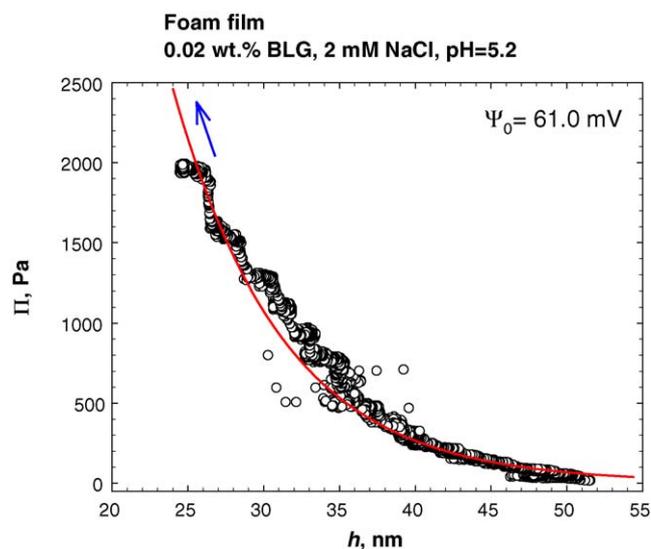


Fig. 8. Disjoining pressure isotherm of foam film stabilized by BLG at pH 5.2, in the presence of 2 mM NaCl. The line represents the best DLVO fit.

A problem that deserves thorough investigation concerns the charge state of the globular protein (BLG), when it is adsorbed on air/water interface (or in foam films). It has been pointed out in the literature that significant difference can exist between the configurations of the protein molecules on air/water and oil/water boundaries. Murray et al. [25] found that BLG appeared to be more unfolded and more flexible at the O-W interface. It is likely that such differences may lead to a concomitant shift in the charge state. In his review on thin films, Clark [6] mentioned a dissimilar behavior of BLG on A-W and O-W surfaces, and quoted smaller thicknesses of foam films compared to emulsion films (25 and 35 nm, respectively, under specific conditions). We did not study systematically the pH dependence of the surface charge in foam films; what can be said from Fig. 8 is that pH 5.2 is definitely not isoelectric at the air/water interface. It remains to be clarified by further studies how the isoelectric point will shift upon adsorption of BLG on the A-W boundary.

5. Conclusions

The interactions in emulsion films stabilized by BLG are in quantitative agreement with the DLVO theory; predominant is the electrostatic repulsion, in superposition with the van der Waals attraction. At the pH of 5.2 (which is the isoelectric point, pI , in the bulk solution), the interface is charged. Possible reason is the conformational change, which the protein undergoes upon adsorption. At bulk pH of 4.0, the BLG-laden oil/water interface is close to isoelectric state. Evidence for this comes from: (i) the determined low surface charge density; (ii) the manifestation of long-range steric repulsion, possibly due to favored aggregation at the interface; and (iii) the formation of spots of very thin Newton Black Films.

Experimental disjoining pressure isotherms, $\Pi(h)$, are fitted with the DLVO theory using one adjustable parameter, the potential at the onset of the diffuse layer, Ψ_0 . From the latter

we estimate the surface charge density, Γ_{ch} , on the two opposing film interfaces. Addition of inorganic salt, NaCl, leads to increase of Γ_{ch} (up to a certain limit). This effect is derived both from results with thin liquid films (measured $\Pi(h)$), and from ζ -potentials of emulsion drops. At the “natural” pH of 6.5, with 150 mM ionic strength, the extent of charging of adsorbed BLG is considerably lower than that in the bulk aqueous solution. This conclusion remains valid when pH is recalculated to account for its local change in the vicinity of the charged liquid boundary. Thus, also at pH 6.5 it can be said that the charge state of the protein molecules residing on the oil/water interface is significantly influenced by the conformational transformations that accompany the adsorption.

One may envisage importance of the latter effect for many biomolecular interactions, which are sensitive to electrostatic forces, in relation to phenomena like ligand associations, molecular recognition, binding rates, etc.

The emulsion films with BLG are less stable than the foam films, and rupture without overcoming a barrier (maximum) in the disjoining pressure isotherm, $\Pi(h)$. This fact indicates that the instability may be connected with certain weakness of the interfacial layer, brought about by the contact with the oil phase (hydrophobization).

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