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2 - 3

156

EMULSION FILMS STABILISED BY PROTEINS

Auteur(s) Author(s)	Nom(s) Name(s)	Prénom(s) First Name(s)	Affiliation
1	Marinova	Krastanka G.	LTPH, Fac. of Chemistry,
2	Gurkov	Theodor D.	Sofia University, Bulgaria;
3	Borwankar	Rajendra P.	Kraft General Foods,
4	Campbell	Bruce	Glenview, Illinois,
5	Oleksiak	Christian	USA

Adresse postale / Mailing address: Laboratory of Thermodynamics and Physico-Chemical Hydrodynamics, Faculty of Chemistry, University of Sofia, James Bourchier Avenue 1, Sofia 1164, Bulgaria

RÉSUMÉ

Nous présentons des résultats obtenus avec des films aqueux de type émulsion, stabilisés par d'albumine de sérum bovin (BSA), de β-lactoglobuline et de la β-caséine. Le comportement du film est lié au temps. En présence de BSA et de β-lactoglobuline, les angles de contact des films noirs Newton obtenus augmentent avec le vieillissement, et présentent une hystérésis prononcée. On observe une lente agrégation réversible du BSA sur la surface (mais pas dans la masse). Il se produit une lente dénaturation de la surface, accompagnée d'une attraction naissante et d'un embrouillement partiel des molécules de BSA, conduisant à terme à l'adhésion de deux interfaces du film. On constate une nette différence dans la mobilité de surface entre les films mousseux et les films d'émulsion stabilisés au BSA. L'hydrophobisation des molécules de protéines en contact avec l'huile peut causer un tel comportement.

Les interactions électrostatiques jouent un rôle important dans les films stabilisés au BSA et à la caséine. Si l'on ajoute trés peu de sel, voire pas de sel du tot, les films restent épais, tandis qu'en présence de 0.15 M NaCl, on obtient des films noirs de Newton. L'addition d'ions Ca^{2+} dans le système stabilisé à la caséine entraîne la diminution de l'épaisseur du film. Il se produit alors une réticulation des deux surfaces de film opposées. Les effets peuvent s'expliquer par l'adsorption de Ca^{2+} sur certains résidus aminoacides particuliers de la caséine, conduisant à la congestion de la couche de protéines adsorbée et à la réticulation. Ainsi, des facteurs spécifiques important peuvent affecter la stabilisation des émulsions par les protéines.

ABSTRACT

We present results with thin aqueous films of emulsion type stabilised by Bovine Serum Albumin (BSA), β-lactoglobulin and β-casein. The film behaviour is time-dependent. In the presence of BSA and β-lactoglobulin the contact angles of the obtained Newton black films (NBF) increase with ageing, and exhibit pronounced hysteresis. Slow reversible aggregation of BSA on the surface (but not in the bulk) is observed. There is a slow surface denaturation, accompanied with developing attraction and partial entanglement of the BSA molecules, which ultimately leads to firm sticking of the two interfaces of the film. Marked difference in the surface mobility is observed with foam and emulsion films stabilised by BSA. The hydrophobisation of the protein molecules in contact with oil may be responsible for this behaviour.

The electrostatic interactions are found to be important. When little or no salt is added the films stay thick, whereas in the presence of 0.15 M NaCl one obtains NBF. Addition of Ca^{2+} ions to the system with casein leads to decrease of the film thickness. A cross-binding of the two opposing film surfaces occurs, which manifests as strong adhesion and very high contact angle. The effects can be explained with adsorption of Ca^{2+} on some particular aminoacid residues of casein. This leads to congestion of the adsorbed protein layer and cross-linking. Thus, important specific factors may affect the stability of emulsions with proteins.

1. INTRODUCTION

The proteins have an important role as emulsion stabilisers in 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. The stability of an emulsion is closely related to the behaviour of the liquid film that forms when two drops approach toward each other. We are interested in the time evolution of the properties of these films, and how they are modified by additives, which interact specifically with the proteins.

We used three different proteins: Bovine Serum Albumin (BSA), β-lactoglobulin and β-casein. The first two are globular, with many disulphide bonds keeping their structure, while β-casein has a disordered flexible molecule, which does not contain any disulphide cross-links [1-3]. The structure of the used proteins is well known in bulk water solution, but has not yet been definitely determined for molecules adsorbed on liquid interfaces. Slow rearrangement, partial denaturation with unfolding and entanglement due to hydrogen bonding accompany the adsorption of globular proteins [4-7]. The surface viscosity of liquid boundaries loaded with such proteins is very high [7], indicating existence of strong attraction between the molecules, and even formation of a network and viscoelastic gel-like structure [4,7].

BSA has isoelectric point at pH about 4.8 [1]. At pH = 7 there are 18 negative charges. One of the most important features of BSA is its strong affinity to hydrophobic ligands, especially fatty acids. In general, the presence of bound fatty acids is believed to stabilise the native form of the protein in the bulk, restraining it from denaturation and aggregation [1]. The isoelectric point of β-lactoglobulin is at pH around 5.2 [3]. The molecule has free -SH group and forms dimers at neutral pH, with about 10 negative charges per dimer [3,6]. β-casein's isoelectric point is at pH around 5, and at pH = 7 it carries about 15 negative charges [6]. The adsorption of casein on oil/water or air/water interface leads to monolayer formation. Dense, thin inner layer (~ 2 nm) and more diffuse, thicker outer layer (~ 5 ÷ 7 nm) were distinguished [6]. The surface shear viscosity is low compared to that of most globular proteins [6,7], which points to the existence of very weak intermolecular interactions in the case of casein.

We have to 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 [8].

This work is devoted to investigation of emulsion films stabilised by BSA, β-lactoglobulin and β-casein. Interferometry in reflected light was used for the film observation and measurements. We monitored the equilibrium and the advancing 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 investigated. The cases with and without addition of inorganic electrolyte (NaCl) are compared. The results provide information for the importance of the electrostatic interactions.

2. MATERIALS AND METHODS

2.1. Materials. Lyophilised BSA, p.a. grade, essentially fatty acid free, β-lactoglobulin and β-casein from bovine milk (all Sigma products) were used as received. All solutions were prepared with deionized water from a Milli-Q system (Millipore). The concentration of BSA was fixed to 0.015 wt%, and that of lactoglobulin and casein was 0.010 wt%. These concentrations correspond to saturated protein layers on oil/water interfaces [2,3]. Xylene (isomeric mixture, p.a.) and soybean oil were chosen as oil phases. Soybean oil was purified by percolating through a column packed with alumina adsorbent [9]. In some experiments oleic acid (p.a., Sigma Co.) was dissolved in the oil, with concentration of 0.1 wt%.

The ionic strength was varied by addition of NaCl (Merck, analytical grade) to the aqueous solutions. When addition of Ca^{2+} ions was desired, $CaCl_2.2H_2O$ from Aldrich (A.C.S. reagent) was used. Two types of systems stabilised by BSA were studied: with 0 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 and equal to 0.15 M. When BSA was dissolved in water (with the above mentioned concentration), the pH was found to be $\approx 6.4 \pm 0.1$, lower pHs were adjusted by addition of HCl (Sigma). With casein we worked at two different pH values, 5.0 (the isoelectric point) and 6.5, regulated by means of HCl and NaOH (Sigma).

2.2. Interferometric Measurements in Thin Liquid Films. We used experimental cell which was a modification of that proposed by Scheludko and Exerowa [10]. Films were formed by sucking out aqueous phase from a biconcave meniscus held in a glass capillary of inner radius 1.49 mm. Microscopic observations were carried out in reflected monochromatic light (with wavelength λ=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. We registered the time changes in the intensity of light, which was reflected from a small piece of area (whose size and position in the film can be adjusted). The thickness, *h*, was calculated according to formula described in Refs. [11] and [12].

The contact angle, θ, was found from the positions of the Newton fringes around the film periphery. Black and white fringes correspond to thickness 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 developed by Dimitrov et al. [12].

The equilibrium contact angle, θ, is an important film characteristic, due to its direct connection with the energy of interaction between the surfaces, *W* (see Eq. (106) in Ref. [13]):

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

Here σ is the interfacial tension, and $\Pi(h)$ denotes the disjoining pressure in the film [13].

Common interference is workable only at small angles. The ***advancing*** angle at the periphery of the investigated protein films is too big, and the interference fringes cannot be distinguished from each other. That is why we applied differential interferometry in reflected light (shearing method) [14]. This method allows to measure angles as big as 30° [15].

3. RESULTS AND DISCUSSION

3.1. Emulsion films containing globular protein and excess inorganic electrolyte, 0.15 M NaCl. In this case well-pronounced ageing effects are operative.

Aqueous films between xylene phases were studied, in the presence of BSA. Irrespective of the pH (varied from 3.8 to 6.4), the films formed immediately after loading of the cell gradually thinned down to Newton black films (NBF), and the final thickness was about 9 ± 3 nm. There were no signs of protein aggregation. When such films were left open for a certain period of time, the contact angle was seen to increase. The initial value was in the range 0.13 - 0.20°, and after ~ 10 minutes it rose up to 0.4 - 0.5°.

30 minutes after loading of the phases in the cell, newly opened films contained protein lumps of micrometer size and irregular shape. We could not detect any clustering in the bulk solution of BSA (using dynamic light scattering). We conclude that big particles appear due to aggregation ***on the surfaces***. This aggregation was seen to be reversible. When films were formed in an aged system, they thinned down and entrapped liquid and protein lumps. The excess material gradually squeezed out, and the particles were destroyed under the action of the capillary

pressure. Finally, the whole film became uniformly thin. Our findings demonstrate that the liquid interfaces remain mobile, at least until the homogeneous NBF forms.

In contrast, in free **foam** films (with BSA, as well as with lactoglobulin) some liquid stayed entrapped in the form of lens. The contained material could not squeeze out and no further thinning was possible. The opposing surfaces had stuck irreversibly and had become completely immobilised. That happened as soon as the protein layers came in contact. The pronounced difference in the surface mobility of foam and emulsion films can be interpreted in view of the protein hydrophobization. The presence of oil, bound in the specific pockets of the protein molecule, reduces to some extent the degree of surface denaturation and unfolding.

We measured the equilibrium contact angle of BSA emulsion films between xylene, 15 minutes after the aggregates had been flattened. The results are shown in Fig. 1 as a function of pH. Two cases, with and without oleic acid in the oil (0.1 wt%) are compared. The angle passes through a maximum, the latter lying close to the isoelectric conditions (pI ~ 4.8). This fact 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 (larger contact angles correspond to stronger attraction, according to Eq. 1). The contact angle is larger in the presence of oleic acid. This fact may be attributed to some 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 most probably replaces the oil molecules from there.

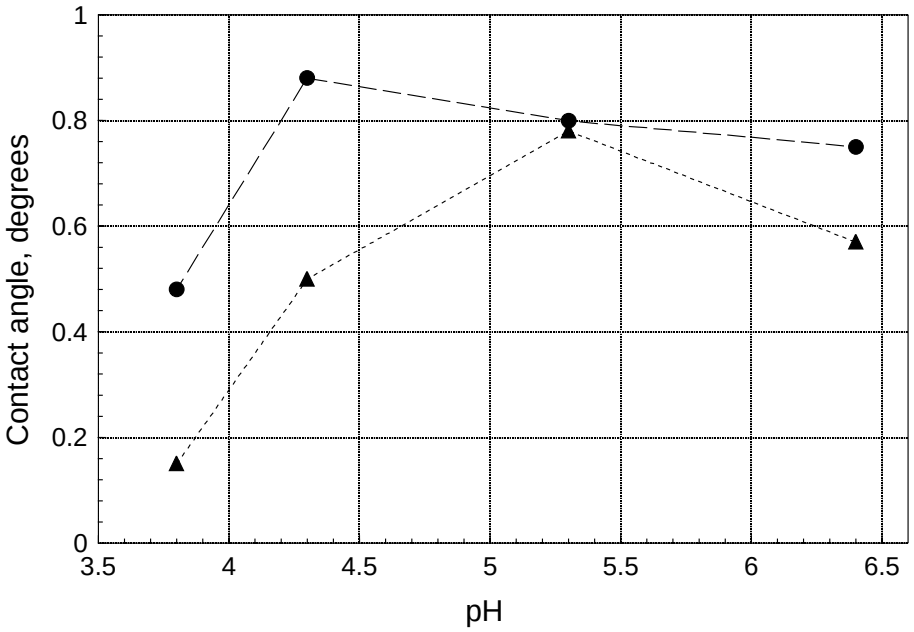

Fig. 1. Contact angles of emulsion films with BSA (0.015 wt%), in the presence of 0.15 M NaCl. The measurements were carried out 15 minutes after uniform NBF had been obtained. (1) Triangles: No other additives; (2) Circles: with 0.1 wt% oleic acid in the oil. The experimental error is within ~0.15°.

The contact angle continued to increase for a time period of about one hour of ageing of an opened film (at fixed capillary pressure). After that a more or less constant value of θ was reached. For instance, without oleic acid, at pH = 6.4, we measured θ = 0.8° after 1 h. This result should be compared with θ = 0.57° at 15 minutes ageing. In addition, well-pronounced contact angle hysteresis develops in these films. (The term "equilibrium angle", which we use, is meaningful as long as the interfaces retain their fluid nature.)

We performed experiments with BSA water films between **soybean oil** phases, at two different pH values: 4.8 and 6.4. The pattern of the film thinning was the same as described above. The thickness of NBF practically coincided (in the frames of the experimental error) with that for

films between xylene phases, 10 ± 3 nm. The equilibrium contact angle, 15 minutes after the black film formation, was measured to be $0.43° \pm 0.10°$, at both pH. This value is smaller compared to the case of films immersed in xylene (see Fig.1). The lower angle suggests weaker attraction between protein molecules adsorbed on the two soybean oil/water interfaces. The contact angle θ increases with time, but the process is slower than that in the system with xylene. Most probably, the presence of triglycerides impedes the partial denaturation, unfolding and entanglement of the BSA molecules, in a greater extent than xylene does.

Emulsion films stabilised with β-lactoglobulin had similar typical behaviour. Ageing effect and aggregation on xylene/ water interfaces were noticed. However, in general the films were unstable: they ruptured soon after spots of NBF appeared (see Fig. 2). We did ***not*** observe squashing and disintegration of lumps, in contrast to the case with BSA. The surfaces covered by lactoglobulin were immobile. It can be understood that β-lactoglobulin is more hydrophobic than BSA, and is more easily denatured on the oil/water boundary.

3.2. Contact angle hysteresis. The equilibrium contact angle that we measure 15 minutes after the formation of the black film corresponds to fluid-like film structure. At that stage the hysteresis of the contact angle is relatively small, as the two protein layers have not yet firmly adhered.

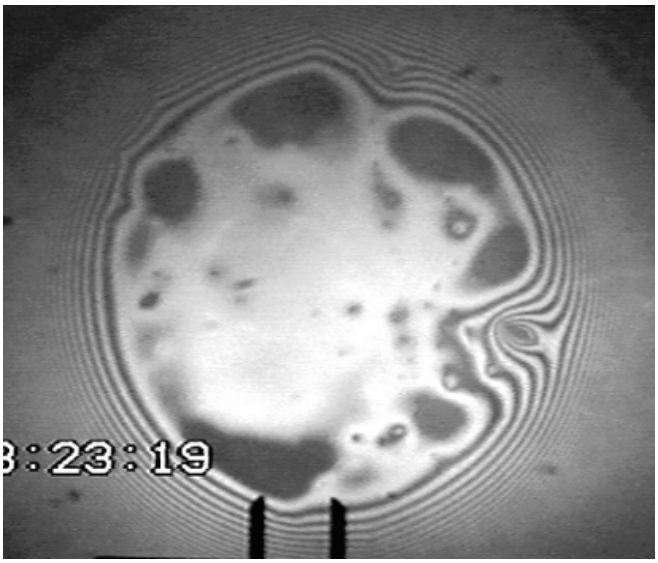

Fig. 2. Emulsion film, stabilised by β-lactoglobulin, shortly before rupture. The distance between the vertical bars is 31.25 μm.

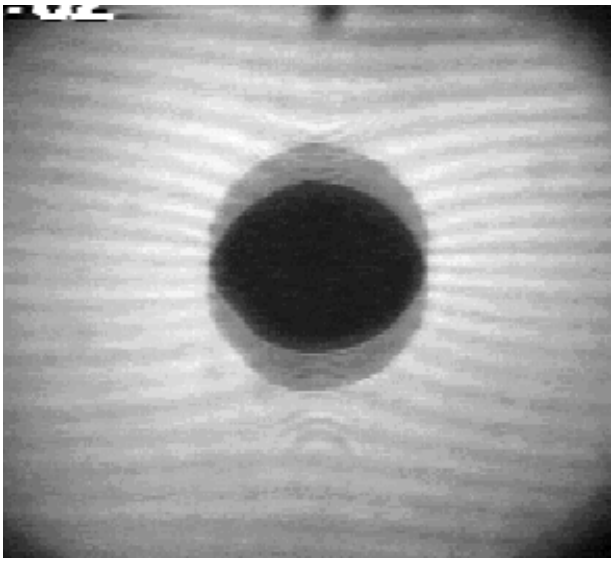

Fig. 3. Differential interference image of a black foam film at pH = 6.4, 0.015%wt. BSA and 0.15 M NaCl. The picture is taken 30 minutes after the formation of NBF.

The ***advancing*** contact angle, θ_{adv}, characterises the short-range attraction between the two opposing protein layers in the NBF. We measured θ_{adv} of BSA **foam** films at pH = pI, and at pH = 6.4. The films were left for "rearrangement" at the stage of NBF for at least 30 minutes. Then we started to decrease the capillary pressure in the meniscus, thus trying to shrink the film diameter (i.e., to detach the two surfaces). The contact angle increased until a certain critical value was reached and the film started contracting. At that moment we applied splitting of the film image until clearly distinguishable interference fringes were seen (Fig. 3). Differential interference microscope (Epival Interphako, Zeiss) was used for this purpose. We determined the positions and the order of interference of the fringes. By fitting the experimental points with the numerical solution of the Laplace equation we obtained the advancing contact angle, θ_{adv}. It was found that $\theta_{adv} = 15.2° \pm 1.6°$ at pH = pI = 4.8, and $\theta_{adv} = 6.8° \pm 1.4°$ at pH = 6.4. The big difference

between the values of θ_{adv} points to a much stronger attraction between the protein molecules when they are uncharged (pH = pI).

The increase of the contact angles of Newton black films with time, the hysteresis, and the surface aggregation represent manifestations of the ageing effect. The latter is connected with partial protein unfolding, accompanied with developing attraction and entanglement of the molecules residing on the interfaces.

3.3. BSA emulsion films without inorganic electrolyte. Our experiments have demonstrated that addition of minor amount of NaCl, even as small as 10^{-3} M, leads to formation of NBF whose behaviour is similar to what is described above. For that reason, we made aqueous films without any salt, and studied the impact of the ageing and the presence of oleic acid.

In the beginning, just after loading of the experimental cell, the films thinned down gradually until black spots appeared near the periphery. In the system without oleic acid such a film ruptured immediately. However, when 0.1 wt% of oleic acid had been initially dissolved in the oil, the black spots expanded and occupied the whole film. The latter remained stable. We proved that the fatty acid alone could not serve as a stabiliser of thin liquid films; they ruptured readily during thinning. One can draw the conclusion that the presence of fatty acid together with the BSA favours the film stability, probably by facilitating the protein adsorption and promoting the formation of more compact surface layer.

30 minutes after loading of the phases a completely different behaviour was observed. The films stayed thick and stable, both with and without oleic acid. The thickness of the films was ~100 nm. These results confirm the importance of the electrostatic interactions. Addition of small amount of inorganic salt leads to NBF 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.

3.4. Emulsion films containing β-casein

3.4.1. No Ca^{2+}. When the electrolyte content was very small, 10^{-3} M NaCl (at pH=6.5), thick films were obtained. The presence of 0.15 M salt led to NBF formation. Hence, electrostatic interactions were operative. The ageing of the system for 72 hours had little effect on the properties of the NBF. The only sign of ageing was found at the isoelectric point, pH = 5: the advancing contact angle increased from 1.5° to 3°. As pointed out by Atkinson et al. [8], slight and slow conformational rearrangement of the β-casein molecule can indeed occur with time.

β-casein strongly aggregates in the bulk. The protein lumps, caught in black emulsion films, are readily squashed by the capillary pressure, which indicates that the interfaces remain mobile and the aggregation is reversible. The presence of oleic acid in the oil phase does not affect the clustering considerably, nor yet the other film properties.

3.4.2. Influence of added Ca^{2+}. Addition of small amount of Ca^{2+} ions, 5×10^{-4} M, was reported in the literature to cause compression in the more diffuse, hydrophilic part of the protein adsorbed on a single air/water surface [8]. At the same time, progressive desorption from the denser, hydrophobic part of the layer took place with rising calcium concentration [8]. Above 5×10^{-4} M Ca^{2+} the total monolayer thickness was ≈ 5 - 6 nm [8]. Our results showed that the emulsion films remained relatively thick up to ionic content of ≈ 0.01 M Ca^{2+}, and after that a compact bilayer was formed, with $h \approx 10$ - 12 nm, which is exactly twice the thickness of the monolayer, as measured by other authors [8]. The decrease of the film thickness with added Ca^{2+} can be explained with compression of the protein adsorption layers, possibly combined with desorption. The presence of calcium ions brings about very strong **adhesion** between the film surfaces. The latter could not even be detached by infusing liquid from the meniscus. These effects may be explained with adsorption of Ca^{2+} on some particular aminoacid residues of casein, which leads to congestion of the adsorbed protein layer and cross-linking (for details see Ref. 16).

4. CONCLUSIONS

With globular proteins (BSA and β-lactoglobulin) the ageing leads to a gradual increase of the equilibrium and the advancing contact angles. This complies with the notion of slow denaturation and unfolding, connected with developing attraction between the two opposing layers, which ultimately leads to firm sticking. Reversible ***surface*** aggregation of BSA is observed after a certain time period, which is a manifestation of the ageing effect.

The oil/water interfaces remain mobile until any excess material is expelled and Newton black film of uniform thickness forms. Only then the surfaces can adhere irreversibly. In foam films the two adsorbed protein layers stick together faster and tighter, compared to the emulsion films (both in xylene and in soybean oil). For that reason, higher values of the equilibrium contact angle were measured with foam films. In the presence of oil the time for configurational rearrangement and entanglement on the interface could be appreciably prolonged.

The electrostatic interactions are very important in protein-stabilised films. Thick films are obtained with little or no added inorganic electrolyte. Considerably higher advancing angles are found at pH= pI than those at pH= 6.4. This difference points to a stronger attraction between the protein molecules when they are uncharged.

Above a certain concentration, the presence of Ca^{2+} ions leads to decreasing thickness of films containing casein. Very strong adhesion of the two opposing film surfaces occurs. The effects can be explained with adsorption of Ca^{2+} on some particular aminoacid residues of casein, which leads to congestion of the adsorbed protein layer and cross-linking.

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