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The role of additives for the behaviour of thin emulsion films stabilized by proteins

Krastanka G. Marinova [a], Theodor D. Gurkov [a,*], Orlin D. Velev [a], Ivan B. Ivanov [a], Bruce Campbell [b], Rajendra P. Borwankar [b]

[a] *Laboratory of Thermodynamics and Physico-Chemical Hydrodynamics, University of Sofia, Faculty of Chemistry, James Boucher Avenue 1, Sofia 1126, Bulgaria*

[b] *Kraft General Foods, Inc., Technology Center, 801 Waukegan Road, Glenview IL 60025, USA*

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Abstract

Experimental results obtained with thin aqueous films of emulsion type stabilized by bovine serum albumin (BSA) and β-casein are presented. The film behaviour is time dependent. The contact angle increases with ageing and exhibits pronounced hysteresis. With BSA one observes slow reversible aggregation on the surface (but not in the bulk) and the protein lumps are gradually squashed by the capillary pressure as the film thins. The findings can be explained by slow surface denaturation, accompanied by developing attraction and partial entanglement of the BSA molecules. These processes are promoted by oleic acid dissolved in the oil phase. Electrostatic interactions were found to be important: without salt the films remain thick, whereas in the presence of 0.15 M NaCl one obtains Newton black films whose contact angle depends upon the molecular charge. A marked difference in the surface mobility is observed with foam and emulsion films stabilized by BSA. Lenses, containing protein aggregates and liquid, when surrounded by an area which has reached the black film stage, remain entrapped in foam films but are slowly squeezed out in emulsion films. Hydrophobization of the protein molecules may be responsible for this behaviour. With β-casein, ageing effects in films are observed only at the isoelectric point. This protein strongly aggregates in the bulk, but the lumps are readily flattened on the film interfaces. Addition of Ca^{2+} ions leads to a decrease in film thickness, depending on the concentration. © 1997 Elsevier Science B.V.

Keywords: Additives; Emulsion films; Proteins; Specific interactions

1. Introduction

Proteins have an important role as emulsion stabilizers in the food industry and in many other practical applications. Their properties and performance can be regulated by the environmental conditions: pH, ionic strength and presence of additives. As pointed out by Voutsinas et al. [1], the emulsifying efficiency of many proteins increases with increasing degree of hydrophobicity of the molecule. On the other hand, the stability of an emulsion is closely related to the behaviour of the liquid film which forms when two drops (of not exceedingly small size) approach each other. The present work is devoted to investigation of aqueous films immersed between oil phases, a system which models the contact between the drops in oil/water (o/w) emulsions. We are interested in the time evolution of the film properties and how it is modified by additives which interact specifically with proteins.

* Corresponding author. e-mail: theodor.gurkov@ltph.cit.bg

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We used two proteins, bovine serum albumin (BSA) and β-casein. The molecular structure of both is well known in bulk water solution, but has not yet been definitely determined for molecules adsorbed on liquid interfaces. BSA is a globular protein with the form of a prolate ellipsoid of revolution whose dimensions are 41.6 × 140.9 Å (at pH 7) [2,3]. The structure consists of three domains held by 17 disulphide bonds. The latter are relatively stable, thus ensuring the ability of the molecule to expand and unfold rapidly and reversibly under denaturing conditions [2]. The isoelectric point is about 4.8 [2]. At pH 7 there are 18 negative charges located in the first and second domains (the third domain is neutral). One of the most important features of BSA is its strong affinity to hydrophobic ligands, especially fatty acids. The surface of hydrophobic amino acid residues faces the interior of each domain, and two hydrocarbon chains can easily penetrate inside the cylindrical hole. Thus, one protein molecule can attach six fatty acids (C_{15}–C_{18}) with very high affinity, and many others more loosely [3]. In general, the presence of bound fatty acids is believed to stabilize the native form of the protein, restraining it from denaturation and aggregation [2].

When adsorbed on a water/air or oil/water interface, BSA usually forms multilayers. Graham and Phillips [4] have shown that the surface load gradually increases with increasing concentration, and the thickness of the layer is about 90 Å at 10^{-3}–10^{-2} wt.% in the bulk. Results from different studies have provided evidence that slow rearrangement, partial denaturation with unfolding and entanglement due to hydrogen bonding accompany the adsorption of globular proteins (in particular BSA) [5–10]. Actually, the process develops in three steps: diffusion, adsorption with overcoming of potential barriers and rearrangement [6]. The last stage is the slowest, with a timescale of several hours [6]. Most tightly bound to the surface is the first layer of molecules [8]. It is dense and partially unfolded, but the disulphide bridges remain intact [9]. The degree of denaturation is not high, which has been confirmed by circular dichroism spectroscopy [9]. In general, the protein layer can be regarded as a dense two-dimensional system of interacting deformable particles [9]. The surface viscosity of liquid boundaries layered with globular proteins is very high [10], indicating the existence of a strong attraction between the molecules, and even the formation of a network and viscoelastic gel-like structure [5,7,10].

In contrast to BSA, β-casein has a disordered flexible molecule which does not contain any disulphide cross-links [9,11]. Its nature is amphiphilic, the isoelectric point is around 5 and at pH 7 it carries about 15 negative charges [9]. The adsorption of casein on water/oil or air/water surfaces leads to monolayer formation. The structure of the latter has been studied by neutron reflectivity [11]. A dense, thin inner layer (~2 nm) and a more diffuse, thicker outer layer (~5–7 nm) were distinguished [11]. These results can be interpreted in the context of the so-called "train–loop–tail model" [9,12]. Much of the adsorbed casein is directly associated with the hydrophobic surface; the remaining hydrophilic segments extend and dangle into the water [9]. The surface shear viscosity is low compared with that of most globular proteins [9,10], which points to the existence of very weak intermolecular interactions in the case of casein.

We should particularly mention the ability of β-casein to bind Ca^{2+} ions with high affinity. Calcium cations attach most easily to the negatively charged phosphoserine residues of the protein, and possibly also to free carboxylate groups [13]. Such binding has important consequences: in the presence of Ca^{2+} the thickness of the protein layer and the amount adsorbed on the air/water boundary turn out to be smaller [13]; the formation of casein aggregates in the bulk solution is highly promoted even by minor quantities of Ca^{2+} [14]; flocculation is induced in o/w emulsions stabilized by casein [15]. No significant ageing effects have been noticed, either in the adsorbed layer on interfaces [13] or with the bulk aggregates [14], with or without Ca^{2+}.

Studies on thin liquid films containing proteins have been reported. Foam films (water in air) were mostly explored. It has been found [16,17] that stable black foam films could be obtained with various proteins if the conditions were such that

the molecule was close to zero charge, i.e. at the isoelectric point, and with a very small salt content. When the proteins were charged, the films stayed thicker and had a grey colour [16,17]. The presence of protein aggregates, entrapped between the surfaces, can significantly change the properties and the stability of thin emulsion films [18]. Comparison with our results for emulsion films in the presence of BSA and casein is discussed below.

Evidence of ageing effects was presented by Proust et al. [19]. Foam films with bovine mucin did not exhibit contact angle hysteresis, but contained aggregates whose quantity and size increased with time. In contrast, free aqueous films with BSA showed pronounced hysteresis of the contact angle, which was within 2–3° initially, and rose to 25–30° after ageing [19]. The Newton black films finally became "solid-like" [19], which meant that any variation of the capillary pressure did not modify the diameter and the film thickness because the two interfaces had stuck firmly and irreversibly. A similar phenomenon led to the entrapment of liquid in the form of a lens in the central region of black foam films [20]. The lens gradually disappeared, the process being slowed with ageing [20]. This behavior was described as typical for very thin films with proteins [20]. In the latter case, in contrast to low molecular weight surfactants, the surfaces are partially or completely immobilized. Mixtures of surfactant and protein normally have an intermediate surface mobility [20].

The interactions between two layers of human serum albumin adsorbed on mica have been studied using the surface force technique [21]. The authors observed attraction and adhesion whose magnitude increased with time of contact. The data were interpreted as a slow denaturation process, induced by the pressure exerted on the layers [21].

This work is devoted to the investigation of emulsion films stabilized by BSA and β-casein. Interferometry in reflected light was used for film observation and measurements. We monitored the equilibrium and the hysteresis contact angles and the film thickness. Particular attention was paid to the ageing effects, connected with the process of protein aggregation. The role of additives was studied. Some experiments were performed in the presence of oleic acid, dissolved in the oil phase. The fatty acid was expected to bind specifically to BSA, and not influence the properties of β-casein significantly. The role of the calcium ions, which interact preferentially with casein, was explored. Results obtained with and without addition of inorganic electrolyte (NaCl) were compared. The results provide information on the importance of the electrostatic interactions. This study may be helpful in view of the stability of emulsions containing proteins, when fine control over the properties of the system is usually required.

2. Materials and methods

2.1. Materials and samples

Lyophilized BSA, p.a. grade, essentially fatty acid free, was purchased from Sigma and was used without further purification. β-casein from bovine milk from Sigma was also used as received. All solutions were prepared with deionized water obtained from a Milli-Q system (Millipore). The concentration of BSA was fixed at 0.015 wt.% and that of β-casein at 0.010 wt.%. Both correspond to saturated protein layers on oil/water interfaces [4]. Xylene (isomeric mixture, p.a.) was chosen as the oil phase. In some experiments oleic acid (p.a., Sigma) was dissolved in the oil at a concentration of 0.1 wt.%. When addition of Ca^{2+} ions was desired, $CaCl_2 \cdot 2H_2O$ from Aldrich (ACS reagent) was used.

The ionic strength was varied by adding NaCl (Merck, analytical grade) to the aqueous solutions. The salt had been preliminarily heated for 5 h at 450°C in order to remove any organic contaminants. Two types of systems stabilized by BSA were studied: without any inorganic electrolyte and with 0.15 M NaCl. Casein solutions free from Ca^{2+} contained either 0.15 or 10^{-3} M NaCl; in the presence of Ca^{2+} the total ionic strength was kept constant at 0.15 M. The pH was adjusted by addition of small quantities of hydrochloric acid (Merck, p.a. grade). When BSA was dissolved in water (at the above-mentioned concentration), the pH was found to be ca. 6.4 ± 0.1. That was the highest pH studied, and no additional reagents were then used for pH adjustment. With casein we

worked at two different pH values, 5.0 (the isoelectric point) and 6.5, which were set by means of HCl and NaOH.

2.2. Interferometric measurements in thin liquid films of emulsion type

The experimental set-up is presented schematically in Fig. 1. The cell is a modification of that proposed by Scheludko and Exerowa [22]. Films were formed by sucking out aqueous phase from a biconcave meniscus held in a glass capillary of inner radius 1.49 mm, immersed in the oil. The cell was mounted on the table of a microscope (Zeiss Axioplan). In order to keep the pressure inside the meniscus constant, we used a pressure control system consisting of two microsyringes and a buffer. The observations were carried out in reflected monochromatic light (wavelength 546 nm) through the optically clear cover of the cell. Images were recorded by means of a CCD camera with linear response to the incoming light (Sony, XC-77). We connected a VCR and a computer supplied with a "Targa+" grabbing board (Fig. 1). Using laboratory developed software, we registered the time changes in the intensity of light which was reflected from a small spot in the film (the size and the position of this spot can be adjusted). The thickness, h, was calculated from the following relationship [23,24]

$$h = \frac{\lambda}{2\pi n_s}(l\pi \pm \arcsin\sqrt{\Delta}) \qquad (1)$$

$$\Delta = \frac{I - I_{min}}{I_{max} - I_{min}}$$

where $\lambda = 546$ nm, l is the order of the interference, n_s is the refractive index of the liquid in the film, I is the instantaneous value of the reflected light intensity in the spot and I_{min} and I_{max} denote the minimum and the maximum values of I. Eq. (1) implies that the film should be conceived as an effective aqueous layer with uniform properties, especially refractive index. Such an assumption may seem dubious for proteins because the liquid interfaces are actually loaded with layers in which the volume fraction changes in the normal direction, and so does n_s. Nevertheless, with thick films the influence of this inhomogeneity is negligible, and for Newton black films one can still accept the uniform layer representation, using different n_s. Besides, the variations of the refractive index are relatively small.

The contact angle, θ, was found from the positions of the Newton fringes around the film periphery (see e.g. Fig. 2). Black and white fringes correspond to thicknesses in multiples of $\lambda/(4n_s)$. We used a computer program that fits the observed fringe locations with the numerical solution of the Laplace equation by the procedure used by Dimitrov et al. [24]. There are three parameters to be varied so as to attain minimum deviation between the experimental data and the calculation. The capillary pressure, the exact film radius, and the contact angle were determined from the best fit. It should be mentioned that the last two quantities are defined at the point of intersection between the extrapolated meniscus profile and the midplane of the film. For a given film, we averaged the results obtained by accounting the positions of the Newton fringes in several directions.

The equilibrium contact angle, θ, is an important film characteristic owing to its direct connection with the energy of interaction between the surfaces, W {see Eq. (106) in Ref. [25]}:

$$W = 2\sigma(\cos\theta - 1) - \Pi h = \int_h^\infty \Pi(z)\,dz \qquad (2)$$

where σ is the interfacial tension and $\Pi(h)$ denotes the disjoining pressure in the film [25].

3. Results and discussion

3.1. Emulsion films containing BSA and excess inorganic electrolyte, 0.15 M NaCl

In this case well pronounced ageing effects are operative.

3.1.1. Experiments carried out immediately after loading of the two phases in the cell

We first made films without ageing. Irrespective of the pH (varied from 3.8 to 6.4), the films

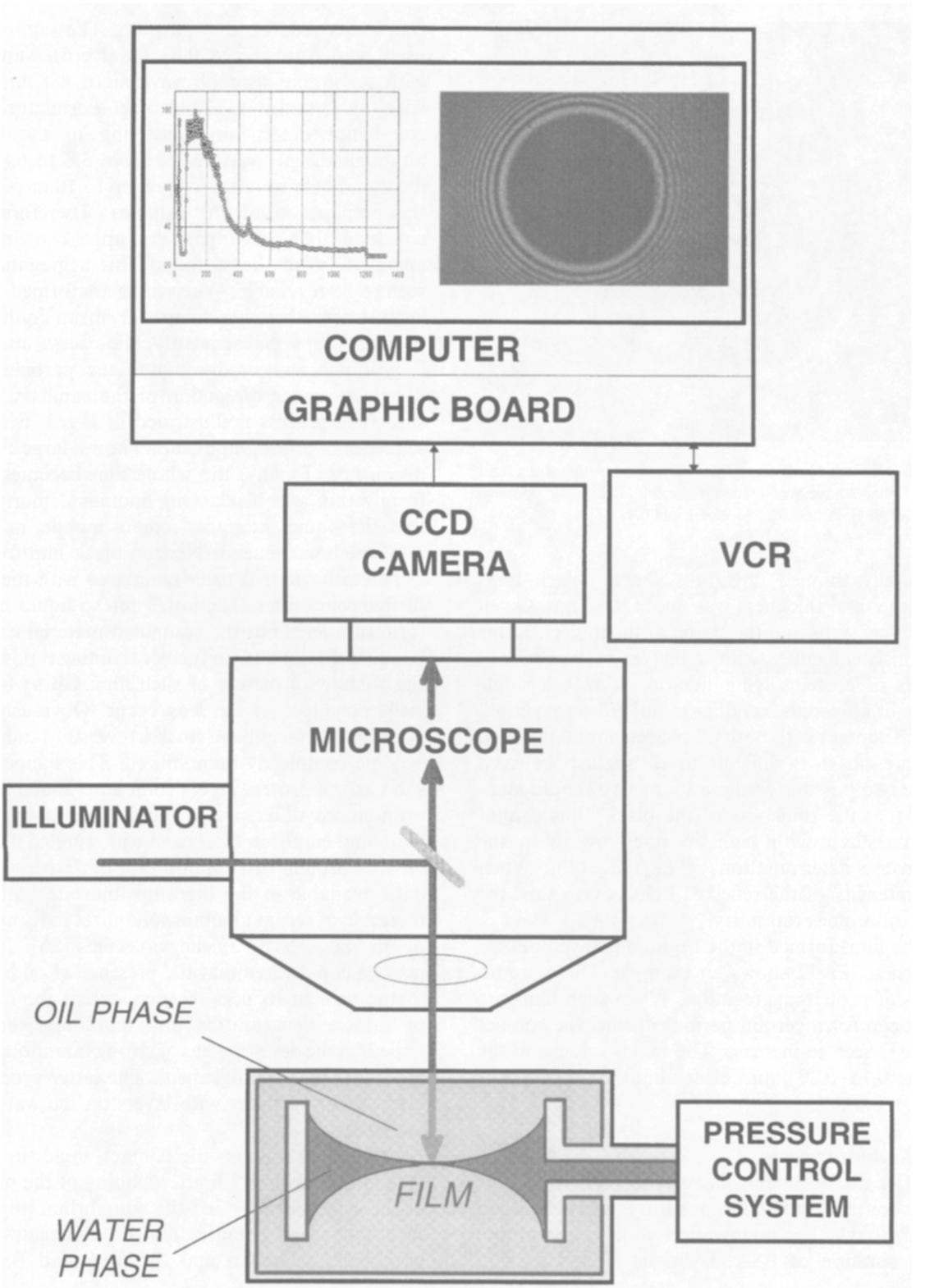


Fig. 1. Sketch of the experimental set-up for microinterferometric investigation of thin emulsion films.

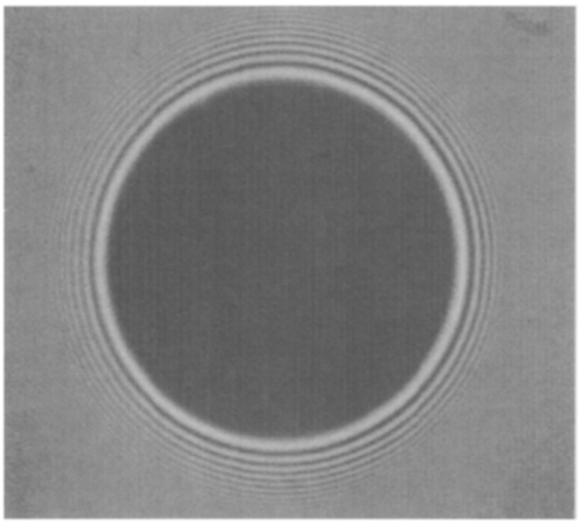


Fig. 2. Newton black film, formed just after the water and xylene phases have been loaded in the cell. The system contains 0.015 wt.% BSA and 0.15 M NaCl, pH 6.4.

gradually thinned down to Newton black films and the final thickness was about 9 ± 3 nm. Given the dimensions and the shape of the molecule, this is consistent either with a bilayer of two monolayers of proteins lying side-on, or with a single layer of ellipsoids, residing in an end-on position, which contact with both oil phases simultaneously. At present it is difficult to distinguish between these two possible configurations. We should mention that the thickness of the black films cannot be measured with high accuracy owing to the imprecise determination of I_{min} [Eq. (1)]. When the intensity of the reflected light is very low, the signal becomes too noisy.

The films formed in the beginning have uniform thickness. Fig. 2 shows an example. There are no signs of protein aggregation. When such films are left open for a certain period of time, the contact angle is seen to increase. The initial value is in the range 0.13–0.20°, and after about 10 min it rises to 0.4–0.5°.

3.1.2. Aged systems

After about 30 min has elapsed, newly opened films contain protein lumps of irregular shape. We checked whether aggregation took place in the bulk solution of BSA. Dynamic light scattering was employed for this purpose. The equipment used was Autosizer 4700C (Malvern), supplied with an argon laser of wavelength 488 nm and with an 8-multibit 128-channel correlator. We could not detect any clustering in the bulk, although the pH was varied from 3.8 to 6.8 and measurements were performed up to 10 days after the preparation of the samples. Therefore, we conclude that large particles appear owing to aggregation on the surfaces. This aggregation is seen to be reversible. When films are formed in an aged system, they thin down and entrap liquid and protein lumps. Subsequently, the excess material is gradually squeezed out and the particles are destroyed under the action of the capillary pressure. The process is illustrated in Fig. 3, where a sequence of photographs shows how a large cluster disappears. Finally, the whole film becomes uniformly thin and black. Our findings demonstrate that the liquid interfaces remain mobile, at least until the homogeneous Newton black film forms.

This behaviour is to be compared with the case of free foam films. The latter entrap liquid in the form of a lens, but the contained material cannot be squeezed out and no further thinning is possible. Fig. 4 shows a picture of such film. Up to 1 h no visible changes in the lens occur. Obviously the opposing surfaces have stuck irreversibly and have become completely immobilized. This happens as soon as the protein layers come into contact. The pronounced difference in the surface mobility of foam and emulsion films can be interpreted in view of the protein hydrophobization. Experimental data available in the literature indicate that one molecule of serum albumin solubilizes in its hydrophobic pockets 23 xylene molecules [26]. It can now be conjectured that the presence of oil bound to the protein reduces to some extent the degree of surface denaturation and unfolding, thereby impeding the development of strong intermolecular attraction and entanglement. The latter processes turn out to be faster with layers on the water/air interface.

Let us now discuss the contact angles in aged systems, more than 1 h after loading of the phases in the cell. We measured the equilibrium angle of emulsion films 15 min after the aggregates had been flattened down and the films had become

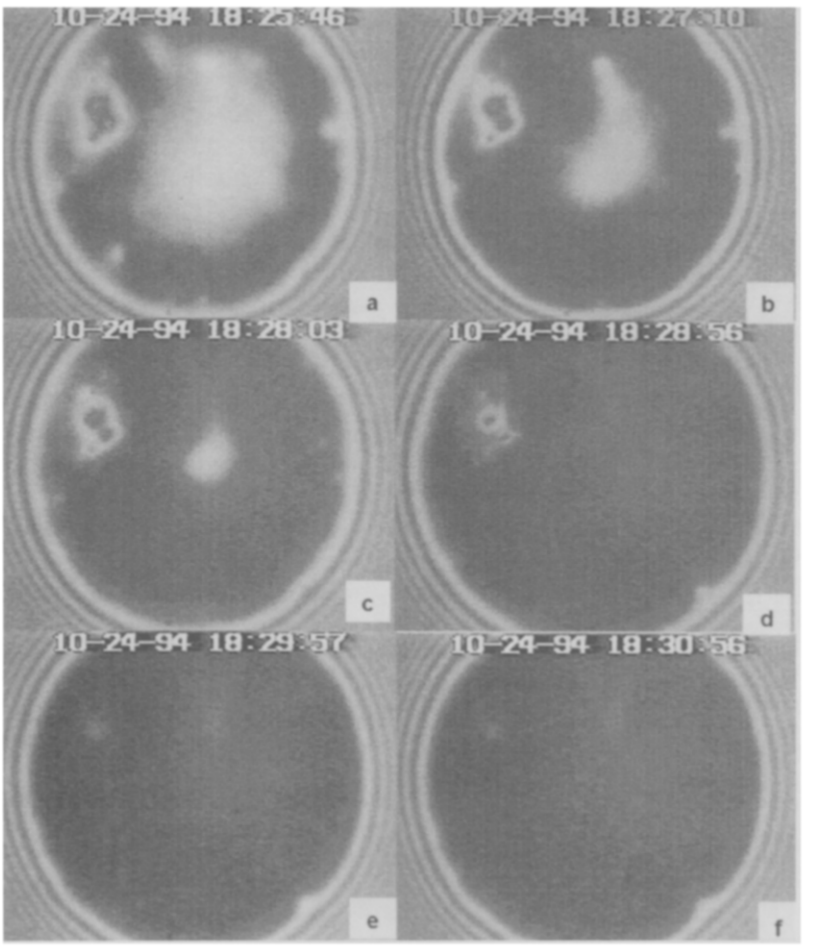


Fig. 3. Interferometric images, taken at six consecutive time moments during thinning of an emulsion film with BSA, in the presence of 0.15 M NaCl. The progressive squashing of a large protein lump can be followed.

uniform and black. The results are shown in Fig. 5 as a function of pH. Two cases, with and without oleic acid in the oil (0.1 wt.%), are compared. First, it is seen that the angle passes through a maximum, the latter lying close to the isoelectric conditions ($pH = pI \approx 4.8$). This points to the role of the electrostatic interactions (inter- or intramolecular), operative even in the presence of 0.15 M inorganic salt. As the pH deviates from the isoelectric point, the protein acquires charges, which may give rise either to repulsion between the molecules or to conformational changes. Anyway, the overall attraction diminishes as θ decreases. (From Eq. (2) it is evident that larger contact angles correspond to stronger attraction.) Similar experimental observations for the pH dependence of θ have been reported with foam films containing α-chymotrypsin [16]. However, in that case deviations from the pI resulted in thicker films and not Newton black films [16].

Second, we note that in the presence of oleic acid the contact angle is larger at the same time of ageing (Fig. 5). This may be attributed to enhancement of the protein rearrangement caused by the fatty acid. The latter has a very strong ability for binding to the protein hydrophobic sites and probably replaces the oil molecules from there.

The contact angle continues to increase for about 1 h of ageing of an opened film (at a fixed capillary pressure). Subsequently a more or less

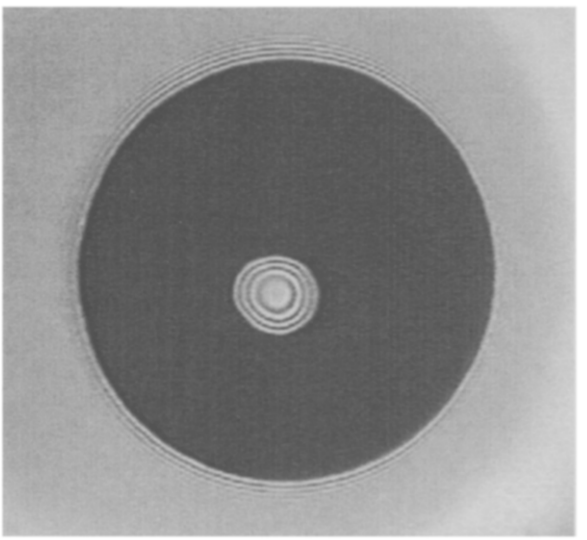


Fig. 4. Thin foam film stabilized by BSA (with 0.15 M NaCl). A lens containing liquid remains irreversibly trapped inside. Note the slightly irregular shape of the lens, which points to the lack of surface fluidity.

constant value of θ is reached. For instance, without oleic acid, at pH 6.4, we measured $\theta = 0.8°$ after 1 h. This result should be compared with $\theta = 0.57°$ at 15 min of ageing (Fig. 5). In addition, well pronounced contact angle hysteresis develops in these films. Throughout the text we use the term "equilibrium angle", which actually refers to mechanical (and not chemical) equilibrium. It should be borne in mind that the term is meaningful as long as the interfaces retain their fluid nature. Only then will the film diameter be able to adjust for the respective values of the contact angle and the capillary pressure in the cell, so that the condition for mechanical equilibrium is met.

We measured the advancing angle, which characterizes the force required to detach the adhered film surfaces. Experimentally, one pushes liquid into the aqueous meniscus (Fig. 1) and the capillary pressure decreases until the contact line starts shrinking. Fig. 6(a) shows a film with little ageing after its formation (about 5 min). The advancing angle can be estimated to be $\theta_{adv} \approx 2°$. A substantial increase in θ_{adv} is observed with time. Thus, after ~ 30 min it rises so much that measurement becomes impossible, since the Newton fringes around the periphery cannot be discerned (Fig. 6(b)). These films are "solid-like", i.e. the two interfaces have stuck firmly and irreversibly.

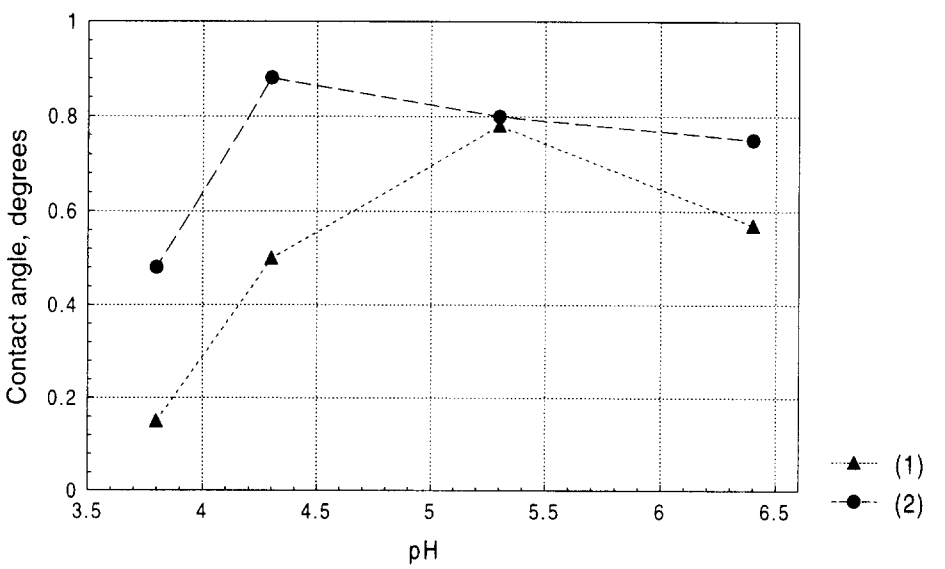
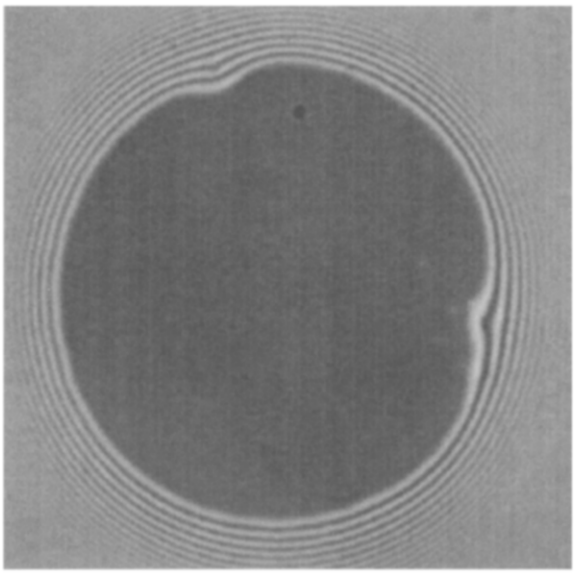
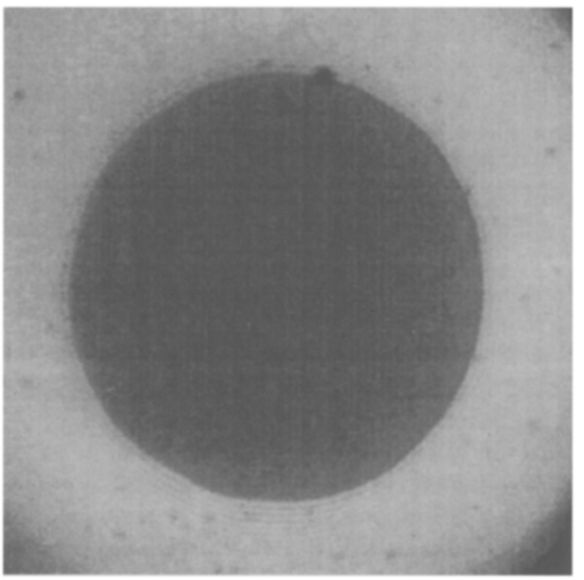


Fig. 5. Contact angles of emulsion films stabilized by BSA (0.015 wt.%) in the presence of 0.15 M NaCl. The measurements were carried out 15 min after uniform Newton black films had been obtained, in aged systems, more than 1 h after loading of the experimental cell. (1) Triangles, no other additives; (2) circles, 0.1 wt.% oleic acid was dissolved in the oil phase. The experimental error is within $\sim 0.15°$.



(a)



(b)

Fig. 6. Emulsion films under decreased capillary pressure, just before shrinking of the contact line starts. The contact angle corresponds to advancing meniscus. (a) 5 min after formation of uniform Newton black film; (b) ~30 min later.

In rheological terms, "yield stress" should be applied in order to detach the surfaces. The corresponding energy of adhesion increases sharply with ageing.

One experiment was carried out in the presence of Ca^{2+} ions. The system contained 0.015 wt.% BSA, 0.05 M $CaCl_2$ and NaCl so that the total ionic strength was 0.15 M (at pH 6.4). Under these conditions, the measured contact angle of the Newton black film, aged for ~15 min, is 0.55°, which agrees well with the value of 0.57° without Ca^{2+} (Fig. 5). Therefore, no influence of Ca^{2+} upon the film properties is observed. This is not surprising because the binding of Ca^{2+} to BSA has been shown to be weak [2].

In summary, the increase in the contact angles of Newton black films with time and the surface aggregation of BSA represent manifestations of the ageing effect. The latter is connected with partial protein unfolding, accompanied by developing attraction and entanglement of the BSA molecules residing on the interfaces. These processes are promoted by oleic acid dissolved in the oil phase.

3.2. Emulsion films with BSA and no inorganic electrolyte

Our experiments have demonstrated that addition of a minor amount of NaCl, even as little as 10^{-3} M, leads to the formation of Newton black films whose behaviour is similar to that described above. For that reason, we made aqueous films without any salt, and studied the impact of ageing and the presence of oleic acid.

3.2.1. Experiments carried out immediately after loading of the two phases in the cell

First, the films thin down gradually until black spots appear near the periphery (Fig. 7). In the system without oleic acid such a film ruptures immediately. However, when 0.1 wt.% of oleic acid has been initially dissolved in the oil, the black spots expand and occupy the whole film. The latter remains stable. We checked whether the fatty acid itself could serve as a stabilizer and found that it cannot: the films rupture readily during thinning. One can draw the conclusion that the presence of fatty acid together with BSA favours the film stability, probably by facilitating protein adsorption and promoting the formation of a more compact surface layer.

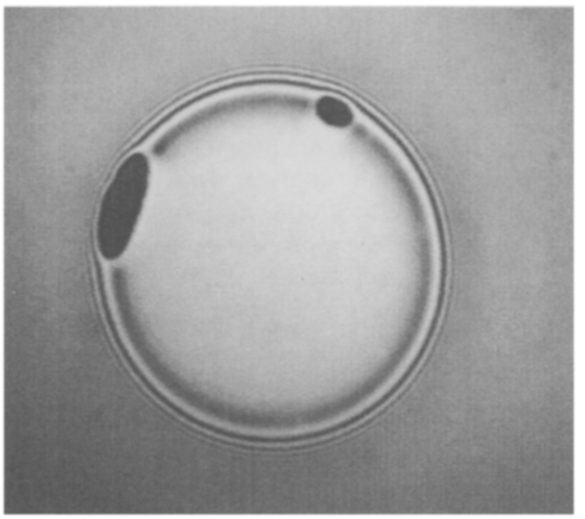


Fig. 7. Emulsion film with 0.015 wt.% BSA at pH 6.6. No inorganic electrolyte was added. The oil phase contains 0.1 wt.% oleic acid.

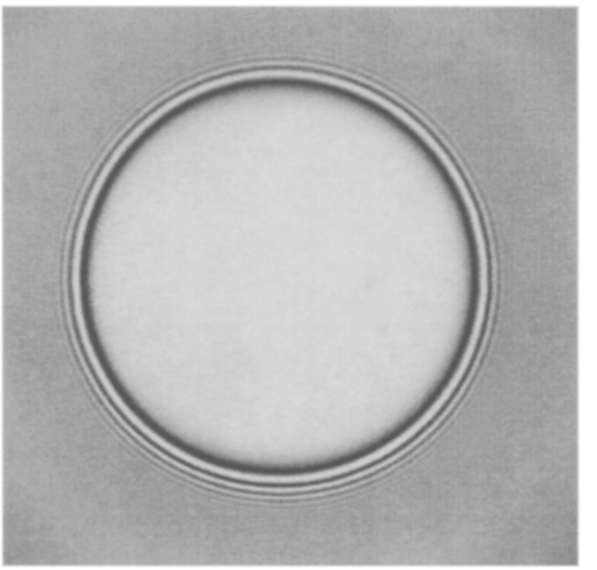


Fig. 8. Thick emulsion film obtained in a system stabilized by 0.015 wt.% BSA at pH 6.6, after about 30 min from loading of the two phases in the cell. Neither NaCl nor oleic acid was added.

3.2.2. Aged systems

After ~ 30 min from loading, completely different behaviour is observed. The films remain thick and stable, both with and without oleic acid. Fig. 8 shows an emulsion film obtained in a fatty acid-free system. It has a bright colour and the first Newton fringe is black, which means that the thickness should be about $\lambda/(4n_s)$ (cf. Eq. (1). Indeed, it is found that $h = 103 \pm 4$ nm in this case. Almost the same is the thickness of the foam film, under identical conditions of pH and protein concentration: $h = 105 \pm 4$ nm.

Similar emulsion films with 0.1 wt.% oleic acid in the oil exhibit a thickness of about 78 ± 3 nm. At present it is difficult to explain the difference in h brought about by the addition of fatty acid. We measured the ζ-potentials (via the electrophoretic mobility) of emulsion drops of xylene in water. The pH was adjusted to 6.5 by means of phosphate buffer with ionic strength 10^{-3} M; the BSA concentration was 0.01 wt.%. A Zetasizer IIC (Malvern) was used. Identical values were obtained, $\zeta = -47 \pm 5$ mV, irrespective of the presence of 0.1 wt.% oleic acid in the oil. Equilibrium was reached within ~ 5 min after preparation of the emulsion, as subsequently ζ did not change with time. In view of these findings, it is likely that the film thickness could be affected by some conformational changes elicited by the fatty acid; e.g. one may think of different screening of the charges existing in the molecule at pH > pI.

Our results confirm the importance of the electrostatic interactions for the behaviour of emulsion films containing protein. We obtained stable films when the surfaces are charged (in contrast to the results reported elsewhere [16,17]). Addition of a small amount of inorganic salt leads to Newton black film formation, whereas without any electrolyte the films remain thick, provided that enough time is allowed for adsorption. The presence of oleic acid is found to enhance the initial film stability (without ageing), probably because the protein adsorption is facilitated.

3.3. Emulsion films containing β-casein

3.3.1. No Ca^{2+}

Table 1 summarizes some results for the thickness and the advancing contact angles at pH 6.5 (far from the isoelectric point of casein, p$I \approx 5$), without added Ca^{2+} ions. First, it is seen that when the electrolyte content is very small

Table 1
Measured advancing contact angles and thicknesses of aqueous emulsion films[a]

System	θ_{adv} (°)	Thickness, h (nm)
With 10^{-3} M NaCl in the aqueous phase	0	70 ± 1.8
With 0.15 M NaCl in the aqueous phase	1.2	20.9
With 0.15 M NaCl in the aqueous phase, after ageing for 72 h at 22°C	1.4	19.3
With 0.15 M NaCl in the aqueous phase and 0.1 wt.% oleic acid in the oil	1.1	21.1

[a]In all cases the water phase contained 0.01 wt.% β-casein at pH 6.5.

(10^{-3} M NaCl), thick films are obtained. On the other hand, the presence of 0.15 M salt leads to Newton black film formation. This agrees with the view of the electrostatic interactions being operative. Ageing of the system for 72 h has only a small effect on the properties of the thin black films. The slightly smaller thickness and higher θ_{adv} are mostly within the limits of the experimental accuracy (rows 2 and 3 in Table 1). Oleic acid also is not influential (last row in Table 1).

We studied the effect of changing the pH, making films at the isoelectric point, pH 5. With 0.15 M NaCl in the aqueous phase, without ageing, the data in the second row in Table 1 were reproduced. After the protein solution had been kept for 72 h at 22°C, the films showed the same thickness as at pH 6.5 (row 3 in Table 1), but the advancing contact angle was higher, about 3°. Hence some evidence of ageing is found at pH p*I*. As pointed out by Atkinson et al. [13], slight and slow conformational rearrangement of the β-casein molecule can occur with time. Testing the system with oleic acid (last row in Table 1) at pH 5 did not lead to values of h and θ_{adv} essentially different from those at pH 6.5. The pH is therefore seen to be of little significance, which probably comes from the fact that the system contains 0.15 M NaCl and the electrostatic interactions are screened to a large extent.

β-Casein strongly aggregates in the bulk, and the process develops when the aqueous solution is kept at room temperature. The protein lumps, caught in black emulsion films, are readily squashed by the capillary pressure, which indicates that the aggregation is reversible and the interfaces are mobile. The presence of oleic acid does not affect the clustering considerably.

3.3.2. *Influence of added Ca^{2+}*

Having in mind the high affinity of β-casein to bind Ca^{2+}, we investigated how the thickness of the black films changed with the ion concentration. The results are plotted in Fig. 9. It is evident that above ~ 0.01 M Ca^{2+} thinner films are obtained. One can compare the values of the film thickness with available literature data for the structure of the adsorbed casein layer on an air/water interface [13] (see also the Introduction). Atkinson et al. [13] found that the overall monolayer thickness without Ca^{2+} is about 8–9 nm. Therefore, the calcium-free Newton black films are close to the state of bilayer (cf. Fig. 9).

Addition of small amount of Ca^{2+} ions (5×10^{-4} M) was reported to cause compression in the more diffuse, hydrophilic part of the protein adsorbed on a single air/water surface [13]. At the same time, progressive desorption from the denser, hydrophobic part of the layer took place with rising calcium concentration [13]. Above 5×10^{-4} M Ca^{2+} the total monolayer thickness was $\sim$5–6 nm [13]. From Fig. 9 we see the trend that the films remain relatively thick up to ionic content of ~ 0.01 M Ca^{2+}, and subsequently a compact bilayer is formed with $h \approx 10$–12 nm, which is exactly twice the thickness of the monolayer. The decrease in the film thickness with added Ca^{2+} can be explained by compression of the protein adsorption layers, possibly combined with desorption. The presence of calcium ions brings about strong adhesion between the film surfaces caused by cross-binding of the overlapping protein layers in the film. These data will be discussed in detail elsewhere [27].

The data on the BSA and β-casein emulsion films obtained by the thin film technique are consistent and complementary with those from ellipsometry, light scattering or interfacial rheology. The thin film method could be used as an

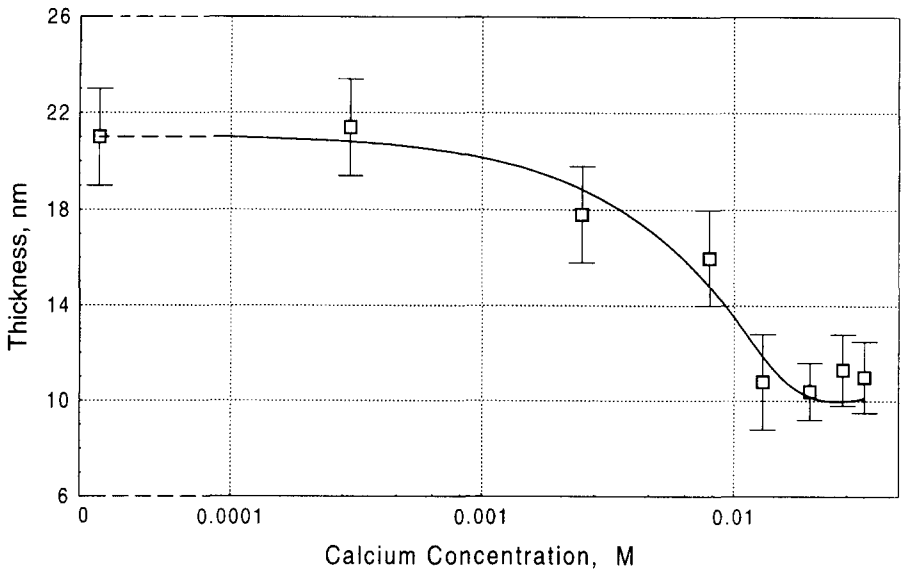


Fig. 9. Thickness of emulsion films with 0.01 wt.% β-casein at pH 5.0 in the presence of Ca^{2+} ions. The total ionic strength, $I=0.15$ M, was adjusted by adding NaCl.

effective tool to study the thickness and interactions between the protein adsorption layers in model emulsions.

4. Conclusion

The present work demonstrates the existence of the following effects in thin emulsion films containing proteins. (i) With BSA the ageing leads to a gradual increase in the equilibrium and the advancing contact angles. This agrees with the notion of slow denaturation and unfolding, connected with developing attraction between the two opposing layers, which ultimately leads to firm sticking. (ii) Reversible surface aggregation of BSA is observed after a certain time period, which is a manifestation of the ageing effect. The lumps disintegrate under the action of the capillary pressure in the film. (iii) The oil/water interfaces remain mobile until the excess material is expelled and a Newton black film of uniform thickness forms. Only then can the surfaces adhere irreversibly. In contrast, with foam films the surfaces stick and become immobile immediately after the thickness of the Newton black film has been reached. The difference is possibly due to hydrophobization of the protein in contact with oil. (iv) The presence of fatty acid enhances the film stability and accelerates to some extent the ageing processes with BSA. This appears to be in contrast with the role of the fatty acids in the bulk, where they are known to stabilize the native structure of BSA against denaturation. (v) The electrostatic interactions are important in the films with BSA as well as with β-casein. Thick films are obtained with little or no added inorganic electrolyte. (vi) Above a certain concentration, the presence of Ca^{2+} ions leads to decreasing thickness of films containing casein. The latter finding is in accordance with the thinner adsorption layers reported in the literature for the same case.

In general, the behaviour of the emulsion films is substantially influenced by additives that interact specifically with the proteins. Such additives are the fatty acids, preferentially attached to BSA, Ca^{2+} ions, prone to binding to β-casein, and electrolytes, which modify the electrostatic interactions depending also on the pH.

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References

[1] L.P. Voutsinas, E. Cheung, S. Nakai, J. Food Sci. 48 (1983) 26
[2] Th. Peters, Adv. Protein Chem. 37 (1985) 161
[3] J.R. Brown, P. Shockley, in: P. Jost, O.H. Griffith (Eds.), Lipid-Protein Interactions, Vol. 1, Wiley, New York, 1982, Chapter 2, p. 25.
[4] D.E. Graham, M.C. Phillips, J. Colloid Interface Sci. 70 (1979) 415
[5] M. Blank, J. Colloid Interface Sci. 29 (1969) 205
[6] D. Cho, G. Narsimhan, E.I. Franses, J. Colloid Interface Sci. 178 (1996) 348
[7] F. MacRitchie, N.F. Owens, J. Colloid Interface Sci. 29 (1969) 66
[8] R.Z. Guzman, R.G. Carbonell, P.K. Kilpatrick, J. Colloid Interface Sci. 114 (1986) 536
[9] E. Dickinson, J. Chem. Soc., Faraday Trans. 88 (1992) 2973
[10] D.E. Graham, M.C. Phillips, J. Colloid Interface Sci. 76 (1980) 240
[11] E. Dickinson, D.S. Horne, J.S. Phipps, R.M. Richardson, Langmuir 9 (1993) 242
[12] D.E. Graham, M.C. Phillips, J. Colloid Interface Sci. 70 (1979) 427
[13] P.J. Atkinson, E. Dickinson, D.S. Horne, R.M. Richardson, J. Chem. Soc., Faraday Trans. 91 (1995) 2847
[14] M.N. Pankratova, L.E. Bobrova, A.V. Bolobova, V.N. Izmailova, Kolloidn. Zh. 36 (1974) 54
[15] E. Dickinson, J.A. Hunt, D.S. Horne, Food Hydrocolloids 6 (1992) 359
[16] Zh.K. Angarska, G.P. Yampol'skaya, L.E. Bobrova, V.N. Izmailova, Kolloidn. Zh. 42 (1980) 424
[17] D. Platikanov, G.P. Yampol'skaya, N. Rangelova, Zh.K. Angarska, L.E. Bobrova, V.N. Izmailova, Kolloidn. Zh. 43 (1981) 177
[18] O.D. Velev, A.D. Nikolov, N.D. Denkov, G. Doxastakis, V. Kiosseoglu, G. Stalidis, Food Hydrocolloids 7 (1993) 55
[19] J.E. Proust, S.D. Tchaliovska, L. Ter-Minassian-Saraga, J. Colloid Interface Sci. 98 (1984) 319
[20] D.C. Clark, in: A.G. Gaonkar (Ed.), Characterization of Food: Emerging Methods, Elsevier, Amsterdam, 1995, Chapter 2, p. 23.
[21] E. Blomberg, P.M. Claesson, C.G. Golander, J. Dispersion Sci. Technol. 12 (1991) 179
[22] A. Scheludko, D. Exerowa, Kolloid-Z. 165 (1959) 148
[23] T.T. Traykov, E.D. Manev, I.B. Ivanov, Int. J. Multiphase Flow 3 (1977) 485
[24] A.S. Dimitrov, P.A. Kralchevsky, A.D. Nikolov, D.T. Wasan, Colloids Surfaces 47 (1990) 299
[25] J.A. de Feijter, in: I.B. Ivanov (Ed.), Thin Liquid Films, Marcel Dekker, New York, 1988, Chapter 1, p. 1.
[26] V.N. Izmailova, P.A. Rebinder, Structure Formation in Protein Systems, Nauka, Moscow, 1974 (in Russian).
[27] O.D. Velev, R.P. Borwankar, in preparation.