Effect of Calcium Ions and Environmental Conditions on the Properties of β-Casein Stabilized Films and Emulsions

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Thin emulsion films stabilized with β-casein were studied by microinterferometry. The data demonstrate that the film thickness and intersurface adhesion are influenced by the electrolyte concentration, the prehistory of the protein solution, pH and the presence of fatty acids in the oil phase. A major shift of the film parameters occurs in the presence of calcium ions. The decrease of the film thickness above 1 mM Ca^{2+} is consistent with earlier neutron reflectivity and hydrodynamic thickness measurements. The most interesting effect is the strong cross-binding of the film surfaces above a threshold concentration of 12 mM of calcium. Electrophoretic mobility data suggest that at this concentration one Ca^{2+} ion per two protein molecules is adsorbed on the outermost segments of the protein layers. The possible origin of the cross-binding is discussed on the basis of the structure of the adsorbed β-casein molecules. Data for the resolution of batch emulsions demonstrate that the effect of calcium observed with emulsion films is directly related to the stability of practical systems. Surface force apparatus measurements indicate that the effect, although attenuated, may also be present in suspensions of casein-stabilized solids.

Introduction

The proteins are among the major stabilizers used in food emulsions. Knowledge of the mechanisms by which the proteins adsorb on the emulsion droplets and stabilize them against flocculation and coalescence is important from both fundamental and practical viewpoints. The general principles of that stabilization, formation of thick adsorption layers that exhibit increased viscoelasticity and steric repulsion, are well-known.[1−4] The recent challenge in colloid and in protein physics is to study in detail the structure of the protein adsorption layers and emulsion films and to correlate these data with the stability of batch emulsions.

One of the four major constituents of the milk caseins is β-casein, a protein widely used in fundamental and applied food emulsion studies.[1,5,6] β-casein is a strongly hydrophobic phosphorylated protein with a disordered ("random coil") structure.[4,7] It has a molecular weight of 24 000 and contains 209 amino acid residues.[8,9] The first 50 amino acids (starting from the N-terminus) are predominantly hydrophilic, while the remaining 159 are of predominantly hydrophobic character.[4,9−12] It could be expected that the hydrophobic part of the molecule adsorbs at the oil−water interface in a "train" configuration, while the hydrophilic chain extends farther away as a "tail" or "loop".[13] Recent studies of the β-casein adsorption layers at different interfaces have shown the existence of a two-layer adsorption structure close to the interface.[4,9,11,12,14−19] Particularly reliable information was provided by neutron reflectivity.[4,10,12,18−20] The thin, compact layer of 1−2.5 nm adjacent to the interface comprises the strongly adsorbed hydrophobic "trains", while the much less dense layer that extends 3−7.5 nm farther away into the aqueous phase includes the dangling hydrophilic portions of the molecules.

Two properties of the β-casein make it distinct from other proteins and of particular interest to the colloid investigator. The first is the strong affinity of casein to divalent ions and to Ca^{2+} in particular.[5,21,22] This affinity is connected with the role of caseins as natural carriers of calcium in milk.[1,5] The main calcium binding sites are

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the five phosphorylated serine residues of the β-casein chain, but binding to some of the other negatively charged residues is also possible.[21,22] It could be expected that the adsorption and the emulsifying properties of the β-casein are strongly affected by the presence and binding of calcium ions. Indeed, the thickness of the less dense adsorption layer in the presence of 2 mM calcium was found to decrease[12] from 4.4 to 2.9 nm, while the volume fraction of the protein chains increases by about 30%. It has also been reported that the presence of calcium ions increases the interfacial viscosity of the β-casein adsorption layers[23] and promotes emulsion flocculation and shear-induced coalescence.[22−25]

Another important property of β-casein is the formation of colloid-sized aggregates in the protein solution. In its natural state in milk, β-casein is present as casein "micelles", aggregates of different caseins of diameter 130−160 nm.[1,5] The size of these aggregates depends on the temperature, calcium concentration, and the prehistory of the casein solutions.[5,26] The presence of protein aggregates or "micelles" in the aqueous phase could have an impact on the mechanism of stabilization of food emulsions by evoking stabilization by solid particles[27] ("Pickering" emulsions). The presence of casein micelles can lead to layering and stratification in the thin films between the emulsion droplets.[28]

The aim of this study is to investigate the effect of calcium ions, environmental conditions, and prehistory of the solutions on the thickness and properties of thin emulsion films in an experimental cell. These films represent a model of the films formed between emulsion droplets during flocculation, creaming, or Brownian collisions (albeit the model films are of much bigger size and lower driving pressure than the real ones). Complementary data to the thin film studies were obtained by ellipsometry, surface force apparatus (SFA), electrophoretic light scattering, and homogenization of batch emulsions. This allows comparing and correlating the model films data with the properties of practical emulsions. The experimental results are discussed in view of their relation to the protein structure in the thin films. The findings are compared to the results from previous β-casein studies.

Experimental Section

Materials. β-Casein from bovine milk was obtained from Sigma, Prod. No. C-6905. The sodium chloride was a Merck product, baked for 4 h at 450 °C before use. $CaCl_2 \cdot 2H_2O$ and the other chemicals were obtained from Aldrich. The water for the solutions was extracted from a Milli-Q Organex system (Millipore). As an oil phase we used xylene, which not only has advantages over aliphatic hydrocarbons in the thin film experiments but also may be a better solvent substitute for vegetable oils due to its higher polarizability.[29] It was a p.a. product twice purified from polar impurities by the method of Gaonkar,[30] i.e., by passing through a column packed with adsorbent (Florisil).

The pH of the solutions was measured via a digital pH-meter, equipped with a protein-and-surfactant resistant electrode, and adjusted by stepwise addition of minute quantities of 1 M solutions of HCl or NaOH. To suppress microbial growth, 20 mg/L of sodium azide was added to all solutions with ionic strength >100 mM. The solutions were stored at 4 °C for no more than 7 days and thermostated 20−40 min before use. The pH drift of the solutions stored under these conditions was negligible. All of the experiments were carried out at 22 ± 0.5 °C.

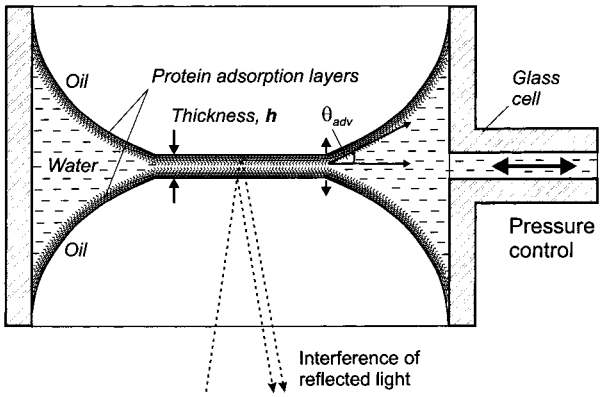

Figure 1. Schematics of the film formation in the model cell and the parameters determined.

Methods. We used several techniques providing information about the properties and the structure of the protein in the adsorption layers and interdroplet films. The main method was the microinterferometric technique for studying thin liquid films, originally introduced by Scheludko and Exerowa[31] and later modified for emulsion systems.[29,32] The films are formed by sucking out liquid from a biconcave aqueous meniscus, held in a glass cell immersed inside the oil phase. The film dynamics and thickness are observed by a microscope in reflected monochromatic illumination (Figure 1). The light pattern and intensity are registered by a CCD videocamera and simultaneously recorded on tape and processed by a computer to extract the film thickness.

The final, equilibrium film thickness was recorded after the thinning of the formed films had stopped and the intensity of the reflected light remained constant. The film diameter was sustained at 100 ± 10 μm. The thickness calculations were made under the assumption that the refraction index of the protein solution inside the film is close to that of pure water. The interfaces were allowed to equilibrate for at least 20 min before every measurement. In each experiment, data from five independently formed thinning films were obtained. To check the reproducibility of the results, some of the measurements were repeated by using a newly prepared protein solution, and the reproducibility of the mean values was better than ±0.5 nm. The measurements of the advancing contact angles were carried out at a rate of meniscus advance ≤10 μm/s. After the coordinates of the interference fringes around the film were recorded, the data were processed according to the previously described procedures[33] to recalculate the angle by extrapolation of the meniscus surfaces to the median plane of the film. The interfacial tension, which was required as a parameter in the calculations, was measured by the du Nouy ring tensiometry method. The protein-covered interface exhibits high dilational viscosity, and to avoid systematic errors arising form stretching during the measurement, the data were collected until 10 h of equilibration and extrapolated to the equilibrium surface state. Ellipsometric experiments were performed by using a homemade null type of ellipsometer with a He−Ne laser beam.

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Table 1. Data on Emulsion Films Stabilized by 0.01 wt % β-Casein, as a Function of Electrolyte Concentration, pH, Solution Prehistory, and Fatty Acid in the Oil

no.	system	pH	h_{final}, nm	θ_{adv}, deg	$-10^3 W_{adh}$, mJ/m^2	protein aggregation
1	1 mM NaCl	6.5	70 ± 1.8	0	0	not visible
2	150 mM NaCl	5	21.0 ± 1.2	1.2	5.0	low
3	150 mM NaCl	6.5	20.9 ± 1.2	1.2	5.3	low
4	150 mM NaCl, 72 h at 22 °C	5	19.8 ± 0.8	≈3	31	high
5	150 mM NaCl, 72 h at 22 °C	6.5	19.3 ± 0.8	1.4	7.2	medium
6	150 mM NaCl, 0.1% oleic acid in the oil	5	25.5 ± 0.9	1.2	5.0	medium
7	150 mM NaCl, 0.1% oleic acid in the oil	6.5	21.1 ± 0.5	1.1	4.4	low
8	150 mM NaCl, 20 mM $CaCl_2$	5	10.4 ± 1.0	≥90	>20000	medium

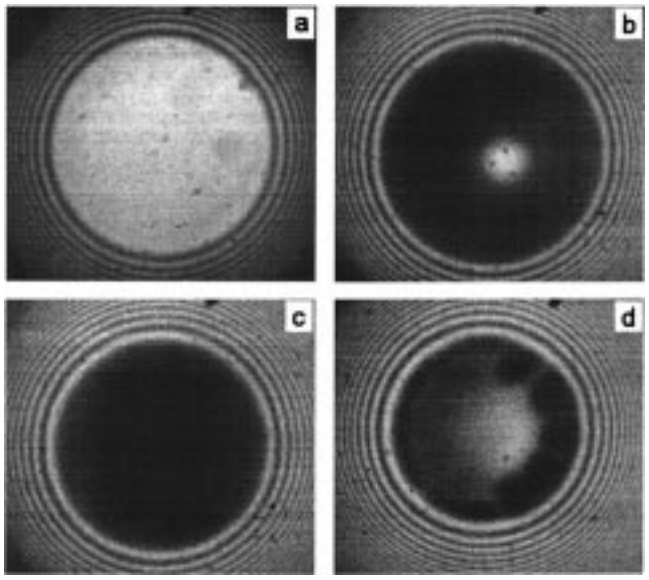

Figure 2. Four distinctive cases of β-casein-stabilized emulsion films. (a) Bright, thick plane-parallel film at low electrolyte concentration (film diameter 110 μm). (b) Film at a high electrolyte concentration during the thinning stage. The bright spot is a remainder from a dimple, which slowly diffuses out. (c) Equilibrium plane-parallel thin film. (d) Thinning to a black film through a spotlike transition in the system with 20 mM of Ca^{2+} at pH 6.5.

The surface force apparatus (SFA) experiments were made with a Mk. II apparatus (Anutech Pty. Ltd.) equipped with a nontilting spring with bath. The equipment, the interferometric measurement of the distances, and the force calibration were as described in the literature.[34,35] Back-silvered mica sheets were glued to the silica disks and the mica surfaces were rendered partially hydrophobic by placing them in a closed container with dry N_2 into which a few droplets of dimethylchlorosilane were added. After 15 min, the container was purged with N_2 and the surface of the hydrophobized mica was multiply washed with pure water. The quality of the hydrophobization procedure was checked by examining the contact angles of water droplets on the treated mica. Best results were obtained at angles ranging in the 60−90° range.

In the course of the SFA experiments, the alignment and the interference pattern of the bare mica surfaces brought into contact in air were checked first, after which the disks were separated and the bath was loaded with casein solution and equilibrated for 30 min. The disks were then brought into contact again and equilibrated for 20 min, and the thickness of the film between them was measured. The surfaces were separated at a speed of ≈25 nm/s and the thickness at the moment of their jump-out of contact was recorded, which allowed the adhesive force to be calculated. The separated disks were equilibrated for another 20 min, before the next approach/separation cycle was started. The solution in the bath was replaced without drying of the film between the disks. The presence of aggregates occasionally led to films of higher thickness and lower adhesion, in which case the obviously wrong data were discarded and the experimental run was repeated, until at least three consistent points obtained in two independent experiments were available for averaging.

The emulsions for the electrophoretic and the resolution experiments were dispersed in a rotor/stator type of homogenizer (Janke and Kunkel Ultra-Turrax T25, equipped with a S25N-10G dispersing head). The crudely predispersed emulsions were subjected to a cycle of 1 min of homogenization at 10 000 rpm, followed by 1 min at rest and an additional 2 min of treatment. The samples for the electrophoretic experiments contained approximately 0.5 vol % of xylene and were prepared 24 h before the measurements to allow the protein adsorption equilibrium to be reached. The electrophoretic mobilities of the drops were measured by a Zetasizer II C electrophoretic laser scattering instrument (Malvern Instruments).

When emulsion resolution was followed, the emulsions were dispersed from 10 mL (40 vol %) of xylene and 15 mL of protein saline. The homogenized samples were immediately poured into graduated tubes of inner diameter 18 mm, which were then left at rest to follow the serum/phase separation.

Results

A. Results for Thin Films in the Absence of Ca^{2+}. The data obtained from the investigation of the β-casein-stabilized emulsion films are presented in Table 1 and interference pictures of emulsion films in some typical cases are presented in Figure 2. The energy of adhesion per unit area, W_{adh}, was estimated from the advancing contact angle, θ_{adv}, based on a formula adapted after de Feijter[36]

$$W_{adh} = 2\gamma(\cos\theta_{adv} - 1)$$

where γ is the interfacial tension. The measured value of γ was 11.3 ± 0.4 mN/m at pH 5 and 12.0 ± 0.2 at pH 6.5. The errors in the contact angle measurements are within ±1 of the smallest digit reported.

The parameters varied in the experiments were the electrolyte concentration, pH, solution prehistory, and presence of additives in the oil or water phase. First, the experimental results for the systems without Ca^{2+} are presented. The effect of calcium ions on the film stability is described in detail in the next subsection.

1. Effect of Electrolyte Concentration. Changing the electrolyte concentration can strongly influence the thickness and behavior of aqueous emulsion films.[29,37,38] Lowering the electrolyte concentration can evoke long-range electrostatic repulsion due to the decreased screening of the surface charges. This effect was observed with films containing only 1 mM of electrolyte (NaCl); the thickness of 70 nm (row 1 of Table 1) was sustained by long-range electrostatic repulsion (Figure 2a). When the

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electrolyte concentration was raised to 150 mM, the electrostatic repulsion was suppressed, and the films thinned down to ca. 20 nm (Figure 2b and c). A numerical estimate of the surface potential is provided in the Discussion.

2. Effect of pH. The pH may influence the film properties by changing the charge of the protein molecules. We worked at two pH values of which the lower (pH 5) is close to the expected isoelectric point of β-casein.[1,7,39] As seen from Table 1, the increase of pH from 5 to 6.5 did not significantly influence the film thickness and surface adhesion, as the measured decrease in these two parameters was mostly within the range of the experimental error. The only notable difference was found for the adhesion energy in the protein solutions stored at elevated temperatures—see below.

3. Effect of Storage Time and Temperature. Storing the casein solutions for more than a few hours at 50 °C strongly increases the aggregation within the system as a possible result of the increased hydrophobic attraction of the molecules.[26] The properties of the emulsion films therefore could depend on the prehistory of the protein solution from which they are formed. In our study, this effect was simulated by storing the solutions at 22 °C in closed sterile containers for 72 h before introducing them into the experimental cell (no microbial growth was observed in these solutions, and their pH drift was below 0.2 units).

As seen from Table 1, rows 4 and 5, the films from such solutions were slightly thinner (in the limit of the experimental error) than the original ones. The storage of the solutions resulted in increased "sticking" of the film surfaces, as demonstrated by the advancing contact angle. As expected, this was accompanied by an increased number of aggregates entrapped between the film surfaces. Both of these effects were particularly notable at pH 5, close to the isoelectric point of the protein.

4. Effect of Aggregates. The presence of aggregated protein could occasionally be observed after the initial formation of thick films in the emulsion cell. The aggregates appeared as thicker (brighter) spots inside the gray film of thickness below 100 nm (for example see Figure 3). In most cases, only a few (one to five) aggregates were present and the film thinning dynamics, thickness, and stability were not affected. Such thin films are denoted in Table 1 as having a "low" number of aggregates. The largest number of aggregates between the film surfaces was observed in the case of the protein solution at pH 5 and stored for 72 h at 22 °C (row 4, denoted as "high" aggregation).

The aggregates entrapped between the film surfaces typically appeared as having a diameter of 1−5 μm and a maximum thickness of 50−400 nm. Therefore, the aggregates that in the bulk solution should be approximately spherical in shape, were significantly flattened after adsorption and compression inside the film. The most striking feature of the entrapped casein particles was their gradual disappearance until the final state of a homogeneous plane-parallel film was reached (Figure 3). This process typically finished after the hydrodynamic stage of film thinning was reached; i.e., the aggregates disappear from within a very thin, black, plane-parallel film. The dissolution therefore is not likely to occur via bulk diffusion of the protein molecules inside the films. A possible mechanism of protein surface transfer is suggested below.

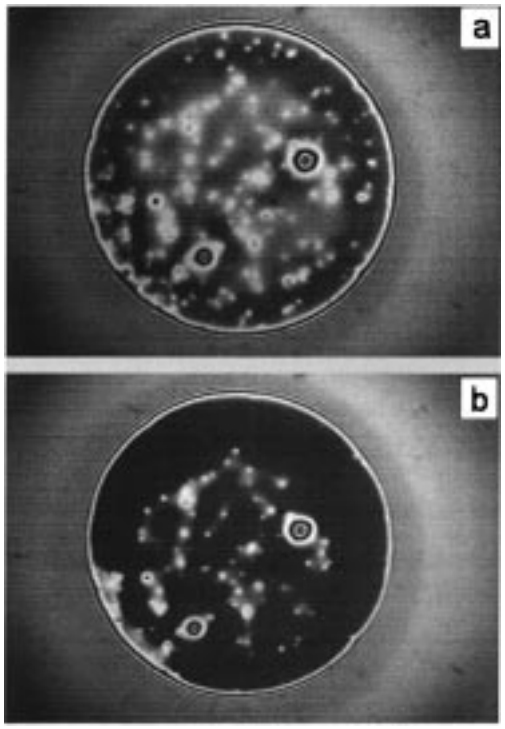

Figure 3. Two consecutive pictures of a thinning film with aggregated protein. The aqueous phase contains 20 mM Ca^{2+}, 150 mM NaCl and is stored for 72 h at 22 °C. The film diameter is approximately 160 μm: (a) picture taken 190 s after the film is formed; (b) picture taken 380 s after the film formation.

5. Effect of Oleic Acid Dissolved in the Oil Phase. The fatty acids are a natural component of the vegetable oils[30] that can cause changes in the state of the adsorption layer by electrostatic or hydrophobic coupling to the adsorbed protein molecules. We carried out experiments after dissolving 0.1 wt % of oleic acid in the xylene phase (rows 6 and 7 of Table 1). The presence of the fatty acid led to slightly increased thickness and aggregation when the protein solution was at pH 5. The acid did not affect the strength of adhesion between the film surfaces.

B. Results for Thin Films in the Presence of Calcium Ions. Both the film thickness and intersurface adhesion changed drastically after the addition of 20 mM of Ca^{2+} (as $CaCl_2$). As shown in Table 1, we observed about a 2-fold decrease of the film thickness, from ca. 21 to 10.4 nm. Even more dramatic was the increase of the adhesion energy from $<10^{-2}$ to >20 mJ/m^2. Phenomenologically this was manifested by an inability to decrease the diameter of the equilibrium films even when the pressure in the cell was increased to the extent where the meniscus surfaces became perpendicular to the film, i.e., when the surfaces were pulled apart with the full force of the oil/water interfacial tension. Notably, this extraordinary strong adhesion was noticed only after the films were allowed to drain fully to their equilibrium thickness.

The film data as a function of the calcium ion concentration are summarized in Figure 4. The film thickness was not affected by Ca^{2+} concentrations up to 0.5 mM, but it decreased from ≈21 to ≈11 nm when the calcium concentration was raised from 1 to 12 mM. Higher Ca^{2+} concentrations did not lead to changes in the measured thickness. The value of 12 mM was also a threshold one for the transition from the mobile contact line (with an advancing angle of only few degrees) to a

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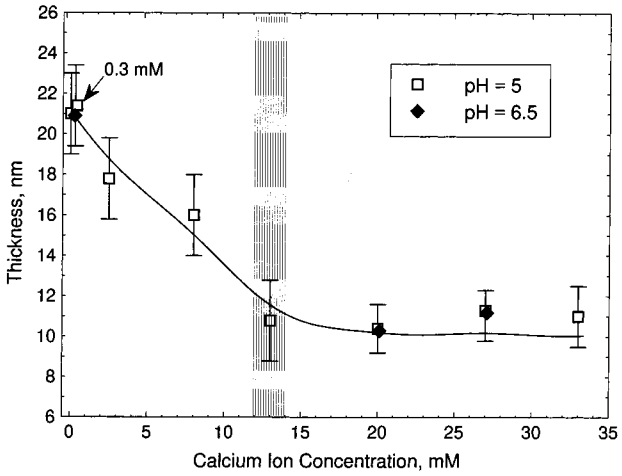

Figure 4. Thickness of the measured emulsion films presented as a function of the concentration of Ca^{2+}. All samples contain 150 mM of NaCl. The shaded area marks the transition from low to high intersurface adhesion.

complete sticking of the film surfaces together. This jumpwise increase of the intersurface adhesion energy may have an important impact on the stability of real batch emulsions.

1. Effect of Electrolyte Concentration, Oleic Acid, Aging, and pH in the Presence of Ca^{2+}. Due to the strong impact of calcium on the film properties, the effects of these parameters were studied separately at a fixed concentration of 20 mM Ca^{2+} (Table 2). The decrease of the NaCl concentration to 100 mM did not significantly change the final thickness and the thinning pattern of the films (row 2). This indicates that the observed 2-fold decrease of the film thickness in the presence of Ca^{2+} is not caused solely by nonspecific interactions due to shrinkage of the counterion atmosphere. The addition of oleic acid in the oil (row 3) did not lead to significant changes in the film thickness either, though a slight increase appeared to be recorded. However, the stability of the final equilibrium film in this case was decreased, probably due the simultaneous presence of calcium ions and fatty acid (which may form some kind of interfacial "precipitate").

The prolonged storage of the β-casein solutions with calcium at room temperature led to pronounced aggregation of the protein, which was apparent from the exceptionally large number of aggregates entrapped in the film (Figure 3a). Although these aggregates gradually "dissolved" (Figure 3b), the film thinning slowed by approximately 30%. The final thickness of the equilibrium films in this case was lowered to 8.6 nm, which indicates that significant changes in the state of the protein adsorption layer had occurred.

The increase of the pH from 5 to 6.5 did not induce any changes in the final film thickness, as seen from both Table 2 and Figure 4. However, the thinning pattern of the films at pH 6.5 was different: instead of a slow approach to the final thickness, we observed spotlike formation, by which mechanism the final film thickness is reached much faster (see right column of Table 2 and compare Figure 2b,d). Such spot-like transitions are common for emulsion films with low molecular weight surfactants[29,37] and may indicate higher interfacial mobility of the adsorbed protein layers.

In relation to the film data, it is necessary to distinguish whether the decrease of thickness with calcium is caused by reconfiguration of the layer or by a sharp decrease in protein adsorption (although the latter hypothesis seems unlikely, as reports indicate that the Ca^{2+}-induced change in the amount of adsorbed casein is approximately 25%[12] or none[16,40]). We used the null type of ellipsometry to investigate the change of the ellipsometric angle Δ after protein injection below a clean O/W interface. The obtained values for $\delta\Delta$ at an angle of incidence of 40.09° were the same for the samples containing 0, 5, and 15 mM $CaCl_2$ and equal to 2.41 ± 0.28°. In the first-order approximation, the $\delta\Delta$ parameter could be considered[41] to be proportional to the product of the layer thickness and the protein volume fraction (which product is also equal to the amount of protein in the layer). Thus, our data provide an indication that there is no substantial change in the amount of adsorbed casein in the presence of calcium. The major change in the thickness therefore occurs via reconfiguration and increase of the adsorption layer density.

SFA Measurements. The results from the surface force apparatus measurements are summarized in Table 3. The equilibrium thickness in the absence of calcium was slightly higher but comparable to the corresponding value from the emulsion film experiments. It is also in good correlation with the value of the distance of contact between the protein tails (25 nm) reported in a study that appeared while this material was in preparation.[42] However unlike that study, we have not observed any transition to a film of lower thickness, which in our case may be attributed to higher surface coverage and lower compression. The thickness of the protein films decreased by about 9 nm in the presence of Ca^{2+}, which is in good correlation with the emulsion data. The energy of adhesion between the surfaces can be estimated from the measured pull-off force, F_p, by the formula[35,43]

$$W_{adh} = -\frac{2F_p}{3\pi R}$$

where R is the curvature of the mounted mica sheets. The calculated values are shown in the third column of Table 3. The adhesion energy in the absence of Ca^{2+} was higher than in the case of fluid films, and the value obtained is close to the one reported in ref 42. The addition of Ca^{2+} led to an ≈6-fold increase of the adhesion energy; however, this adhesion was much lower than in the corresponding emulsion case. Thus, the effect of calcium-induced adhesion was present with films between β-casein-covered solid surfaces, but it was not as drastic as in the case of emulsion films.

Electrophoretic Measurements. The data obtained on the electrophoretic mobility of protein-covered oil droplets are presented in Table 4. It has been reported in the literature that the presence of calcium ions leads to a decrease in the magnitude of the measured mobility and the respective negative ζ potential. The same effect can, however, occur in principle with increasing concentrations of NaCl due to the shrinkage of the counterion atmosphere. To quantify the specific effect of the calcium ions, we carried out measurements (rows 2−6) by keeping

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Table 2. Data on the Final Thickness and Thinning Pattern of Emulsion Films Stabilized by 0.01 wt % of β-Casein, in the Presence of 20 mM $CaCl_2$

no.	system	h_{final}, nm	thinning rate and pattern
1	150 mM NaCl, pH 5	10.4 ± 1.0	thins to final thickness in ca. 6.5 min
2	100 mM NaCl, pH 5	9.8 ± 0.7	rate of thinning as above
3	150 mM NaCl, pH 5, 0.1% oleic acid in the oil	11.1 ± 1.4	same rate of thinning but the final thin films are unstable
4	150 mM NaCl, pH 5 72 h at 22 °C	8.6 ± 0.6	very high aggregation, slows film thinning to ca. 8.5 min
5	150 mM NaCl, pH 6.5	10.3 ± 0.7	quick thinning in 2.0 min by spotlike transition to black films

Table 3. Final Thickness and Energy of Adhesion of β-Casein Adsorption Layers on Hydrophobized Mica Measured by SFA[a]

Ca^{2+} concn, mM	lowest thickness, nm	energy of adhesion, mJ/m^2
0	22.2 ± 5	0.14 ± 0.02
20	13.0 ± 5	0.72 ± 0.04

[a] The solutions also contain NaCl to a total ionic strength of 150 mM.

Table 4. Measured Electrophoretic Mobilities of β-Casein-Covered Emulsion Drops at Different Ionic Strengths and Ca^{2+} Concentrations

ionic strength, mM	Ca^{2+} concn, mM	electrophoretic mobility, $m^2\,V^{-1}\,s^{-1}$	peak half-width, %
1	0	−3.89	16
45	0	−2.55	17
45	5	−1.66	23
45	9	−1.24	26
45	12	−1.01	30
45	15	−0.92	36

the ionic strength of the saline environment,[44] I, constant at 45 mM

$$I = \frac{1}{2}(2[\mathrm{NaCl}] + 6[\mathrm{CaCl_2}])$$

where [NaCl] and [$CaCl_2$] are the respective concentrations of the salts dissolved. When I is kept constant, the Debye screening length,[44,45] $(\kappa)^{-1}$, for all of the samples remains the same at 1.43 nm

$$(\kappa)^{-1} = \left(\frac{\epsilon\epsilon_0 kT}{2000F^2N_a}\right)^{1/2}\frac{1}{\sqrt{I}}$$

where $\epsilon\epsilon_o kT$ is the product of the dielectric permittivity of water, permittivity of free space, Boltzmann's constant, and the temperature, F is the Faraday constant, and N_a is the Avogadro constant. This experimental procedure provided data on the specific adsorption of calcium ions onto the surface of the protein layer. The ζ potential was calculated from the electrophoretic mobility data using the Smoluchowski equation.[44] The electrokinetic charge density of the protein layer, σ, was estimated on the basis of the Grahame equation[45] by substituting ζ for the surface potential Ψ (e is the electronic charge)

$$\sigma = \sqrt{8\epsilon\epsilon_0 kT}\,\sinh(-e\Psi/2kT)([\mathrm{Na^+}] + [\mathrm{Ca^{2+}}](2 + \exp(-e\Psi/kT))^{1/2}$$

This equation is strictly valid for flat surfaces and does not take into account the effect of the droplet curvature on the calculated potential. Since $(\kappa)^{-1} \ll r_d$ (r_d is the droplet radius), this effect was small and was estimated to be below 2% on the basis of the equation of Loeb et al.[44] It should be emphasized, that as the value of the ζ potential is used in the calculations, the above estimate gives only the charges affecting the potential in the shear plane around the droplets. As pointed out in the discussion, there are strong reasons to believe that the shear plane is situated close to the outer side of the protein adsorption layer. The mean number of ions adsorbed on a single molecule can be estimated by multiplying the values of the surface charge by the expected area per β-casein entity adsorbed (14.5 nm^2).[10,11,13,15] The results from the ζ potential and surface charge calculations are presented in Figure 5. This estimation suggests that close to the threshold of the strong intersurface adhesion (0.12 mM Ca^{2+}), the registered change in the positive charges adsorbed per molecule reaches unity.

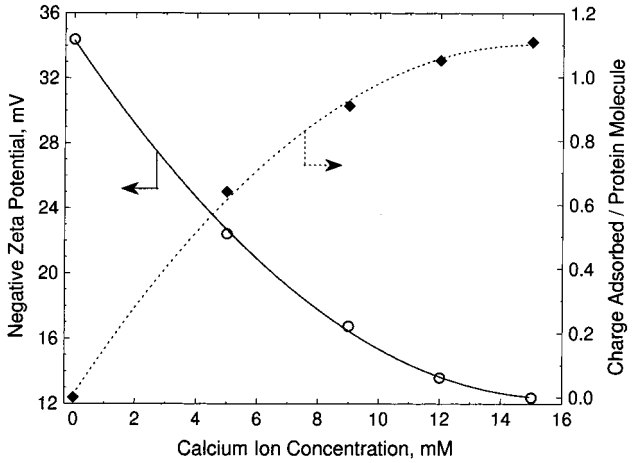

Figure 5. Dependence of the ζ potential and estimated charge adsorbed per protein molecule on the Ca^{2+} concentration.

Resolution of Batch Emulsions. We followed the resolution within real batch emulsion samples of varying Ca^{2+} concentration at fixed ionic strength (45 mM), to check out whether a flocculation threshold exists due to increased adhesion between the droplets. The results are presented in Figure 6. It is seen that the speed of resolution was lowest with the sample containing only NaCl as electrolyte and did not change significantly in the presence of 5 mM $CaCl_2$. Further addition of Ca^{2+} led to a gradual increase of the resolution speed. Though the data do not demonstrate a discontinuous transition in the creaming rate and flocculation, it is evident that emulsion destabilization is encountered only above a certain, not very low, concentration of calcium ions (5 mM in this case). The creamed layer in the samples of high calcium concentration (≥9 mM) was highly viscoelastic and could not be redispersed by mild shaking. The release of oil after storage of the samples for 12−24 h was 30−60% higher for the three emulsions of high calcium concentration. Consistent with our film data, the calcium desta-

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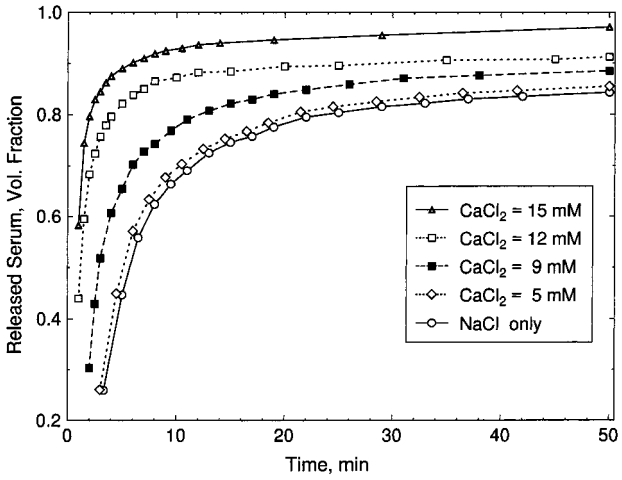

Figure 6. Resolution curves for emulsion samples with different Ca^{2+} concentrations. The ordinate is the normalized volume of the serum released from the creamed emulsion.

bilizes the emulsions by increasing both their flocculation and coalescence rates.

Discussion

Film Thickness in the Absence of Ca^{2+}. The emulsion films at low electrolyte concentration are much thicker (70 nm, Table 1) than the sum of the thicknesses of the two β-casein adsorption layers. The surfaces are separated by long-range electrostatic repulsion.[27,29,37] The film thinning is driven by the capillary pressure in the model cell, P_{cap}, which in our case can be estimated as

$$P_{cap} = \frac{2\gamma R_c}{R_c^2 - R_f^2} = 15.1 \text{ Pa}$$

where R_c and R_f are the cell and film diameters.[46] When the film thickness reaches equilibrium, the capillary pressure is counterbalanced by the electrostatic repulsion between the charged surfaces.

The formulas, based on the DLVO theory, which relate the interfacial potential to the measured film thickness are well-known.[47] By these formulas, the effective surface potential at 1 mM of electrolyte was numerically calculated as −41.3 mV. This value is 20% lower than that from electrophoretic mobility measurements (−51.9 mV), but because of the complexity of the electrostatic interactions in the system we find the obtained correlation reasonable. Low concentrations of electrolyte are seldom encountered in food emulsions, but the data demonstrate that the importance of long-range electrostatics in some cases should not be underestimated.

In the more common case when the films contain 150 mM of electrolyte, the long-range repulsion is suppressed and the capillary pressure is opposed by the osmotic pressure of the overlapping chains of the protein adsorption layers ("steric repulsion").[1,38,45] The magnitude of the steric repulsion is usually quite high, and because of the low capillary pressures generated in the model cell, a minor overlap of the extended casein chains could be expected. It has been shown[48] that the thickness of polymer-stabilized liquid films is approximately twice the thickness of the most extended protein chains in the adsorption layers. Our data are in good correlation with the literature reports, which show that the "steric" thickness thus obtained is equal to, or slightly lower than, the "hydrodynamic" thickness obtained by flow experiments and higher than the thickness obtained by methods sensitive to the density of the adsorbed layer.[49,50] The value of 21 nm for the thickness of two β-casein layers (rows 2 and 3 of Table 1) is higher than *twice* the thicknesses measured by neutron reflectivity (a method that is sensitive to the protein density), 8−16 nm.[4,10,12,18−20] On the other hand, our value is a little lower than *twice* the casein layer thickness determined through hydrodynamic radii (which is sensitive to the length of the longest extended protein chains), 20−40 nm.[11,15,16]

Film Thickness in the Presence of Ca^{2+}. Our data suggest that above a certain concentration, the calcium ions cause compression of all of the β-casein tails toward the compact adsorption layer in the vicinity of the interface. The values obtained for the layer thickness in the presence of Ca^{2+} (10.4 nm for two layers, Table 1 and Figure 4) are in good correlation with twice the single layer thickness from literature reports, 8−10 nm obtained through neutron reflectivity[12] and 12−16 nm obtained by light scattering.[16]

To understand this behavior of the β-casein, it is worth considering the molecular configuration and the binding ability of the protein molecules adsorbed at the oil/water interfaces. It has been firmly established that the segment of the β-casein molecule that extends into the water phase is the chain of 48−50 hydrophilic residues at the N-terminus end.[9,11,12,16,18,20] Two alternative models for the conformation of this chain have been proposed. The first model assumes that the chains extend as "tails", the N-terminus being farthest from the interface,[11,12,20] while the second one suggests a "loop" configuration into which the terminus is adsorbed at the interface.[9,14,16] As it has been established[15] that at the concentration studied β-casein adsorption is monolayer in nature, assuming a 50 residue "loop" extended 10 nm away from the surface gives a vertical increment per residue of ≈0.4 nm. This value is approximately equal to that in a fully extended polypeptide chain[51] and is unrealistic for a hydrated protein due to entropic reasons. Therefore, our data suggest that some, or all, of the adsorbed molecules are in the "tail" configuration. A schematic presentation of the terminal chain of 40 amino acids in a "tail" configuration is given in Figure 7.

In the presence of Ca^{2+} ions, specific adsorption of five divalent ions onto the phosphorylated serine residues, situated at positions 15, 17−19, and 35 along the chain, is likely to take place. Previous studies[21,24] have shown that the amount of calcium ions bound by β-casein gradually increases with the calcium concentration, reaching the value of 5 mol of Ca^{2+}/mol of casein at approximately 10 mM dissolved Ca^{2+}. This concentration corresponds to the one at which we record a collapse of the layer thickness and cross-binding of the film surfaces. Therefore, the reconfiguration of the tails follows the saturation of the phosphorylated residues with calcium. It is suggested in the literature that such a reconfiguration occurs due to a decrease in the electrostatic repulsion between the strongly charged phosphoseryl residues and the interface.[16]

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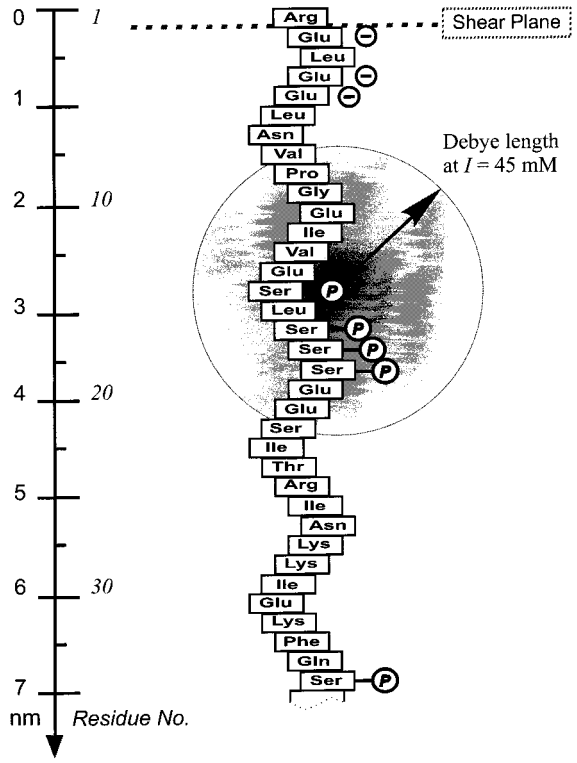

Figure 7. Schematics of the first 40 residues at the N-terminus of the β-casein tail. The phosphorylated residues that are able to bind Ca^{2+}specifically are denoted by "p". The radius of the circle around the first phosphoseryl residue is equal to the Debye screening length at $I = 45$ mM. Three glutamic acid residues near the outer end of the tail that can eventually participate in calcium adsorption are marked with "—".

Calcium-Induced Adhesion. The most interesting and practically important result from the film measurements is the jumpwise increase of intersurface adhesion with the increase in Ca^{2+} concentration. The calcium ions obviously cross-link the casein molecules, forming a gellike structure in the film. The exact protein conformation in these cross-linked layers does not appear so transparent in the context of literature and our data. One is tempted to assume that this adhesion arises from calcium cross-binding of the phosphorylated groups of the molecules from the opposing film surfaces. The phosphorylated sites in the extended tails are buried 2.8 nm deep into the adsorption layer (Figure 7), in which state they are sterically hindered from participation in cross-linking. The phosphorylated residues may become exposed on the surface of the adsorption layer only after the tails collapse when calcium is added. If true, this hypothesis provides a simple and straightforward explanation for the threshold increase of the adhesion energy in the region of layer shrinkage.

The electrophoretic data may shed some light on the amount of calcium available for cross-linking onto the surface of the protein layer. The electrokinetic description of a polyelectrolyte-covered interface is a complex problem; however a simple assumption can be used in the discussion here. It has been established that the hydrodynamic thickness of an adsorbed polymer layer is approximately equal to the length of the extended tails, even when the density of these chains is very low.[49,50] Thus, in our case we may assume that the shear plane is situated close to the terminus of the casein tails extended out into the solution. While on the basis of the literature, we are certain that in the investigated concentration region each casein molecule will bind ca. 5 Ca^{2+} ions (10 positive charges); by the electrophoretic method we register specific adsorption of only one positive charge per molecule. This could be explained by keeping in mind that the phosphorylated residues in the extended chains are buried at a thickness bigger than the Debye screening length of the counterion atmosphere (Figure 7). These charges can be considered as effectively screened from contributing to the potential in the plane of shear. Therefore, as long as the casein tails remain extended, the electrophoretic method should not register the charges contributed by the adsorption of Ca^{2+} at the phosphorylated residues.

The value of one positive charge adsorbed per protein molecule obtained by the electrophoretic data could be considered as indicative of the amount of calcium present only in the close vicinity of the outer side of the protein layer and accessible for cross-linking with the opposite molecules in the film. It is well-known that the optimal conditions for flocculation via cross-linking occur when the binding sites are only half-saturated with linking agent.[52] Though this may be somewhat coincidental, our data point out that the optimal condition for cross-binding (1 accessible Ca^{2+} ion per two β-casein molecules) is reached exactly in the concentration region where we registered strong intersurface adhesion. As the surfaces stick together at a concentration of calcium where the phosphorylated binding sites are fully saturated, the calcium ions that participate in this cross-binding may be adsorbed not only on the phosphorylated residues but also on the other negatively charged amino acids close to the end of the tail (Figure 7).

It has been demonstrated previously,[22−25] as well as in this study, that the presence of Ca^{2+} is a major destabilizing factor in the β-casein-containing emulsions. The source of this destabilization is possibly the strong adhesion of the protein adsorption layers, which leads to quick flocculation, after which the thin, gellike interdroplet films formed may be unable to resist shear and thermal fluctuations. The use of cross-binding agents and ones that bring about specific interactions is a promising field of study,[53] as these could in principle be used as a powerful tool in the design of protein containing foams and emulsions with defined stability and rheology.

The SFA data indicate that the thicknesses of the casein film between the mica sheets with and without Ca^{2+} are approximately the same as in the emulsion systems. This is not surprising, as long as in both cases the strong steric repulsion should be the main factor determining this thickness. Even though a variety of configurations have been reported for the casein layers between mica sheets,[42] having in mind the heavy hydrophobization of the mica surfaces, we expect that the protein molecules in our liquid and solid films are in similar configurations. Despite that, the measured effect of the Ca^{2+} on the SFA intersurface adhesion was much weaker than in the emulsion case. The relatively weak calcium-induced adhesion can be explained by the lack of tangential mobility of the molecules adsorbed on the opposing solid surfaces, which does not permit the optimal cross-linking conditions to be achieved. It is interesting, whether the addition of Ca^{2+} to a casein-stabilized suspension of solid particles would induce flocculation in a manner similar to that observed in emulsion systems. Our data suggest that a certain degree of suspension flocculation may occur, though the significance of that effect appears questionable.

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The presence of calcium and the prehistory of the solution were able to influence the properties of the thin films not only by changing the molecular configuration of the single protein molecules but also by influencing the aggregation state of the protein in the bulk solution. As suggested earlier,[27] and indicated by our data, the aggregates can significantly slow the film thinning rate and change the mechanism of film stabilization. One interesting, but still poorly understood, phenomenon is the disappearance of the aggregates compressed in the thin films. The only mechanism that appears plausible for this process is the disaggregation of the protein clusters, followed by adsorption and interfacial transport. However, this mechanism contradicts the current concepts of retarded, sterically hindered adsorption and immobile, viscoelastic adsorption layers of protein. Thus, the role of the aggregates and their dynamics in the emulsion appears still to hold some intriguing questions.

Conclusions

Our results demonstrate that the thin emulsion film method as applied in this work can provide data that is complementary and consistent with the available information obtained by other methods. By this method we show that the protein aggregation, hydrophobization upon storage, presence of fatty acids and pH could influence the properties and the stability of the β-casein-containing films and emulsions. The most dramatic effect is observed when Ca^{2+} ions above a concentration of 12 mM are dissolved in the film. This leads to a 2-fold decrease of the film thickness and formation of a gellike protein structure in the film that strongly binds the film surfaces together. This change in the structure results from the calcium adsorption on the phosphorylated and possibly on the nonphosphorylated amino acid residues in the N-terminal protein tail that extends into the aqueous phase. The data provide additional information on the mechanism of calcium-induced destabilization of food emulsions.

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