# Coalescence in β-Lactoglobulin-Stabilized Emulsions: Effects of Protein Adsorption and Drop Size

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The effects of two factors on the coalescence stability of protein containing, oil-in-water emulsions are studied: (1) protein adsorption on the drop surface and (2) drop size. The experiments are performed with β-lactoglobulin (BLG) solutions, with protein concentration spanning the range between $2 \times 10^{-4}$ and 0.5 wt %. A combination of several experimental methods is applied: centrifugation for quantitative assessment of the emulsion stability, the Bradford method to determine protein adsorption, and the film trapping technique (FTT) to quantify the effect of drop size. An analysis of the conditions for coalescence in the cream during centrifugation is performed to interpret properly the experimental data. The obtained results convincingly show the presence of a well-defined threshold value of the BLG adsorption, $\Gamma^* \approx 1.55$ mg/m$^2$, which is required for obtaining stable emulsions: the emulsions are very unstable at $\Gamma < \Gamma^*$, a stepwise increase of stability is observed at $\Gamma \approx \Gamma^*$, and a further, much slower increase is observed at $\Gamma > \Gamma^*$. The value of $\Gamma^*$ is slightly lower than the one for a dense monolayer of adsorbed BLG molecules ($\Gamma_M \approx 1.65$ mg/m$^2$). The experiments show that the emulsion stability strongly decreases with the increase of the drop size (at constant protein adsorption). A simple relation between the value of the critical pressure, $P_{CR}$, which characterizes the coalescence barrier (measured by FTT or centrifugation) and the drop size, is found experimentally: $1/P_{CR}$ is a linear function of the drop radius. The same relation has been found applicable to two other systems, as well (emulsions stabilized by whey protein concentrate and single oil drops stabilized by an anionic surfactant of low molecular mass). Thus, we have been able to separate the effects of the protein adsorption and the drop size from each other.

## 1. Introduction

In this paper we study the effect of two factors on the coalescence of protein stabilized, oil-in-water emulsions: (1) protein adsorption on the drop surface and (2) drop size. Although the effect of these factors is well-known to scientists and practitioners[1−6] (larger adsorption and smaller drops correspond to more stable emulsions under equivalent other conditions), their systematic quantitative analysis is still missing in the literature.

The main difficulty in the analysis of these effects is that they usually interfere in the real emulsions. In virtually all studies of protein-stabilized emulsions, the used emulsification methods produce smaller drops at higher protein concentrations (when the adsorption is expected to be larger), so the relative contributions of the aforementioned two effects cannot be separated.[3−6]

An additional difficulty arises from the tedious and not very accurate procedures for determination of the protein adsorption in emulsions. This is probably one of the reasons that the emulsion stability data is usually plotted as a function of protein concentration in the aqueous phase. However, it is intuitively expected that the stability to coalescence should depend primarily on the protein adsorption at the surfaces of the emulsion films, rather than on the protein concentration in the bulk solution.[1−3]

An uncomfortable consequence of the absence of systematic data relating the protein adsorption to emulsion stability is the following: It is well-known that the emulsion stability toward coalescence steadily increases with protein concentration in a very wide range of concentrations (from ca. $10^{-4}$ up to several weight percent).[3−6] However, several measurements of β-lactoglobulin (BLG) adsorption, Γ, on a single air−water interface showed that Γ was virtually independent of the bulk BLG concentration in the range between $10^{-3}$ and $2 \times 10^{-2}$ wt %.[7,8] For example, Miller et al.[8] reported that the adsorption, Γ (measured by ellipsometry), remained practically constant, 1.6−1.7 mg/m$^2$, in the concentration range between $1.5 \times 10^{-3}$ and $1.8 \times 10^{-2}$ wt %. Very similar values were obtained by Atkinson et al.[7] by using the method of neutron reflectivity for the concentration range between $10^{-3}$ and $10^{-2}$ wt % BLG. It is still unclear whether the emulsion stability in the latter concentration range increases just because of a reduced drop size, or alternatively, the measurements at single interfaces are not representative for the protein adsorption on the drops' surface in batch emulsions. Indeed, a considerably larger

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protein adsorption is often reported in experiments with batch emulsions, as compared to the results from ellipsometrical measurements with single interfaces at the same pH and bulk protein concentration.[9] Note that the measurements at single interfaces are made typically under mild hydrodynamic conditions (the protein is transported toward the interface mainly by diffusion),[8,10] whereas the emulsions are produced by vigorous stirring, so it is uncertain whether the adsorption should be the same in these two very different experimental situations. Let us note that the protein adsorption is often considered[4,5,10] as an irreversible process, so the final adsorption could strongly depend on the conditions under which the process takes place. In addition, there is a considerable variation between the data obtained for BLG by various authors[8,10,11] (under apparently similar conditions), which makes the analysis even more subtle.

To clarify further the effects of protein adsorption and drop size on the stability of protein emulsions, we combine in the present study several experimental methods that seem particularly suitable for our aims: (1) Like in refs 1, 3, 6, 12−16, centrifugation is used for quantification of the emulsion stability to coalescence, (2) the method of Bradford[17] is used to determine the protein adsorption in batch emulsions, and (3) the film trapping technique[18−20] (FTT) is used to quantify the effect of drop size by measuring the coalescence barrier for single emulsion drops of various diameters. All experiments are performed with BLG solutions, with protein concentration spanning the range between $2 \times 10^{-4}$ and 0.5 wt %.

The obtained results convincingly show the presence of a well-defined threshold value of the protein adsorption, Γ*, which is required for obtaining stable emulsions. The value of Γ* is slightly lower than the value measured for a dense monolayer of adsorbed BLG molecules at the air−water interface.[8] We find also that the coalescence barrier strongly decreases with the oil drop size, and a simple relation between the value of the critical capillary pressure (which characterizes the coalescence barrier) and the drop radius is established. The effects of other factors, such as pH, thermal treatment, and emulsion aging, present the subject of a separate study (manuscript in preparation).

## 2. Materials and Methods

**2.1. Materials.** The protein β-lactoglobulin (BLG), from bovine milk, was a product of Sigma (Cat. No. L-0130, Lot No. 124H7045) and was used as received. Soybean oil (used as an oil phase) was purified from polar contaminants by passing through a glass column filled with Florisil adsorbent.[21]

The aqueous solutions were prepared with deionized water, purified by a Milli-Q Organex system (Millipore). Along with the protein, all solutions contained 0.15 M NaCl (Merck, analytical grade, heated for 5 h at 450 °C) and 0.01 wt % $NaN_3$ (Riedel-de Haën). The experiments were carried out at the natural pH ≈ 6.2 for BLG solutions, without additional adjustment.

Sodium dodecyl sulfate (SDS, product of Sigma), was used for deflocculation of the oil drops after centrifugation, when specimens for determination of the drop size distribution were prepared (see section 2.3 for the respective procedure).

**2.2. Emulsion Preparation.** Oil in water emulsions were prepared by intensive stirring of 35 mL of protein solution and 15 mL of soybean oil (30 vol. %) with a rotor-stator homogenizer (Ultra Turrax T25, Janke & Kunkel GmbH & Co, IKA-Labortechnik) operating at 13 500 rpm. The duration of stirring was fixed at 3 min for all emulsions. Our experiments showed that this procedure of homogenization gives emulsions with similar drop size distributions and almost the same mean drop radius for all of the studied emulsions, which allows us to separate the drop size effect from the other effects on emulsion stability.

**2.3. Determination of the Drop Size Distribution.** The drop size distribution before and after centrifugation of the emulsions was determined by optical microscopy. The emulsions were always well mixed, before taking specimens for optical observations, to avoid the vertical segregation of the drops by size, which would distort the measured drop size distributions.

The specimens from noncentrifuged emulsions were taken immediately after emulsion preparation. The oil drops were observed in transmitted light with a microscope (Axioplan, Zeiss, Germany) equipped with objective Epiplan ×50 and connected to a CCD camera (Sony) and video recorder (Samsung SV-4000). The diameters of the recorded oil drops were afterward measured (one by one) by using a custom-made, image analysis software operating with a Targa+ graphic board (Truevision). The diameters of at least 5000 drops (from 3 to 10 independently prepared emulsions) were measured for each system.

The procedure for preparation of samples for drop size determination of centrifuged emulsions was slightly different: After centrifugation, the serum (the separated protein solution below the cream) was gently removed by a syringe and replaced by 20 mM SDS solution. The latter facilitated the deflocculation of the emulsion drops. Afterward, the emulsion was gently shaken by hand to disperse the drops throughout the entire emulsion volume and a sample for optical observation was taken. It was reported in the literature[3,22] that this procedure does not change the size distribution for drops with radii below 100 μm.

The mean volume-surface radius, $R_{32}$, was calculated from the size-distribution histogram by using the relation

$$R_{32} = \frac{\sum_i N_i R_i^3}{\sum_i N_i R_i^2} \qquad (1)$$

where $N_i$ is the number of drops with radius $R_i$. One can calculate the specific surface area of the drops, $S$ (area per unit volume

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of dispersed oil), from $R_{32}$ by the equation

$$S = \frac{4\pi {R_{32}}^2}{(4/3)\pi {R_{32}}^3} = \frac{3}{R_{32}} \quad (2)$$

If $R_{32}$ is expressed in microns, $S$ has the dimension of square meters per mL of oil.

Let us note that the used procedure of optical observation detects all of the droplets present in the sample (even the smallest ones). Therefore, the main error in the calculation of $R_{32}$ could come from inaccurate measurement of the size of drops whose diameter is comparable to the optical resolution of the microscope ($\approx$0.6 $\mu$m). The number fraction of these smallest drops (ca. of diameter $< 1\ \mu$m) was estimated from the experimental data to be <0.3%, which means that the contribution of the small drops in the calculated value of $R_{32}$ is negligible. From eq 1, one can estimate that the contribution of these small drops could be significant only if their number fraction was strongly prevailing (e.g., >80%), which was not the case in our systems—the main number fraction of the drops fall in the range between 2 and 5 $\mu$m.

**2.4. Determination of Protein Adsorption.** The protein adsorption was calculated from the specific surface area of the oil drops, $S$, and from the change of the protein concentration in the aqueous phase (the serum) as a result of the emulsification process.

The protein concentration in the serum was determined by the method of Bradford,[17] which involves the binding of a dye (Coomassie Brilliant Blue G250) to the protein. The formation of the protein−dye complex causes a change in the light absorption spectrum of the dye, which is used to determine the protein concentration by a spectrophotometer.

The procedure was as follows: After preparing the emulsions, they were kept immobile in a gravity field for 30 min. During this period, the oil drops floated up under the action of buoyancy, forming a cream. The serum remaining below the cream was taken out from the vial by using a syringe. At this stage, the serum was slightly turbid, because it contained a small fraction of dispersed tiny oil drops. To remove these drops, which could affect the protein concentration determination, the serum was centrifuged for 1 h at 5500 rpm; the lower half of the serum (deprived of drops) was used for further analysis. A fraction of the centrifuged protein serum was diluted to a protein concentration falling in the range between 1 and 100 $\mu$g/mL. The protein concentration in this solution was determined after addition of the colored reagent of Bradford[17] by means of UV−vis spectrophotometer (UNICAM 5625) at a light wavelength of 595 nm. The spectrophotometric measurements were always performed 2 min after mixing the protein solution and the Bradford reagent.

Two calibration curves were prepared (to avoid dilution error) by following the method from ref 17 (see Figure 1): If the protein concentration was between 10 and 100 $\mu$g/mL, 100 $\mu$L of the protein solution was mixed with 5 mL of the dye solution (standard Bradford method). Alternatively, if the protein concentration was between 1 and 10 $\mu$g/mL, 500 $\mu$L protein solution was mixed with 5 mL of the dye solution (so-called microprotein assay[17]). The appropriate calibration curve was used in the actual measurements, depending on the protein concentration in the serum, $C_{SER}$.

The protein adsorption, $\Gamma$, was calculated from the relation

$$\Gamma = \frac{V_C(C_{INI} - C_{SER})}{S V_{OIL}} = \frac{V_C(C_{INI} - C_{SER})R_{32}}{3V_{OIL}} \quad (3)$$

where $V_C$ and $V_{OIL}$ are the volumes of the continuous and oil phases, while $C_{INI}$ and $C_{SER}$ are the protein concentration in the initial solution (prior to emulsification) and the concentration in the serum after the emulsification procedure, respectively. Whenever $C_{INI} = C_{SER}$, we use the notation $C_P$ for the protein concentration.

**2.5. Evaluation of the Emulsion Stability by Centrifugation.** The emulsions were centrifuged at 20 °C in 3K15 centrifuge (Sigma Laborzentrifugen, Germany), by using a swinging-bucket rotor. The centrifugal acceleration was varied between 10 and 5000$g$, depending on the experiment ($g$ is the gravity acceleration). Due to the centrifugal force, the emulsion drops (with lower density than the continuous phase) move toward the axis of rotation and form a cream. For reasons explained in sections 3.5 and 3.6 below, we characterize the stability of the emulsions subjected to centrifugation by the critical osmotic pressure, $P_{OSM}^{CR}$, at which a bulk oil layer is released on top of the emulsion cream (see eq 4′ below for an explicit definition).

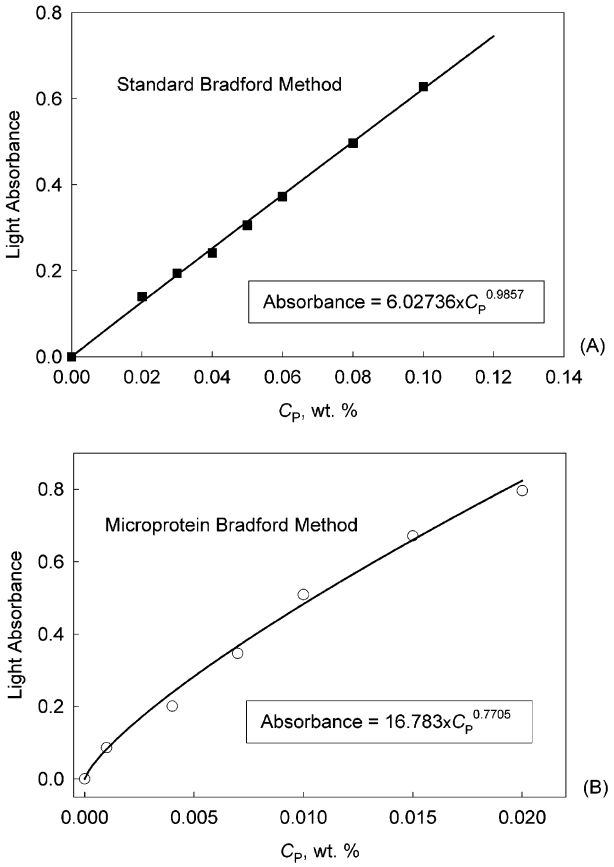


**Figure 1.** Calibration curves of light absorbance at 595 nm as a function of the bulk BLG concentration: (A) standard Bradford assay and (B) microprotein Bradford assay.[17] The experimental points and the curves used for determination of the protein concentration in the serum are shown.

The osmotic pressure of an emulsion, $P_{OSM}$, is defined[23,24] as the pressure difference that should be imposed on the emulsion to maintain it in equilibrium with its bulk continuous phase (the aqueous phase for oil-in-water emulsions), if the latter is separated by a semipermeable membrane (see Figure 2A). As shown by Princen,[23,24] the equilibrium of an emulsion column in a gravity field requires a balance between the osmotic pressure $P_{OSM}(H)$ (which acts to suck in water from the continuous phase below the cream) and the buoyancy force exerted on the oil drops in the cream (which acts to squeeze water from the cream):

$$P_{OSM}(H) = \Delta\rho g \int_0^H \Phi(z)\, dz = \Delta\rho g H \bar{\Phi} \quad (4)$$

where $\Delta\rho$ is the difference between the mass densities of the oil and the aqueous phase, $\Phi(z)$ is the volume fraction of oil in the cream, and $\bar{\Phi}$ is the respective average volume fraction (see Figure 2B).

In our experiments, we denote as critical osmotic pressure, $P_{OSM}^{CR}$, the osmotic pressure at the top of the cream during centrifugation, after the coalescence process has been completed,

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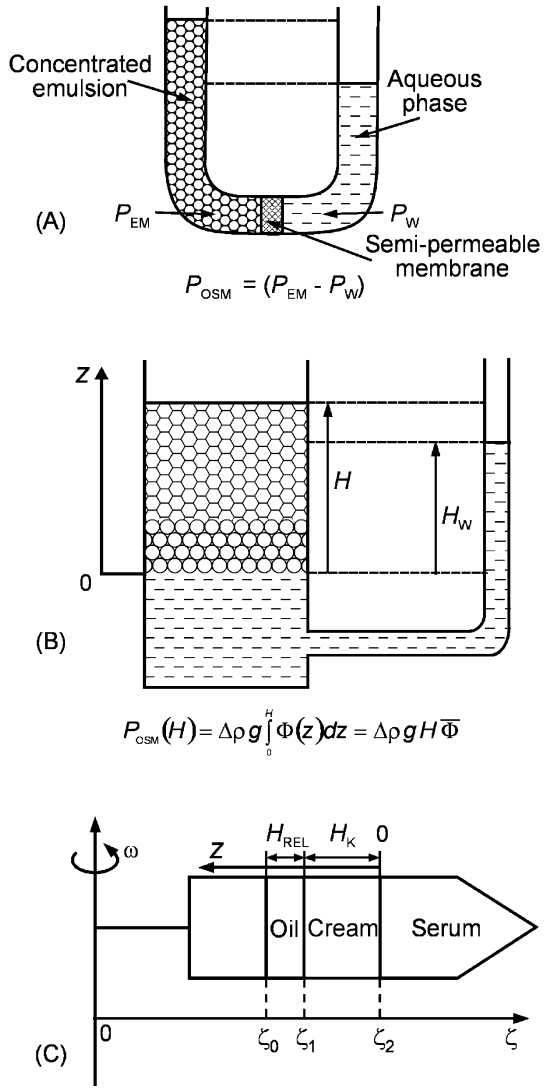


**Figure 2.** (A) The mechanic equilibrium between a concentrated oil-in-water emulsion and an aqueous phase (separated by a semipermeable membrane) requires a pressure difference ($P_{EM} - P_W$), which can be considered as an osmotic pressure, $P_{OSM}$.[23,24] (B) The equilibrium of an emulsion column in a gravity field is achieved as a balance of $P_{OSM}$ (which acts to suck water into the cream) with the buoyancy force acting on the oil drops (thus squeezing water from the cream).[23,24] (C) The same concepts can be used to describe the equilibrium in a centrifugal field: $H_K$ is the equilibrium height of the cream and $H_{REL}$ is the height of the layer of released oil as a result of drop coalescence in the uppermost layer of the cream.

that is, after some oil layer has been released on top of the cream and the system has attained a mechanical equilibrium at the given centrifugal acceleration, $g_k = kg$ (see Figure 2C). As explained in section 3.6 below, the osmotic pressure, defined in this way, is a measure of the stability of the emulsion films formed between the oil drops in the uppermost layer of the cream and the layer of released oil on top of the cream (the possibility for drop−drop coalescence within the cream is discussed in sections 3.5 and 3.6). Let us note that millimeter-sized oil lenses were always present on top of the cream, even prior to centrifugation. These lenses (whose volume was negligible in comparison with the total volume of dispersed oil) remained from the nonperfect procedure of homogenization by the rotor-stator device and could serve as nuclei for initiation of the bulk oil formation during centrifugation.

In principle, the centrifugal acceleration depends on both the frequency of rotation [expressed, e.g., by the angular velocity, $\omega$ (rad/s)] and the distance to the axis of rotation, $\zeta$ (see Figure 2C). $P_{OSM}^{CR}$ can be easily calculated from the experimental data if

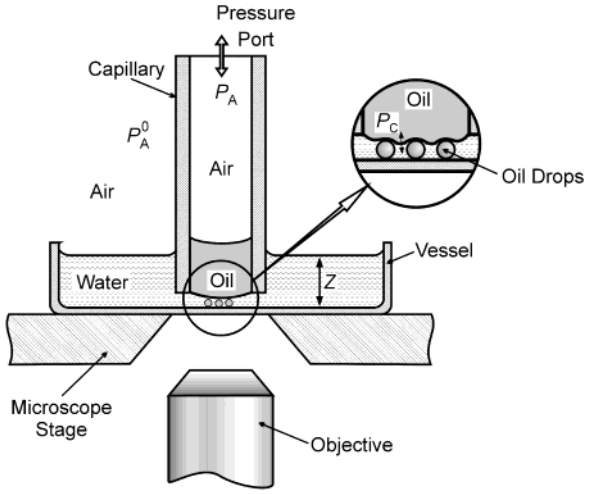


**Figure 3.** Basic scheme of the film trapping apparatus. Scheme of the droplets trapped between the oil−water interface and the substrate (see the magnification lens).

one assumes that the centrifugation field is homogeneous throughout the emulsion column: $g_k \approx \omega^2(\zeta_1 + \zeta_2)/2 = \text{constant}$. Under this assumption, $P_{OSM}^{CR}$ can be calculated from the following relations

$$P_{OSM}^{CR} = \Delta\rho g_k \int_0^{H_k} \Phi(z)\, dz = \Delta\rho g_k H_k \bar{\Phi} = \Delta\rho g_k (V_{OIL} - V_{REL})/A = \Delta\rho g_k (H_{OIL} - H_{REL}) \quad (4')$$

Here $H_k$ is the cream height after the coalescence process has been completed and the equilibrium in the centrifuged test tube has been established, $V_{OIL}$ is the total volume of oil in the centrifuged emulsion, $V_{REL}$ is the volume of the released oil on top of the cream, $A$ is the cross-sectional area of the test tube, $H_{OIL} = (V_{OIL}/A)$, and $H_{REL} = (V_{REL}/A)$. Equation 4′ shows that one can determine $P_{OSM}^{CR}$ from the known values of $\Delta\rho = 0.08$ g/cm$^3$ and $H_{OIL}$, and from the measured values of $g_k$ and $H_{REL}$.

In the Appendix we estimate the error created by the used approximation for a homogeneous centrifugal field in the cream. It turned out that a more precise calculation, which accounts for the variation of the centrifugal acceleration along the cream height, gives the same result in the framework of our experimental accuracy (±10%).

**2.6. Film Trapping Technique (FTT).** The critical capillary pressure, at which oil drops coalesce with a large oil phase, can be measured by the FTT.[18−20] The principle of the method is the following: A vertical glass capillary, partially filled with oil, is held at a small distance above the flat bottom of a glass vessel (Figure 3). The lower edge of the capillary is immersed in the working protein solution, which contains dispersed oil drops. The capillary is connected to a pressure control system, which allows one to vary and to measure precisely the difference, $\Delta P_A$, between the air pressure in the capillary, $P_A$, and the ambient atmospheric pressure, $P_A^0$ (from $\Delta P_A$ one can determine the capillary pressure at the oil−water interface in the capillary, $P_C$; see eq 5 below). The data acquisition equipment includes a pressure sensor (PX163-005BD5V, Omega Engineering Inc.; pressure range ±125 Pa, accuracy ±1.25 Pa) and a digital multimeter (Metex M-4660A, Metex Instruments), connected to a personal computer.

Upon the increase of $P_A$, the oil−water meniscus in the capillary moves downward against the substrate. When the distance between the oil−water meniscus and the glass substrate becomes smaller than the drop diameter, some of the drops remain entrapped in the formed glass−water−oil layer (Figure 3). The capillary pressure of the oil−water meniscus, formed around the trapped drops, is calculated from the relationship

$$P_C = \Delta P_A - \Delta P_{OIL} - \rho_W g Z \quad (5)$$

where $\Delta P_{OIL}$ is the pressure jump across the oil column in the capillary. $\Delta P_{OIL}$ includes contributions from the hydrostatic

pressure of the oil column and the capillary pressure of the air−oil meniscus. $\Delta P_{OIL}$ is measured after filling the FTT capillary with oil but before immersing the capillary into the water pool ($\Delta P_{OIL} = \Delta P_A$ at that moment). The term $\rho_W g Z$ in eq 5 is the hydrostatic pressure in the aqueous phase; $Z$ is the depth of the water pool outside the capillary, and it is measured by a micrometer translator.

During the FTT experiment, one increases the pressure $P_A$ until coalescence of the entrapped oil drops with the upper oil phase is observed. The capillary pressure in the moment of drop coalescence, $P_C^{CR}$, is called the critical capillary pressure or the barrier to coalescence. Higher values of $P_C^{CR}$ correspond to more stable emulsion films and vice versa.

The trapped oil drops were observed from below, through the substrate, with an inverted optical microscope (Carl Zeiss, Jena, Germany), equipped with objective LD Epiplan ×20/0.40, in reflected monochromatic light, $\lambda = 546$ nm, or in transmitted white light. A digital CCD camera (Kappa CF 8/1 DX) and video recorder (Panasonic NV-HD680) were used to record the observed picture. The observation in transmitted light allowed us to measure the equatorial drop radius, $R_E$. Due to the deformation of the entrapped drops, $R_E$ is somewhat larger than the initial drop radius before deformation, $R_0$. By using the numerical procedure from ref 18, we calculated $R_0$ from the values of $R_E$, $P_C$, and the interfacial tension, $\sigma_{OW}$. The value of $\sigma_{OW}$ was measured by the de Nouy ring technique on a Kruss K10T digital tensiometer. One should note that the calculated value of $R_0$ slightly depends on the exact value of $\sigma_{OW}$, and a precision of about ±20% (which is well above the experimental accuracy) is sufficient to calculate $R_0$.

In reflected light, we observed an interference picture, caused by the curved oil−water meniscus around the trapped drops. After each incremental increase of the pressure in the capillary, $P_A$, we waited until the interference pattern stopped changing, which indicated the establishment of mechanical equilibrium in the system.

The following procedure for preparing emulsion drops for these experiments was used: Several small portions of oil (≈1 μL each) were first deposited on the dry bottom of the working vessel (small Petri dish). Afterward, a protein solution was carefully poured into the vessel. The oil was partially washed out from the glass substrate, but the remaining parts of the oily deposit formed drops of diameter varying between 20 and 500 μm, which were attached to the substrate. These oil drops and the large oil−water interface were kept in contact with the protein solution for a certain period of time $t_A$ (varying from 1 to 30 min) before starting the actual FTT measurements, that is, before increasing the pressure in the capillary and thus forming the oil−water−oil films. This procedure allowed us to measure $P_C^{CR}$ as a function of protein concentration, drop size, and time of protein adsorption, $t_A$. All experiments were carried out at the ambient room temperature ($T = 22 \pm 2$ °C).

## 3. Results and Discussion

**3.1. Effect of Protein Concentration and Adsorption on the Coalescence Barrier, Studied by FTT.** Due to the small interfacial area of the protein solution in the FTT experiments, no exhaustion of the solution (as a result of protein adsorption on the interfaces) is expected to take place. Hence, the protein concentration during the experiment, $C_P$, is practically equal to the initial concentration, $C_{INI}$. FTT experiments showed that the critical coalescence pressure, $P_C^{CR}$, was too high to be measured by the used equipment at $C_P > 10^{-2}$ wt % (at adsorption times $t_A > 5$ min). Hence, the stability of emulsions containing BLG of concentrations higher than $10^{-2}$ wt % was evaluated only by centrifugation (section 3.4). On the other hand, for protein concentrations $C_P < 2 \times 10^{-4}$ wt %, the barrier to drop coalescence was below 8 Pa, which was the lowest accessible limit in the used equipment. Therefore, only data for solutions with $C_P$ varying in the range from $2 \times 10^{-4}$ to $10^{-2}$ wt % are reported and discussed in this section.

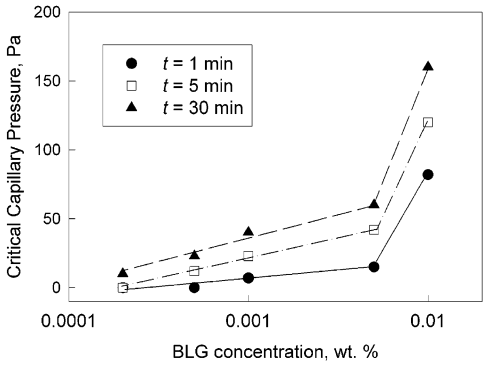


**Figure 4.** Critical capillary pressure, $P_C^{CR}$, measured by FTT for drops of equatorial diameter, $2R_E \approx 20$ μm, as a function of BLG concentration for three different adsorption times (natural pH ≈ 6.2, 0.15 M NaCl, 0.01 wt. % $NaN_3$).

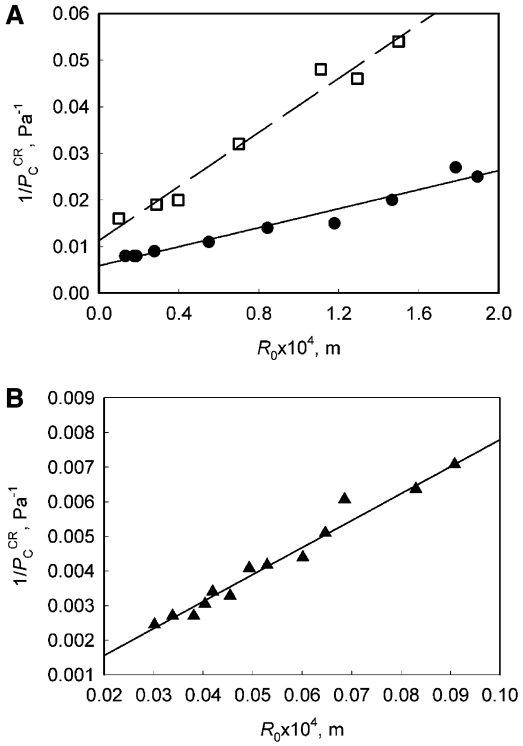


**Figure 5.** Inverse value of the barrier to drop coalescence, $1/P_C^{CR}$, as a function of drop radius, $R_0$, as measured by FTT: (A) Drops stabilized by 0.01 wt. % (full circles) and $5 \times 10^{-3}$ (empty squares) of BLG (0.15 M NaCl, 0.1 g/L $NaN_3$, natural pH). (B) Drops stabilized by 0.1 M SDP3S. The lines are linear fits according to eq 6 for panel A and to eq 6′ for panel B.

The obtained results for $P_C^{CR}$ versus $C_P$ for three different adsorption times ($t_A \approx 1$, 5, and 30 min) are shown in Figure 4. To eliminate the effect of the drop size on the value of $P_C^{CR}$, only results obtained with drops of approximately the same equatorial diameter, $2R_E \approx 20$ μm, are presented.

As seen from Figure 4, the coalescence barrier increases almost linearly with the logarithm of protein concentration in the range between $2 \times 10^{-4}$ and $5 \times 10^{-3}$ wt % (at fixed value of the adsorption time, $t_A$), followed by a much steeper increase at $C_P \approx 0.01$ wt %. The data indicate also a significant increase of the coalescence barrier with the time allowed for adsorption, $t_A$ (cf. the different curves in Figure 4). Additional data, obtained at longer $t_A$ (1−3 h; not shown in Figure 4), confirmed that $P_C^{CR}$ continued to increase even after 1 h of protein adsorption.

The observed strong dependence of $P_C^{CR}$ on $t_A$ shows that the adsorption process had not been completed before starting the FTT experiments, and the proper analysis of these data should include the kinetics of protein adsorption. Indeed, the results presented in several experimental papers[8,11] show that the BLG adsorption in the studied concentration range is a relatively slow process and that the adsorption layer is incomplete in the time scale of interest (up to 30 min) due to kinetic reasons. In other words, we explain the observed increase of $P_C^{CR}$ with the protein concentration at fixed $t_A$ (see Figure 4), as a result of a faster adsorption at higher protein concentrations.

**3.2. Effect of Oil Drop Size on the Coalescence Barrier, Investigated by FTT.** To check how the coalescence barrier depends on the drop size at a given protein concentration, FTT experiments with drops having an equatorial radius between 10 and 200 $\mu$m were performed at two different protein concentrations (0.005 and 0.01 wt %) (see Figure 5A). The drops were kept in contact for 30 min with the protein solution before trapping them in a film to ensure similar protein adsorption on the drop surface (at a given BLG concentration), independent of the drop size.

The results clearly show that the larger drops coalesce at lower capillary pressure, $P_C^{CR}$, that is, the barrier to coalescence decreases with the increase of drop size. The results can be represented by a simple empirical expression, which implies that $1/P_C^{CR}$ is a linear function of the drop radius, $R_0$ (see Figure 5A):

$$1/P_C^{CR} = A + BR_0 \qquad (6)$$

where

$$A = 1.13 \times 10^{-2}\ \mathrm{Pa}^{-1} \quad B = 290\ \mathrm{Pa}^{-1}.\mathrm{m}^{-1} \quad C_P = 5 \times 10^{-3}\ \text{wt \%}$$

$$A = 5.88 \times 10^{-3}\ \mathrm{Pa}^{-1} \quad B = 102\ \mathrm{Pa}^{-1}.\mathrm{m}^{-1} \quad C_P = 10^{-2}\ \text{wt \%}$$

For large drops, eq 6 predicts that $P_C^{CR}$ is a linear function of $1/R_0$:

$$P_C^{CR} \approx 1/(BR_0) \qquad (6')$$

We found that the observed dependence of the critical pressure for drop coalescence on drop size (eq 6′) describes very well data obtained in separate experiments with two rather different systems.

First, we found that eq 6′ (with $B = 779\ \mathrm{Pa}^{-1}\ \mathrm{m}^{-1}$) describes very well the FTT results, presented in ref 25, for drops of radius varying between 3 and 10 $\mu$m, which were stabilized by low-molecular mass surfactant (sodium dodecyl trioxyethylene sulfate, SDP3S) (see Figure 5B).

Second, in a separate experimental series we studied the coalescence stability of soybean oil-in-water emulsions stabilized by whey protein concentrate (WPC).[26] A high-pressure homogenizer was used to prepare these emulsions, and the protein adsorption on the drop surface was determined by the Bradford method (section 2.4), whereas the emulsion stability was evaluated by centrifugation (section 2.5). The obtained results for the critical osmotic pressure, $P_{OSM}^{CR}$, as a function of the mean drop radius, $R_{32}$, are presented in Figure 6 along with a linear fit according to eq 6′ ($B = 17.3\ \mathrm{Pa}^{-1}.\mathrm{m}^{-1}$). We found that at low WPC concentration, $C_{WPC}$ between 0.02 and 0.05 wt %, the high-pressure homogenizer produced emulsions of different mean drop size (varying from 8 to 15 $\mu$m) at virtually constant value of the protein adsorption on the drop surface, $\Gamma \approx 1.9\ \mathrm{mg/m^2}$. Therefore, the variation of the emulsion stability, demonstrated in Figure 6, is due exclusively to the different mean drop size in the studied emulsions.

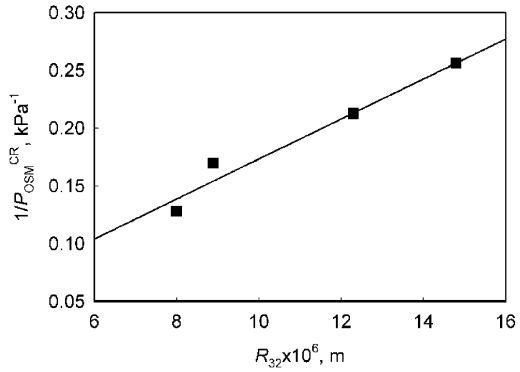


**Figure 6.** Inverse value of the critical osmotic pressure, $1/P_{OSM}^{CR}$, as a function of the volume-surface radius, $R_{32}$ at constant protein adsorption $\Gamma = 1.9\ \mathrm{mg/m^2}$ for WPC-containing emulsions. The points are experimental data, whereas the solid line represents the best fit by means of eq 6′.

The results presented in this section allow us to speculate that eqs 6 and 6′, though empirically established at that moment, are more general and could describe adequately a variety of experimental systems.

It is worthwhile noting that Bibette et al.[27] found experimentally a different trend for silicone oil-in-water emulsions stabilized by sodium dodecyl sulfate: in their experiments, the critical osmotic pressure leading to drop−drop coalescence increased with the drop size. This different trend could indicate a different mechanism of emulsion film rupture in the various systems. Further experimental and theoretical work is needed to clarify the reasons for these differences.

**3.3. Drop Size Distribution and Protein Adsorption in Emulsions.** A series of experiments was performed, by using the procedure from section 2.4, for determination of the protein adsorption, Γ, on the surface of the oil drops in batch emulsions.

The drop size distribution was determined immediately after preparation of the emulsions. As an example, the drop size distributions of an emulsion, stabilized by 0.1 wt % BLG, are presented in Figure 7—the size distributions by both number and volume of the dispersed drops are shown. As seen from Figure 7, the drops of radius above 12 $\mu$m present a negligible *number* fraction, but their contribution is very significant in the *volume* fraction of the dispersed oil. Respectively, the drop radius averaged by drop number was $R_N = 2.7\ \mu$m, whereas the volume-surface radius was much larger, $R_{32} = 18\ \mu$m. Therefore, the samples had relatively wide size distribution and the reliable determination of the protein adsorption required a precise measurement of the specific drop surface, $S = 3/R_{32}$ (eqs 1 and 2), by counting a large number of drops (more than 5000 for each sample).

(25) Hadjiiski, A.; Denkov, N. D.; Tcholakova, S.; Ivanov, I. Role of entry barriers in the foam destruction by oil drops. In *Adsorption and Aggregation of Surfactants in Solution*; Mittal, K., Shah, D., Eds.; Marcel Dekker: New York, 2002; Chapter 23, pp 465−500.

(26) Denkov, N. D.; Tcholakova, S.; Ivanov, I. B.; Campbell, B. Methods for evaluation of emulsion stability at a single drop level. In *Proceedings of the 3rd World Congress on Emulsions*, 24−27 September, Lyon, France; paper 198 (in press).

(27) Bibette, J.; Morse, D. C.; Witten, T. A.; Weitz, D. A. Stability criteria for emulsions. *Phys. Rev. Lett.* **1992**, *69*, 2439.

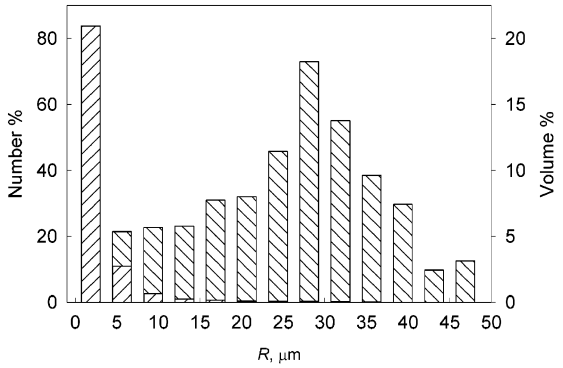


**Figure 7.** Histogram of the drop radius in soybean oil-in-water emulsion, stabilized by 0.1 wt % BLG (0.15 M NaCl, 0.01 wt. % $NaN_3$, natural pH), immediately after preparation of the emulsion. The number-averaged drop radius is $R_N = 2.7\ \mu$m, whereas the volume-surface radius $R_{32} = 18\ \mu$m.

**Table 1. Mean Volume-Surface Radius, $R_{32}$, Mean Number Radius, $R_N$, Protein Concentration in the Serum, $C_{SER}$, Calculated Adsorption, Γ, and Critical Osmotic Pressure, $P_{OSM}^{CR}$ for Emulsions Prepared with BLG Solutions of Various Initial Concentrations, $C_{INI}$[a]**

| $C_{INI}$, wt % | $R_{32}$, μm | $R_N$, μm | $C_{SER}$, wt % | Γ, mg/m² | $P_{OSM}^{CR}$, Pa |
|---|---|---|---|---|---|
| 0.01 | 24 ± 3 | 2.9 | (1.7 ± 0.3) × $10^{-3}$ | 1.5 ± 0.2 | 360 ± 20 |
| 0.02 | 21 ± 2 | 3.3 | (1.1 ± 0.1) × $10^{-2}$ | 1.5 ± 0.2 | 920 ± 70 |
| 0.04 | 21 ± 2 | 4.4 | (3.0 ± 0.1) × $10^{-2}$ | 1.6 ± 0.2 | 3500 ± 500 |
| 0.05 | 21 ± 2 | 3.5 | (3.9 ± 0.1) × $10^{-2}$ | 1.8 ± 0.2 | 5500 ± 1000 |
| 0.08 | 18 ± 2 | 3.1 | (6.2 ± 0.1) × $10^{-2}$ | 2.5 ± 0.3 | 8000 ± 1000 |
| 0.1 | 18 ± 2 | 2.7 | (7.9 ± 0.4) × $10^{-2}$ | 2.9 ± 0.6 | 9700 ± 1400 |

[a] The results are obtained from 3 to 10 independently prepared emulsions for each system.

The results for the size distribution in the other emulsions (protein concentration varied between 0.01 and 0.5 wt %) were very similar to those shown in Figure 7. In Table 1 we present the mean volume-surface radius, $R_{32}$, calculated from the respective size distributions. The data indicate a slight reduction of the average drop size with the protein concentration, from 24 ± 3 μm at 0.01 wt %, through 21 ± 2 μm in the range between 0.02 and 0.05 wt %, to 18 ± 2 μm at 0.08 and 0.1 wt % of BLG. Though not negligible, the drop size reduction with protein concentration is relatively small in the studied emulsions (within 25%). According to eqs 6 and 6′, the effect of drop size on the critical pressure to emulsion coalescence in these emulsions is also expected to be relatively small (below 25%). On the other hand, the emulsion stability, indicated by the values of $P_{OSM}^{CR}$, changes almost 30 times in the studied range of protein concentrations. Therefore, we can analyze the emulsion stability with respect to the protein adsorption and concentration without the necessity for an explicit account of the drop size effect (the latter can be neglected).

Let us note that we have not detected any change of the drop size in the studied emulsions as a function of the storage time in a gravity field. The measurements of the drop size distribution for a given type of emulsion immediately after preparation and 5 min, 30 min, 1 h, and 24 h after the emulsion preparation gave practically the same result.

For determination of the protein adsorption, we measured the protein concentration in the serum taken from emulsions, which were stored for 30 min in a gravity field (section 2.4). The obtained values are presented in Figure 8 as an adsorption isotherm of Γ($C_{SER}$). It was impossible to determine the value of Γ at protein concentrations higher than 0.1 wt %, because the measured values of $C_{SER}$ were

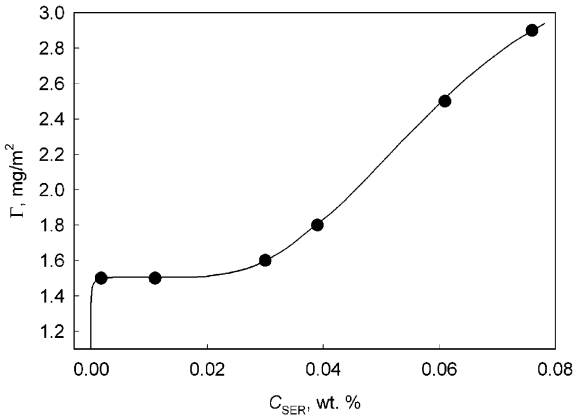


**Figure 8.** BLG adsorption, Γ, on the surface of the emulsion drops, plotted as a function of the protein concentration in the serum, $C_{SER}$.

equal (in the framework of our accuracy) to the initial concentration, $C_{INI}$. Thus it was impossible to apply eq 3 for calculation of the adsorption. It was impossible also to determine Γ for emulsions containing less than 0.01 wt % BLG, because these emulsions were not sufficiently stable.

As seen from Table 1 and Figure 8, the adsorption remained the same in the framework of our experimental accuracy at $C_{INI} = 0.01$ and 0.02 wt %, Γ = 1.5 ± 0.2 mg/m². A slight increase, Γ = 1.6 ± 0.2 mg/m², was observed at 0.04 wt % of BLG. These results are in a good agreement with the data obtained by ellipsometry and radiotracer method for the air−water interface (between 1.64 and 1.7 mg/m²) at low protein concentration.[7,8] Note that the protein concentration in the serum, $C_{SER}$, obtained after the emulsion preparation, was significantly reduced in comparison with $C_{INI}$ for these samples (Table 1). In fact, the adsorption Γ ≈ 1.5 mg/m² is the smallest coverage of the drop surface with BLG molecules, which ensures stable emulsions.

We found that Γ significantly increased at a higher protein concentration and reached 2.9 mg/m² at 0.1 wt % of BLG. The latter value is well above the value for the adsorption in a compact BLG monolayer, $\Gamma_M \approx 1.65$ mg/m². Such higher values for BLG adsorption, $\Gamma > \Gamma_M$, have been already reported in the literature for BLG solutions of higher concentration. For instance, at 0.1 wt %, one can find in the literature the following values: 2.0 mg/m² (ref 11), 2.4 mg/m² (ref 7), and 3.8 mg/m² (refs 4 and 5).

The excess of the protein adsorption over $\Gamma_M$ most probably corresponds to the formation of a second adsorption layer over the first one. The protein adsorption in the first monolayer (in which the protein molecules are in direct contact with the oil−water interface) is known to be practically irreversible, because the adsorption energy per protein molecule is rather large.[10] On the other hand, the protein molecules in the second adsorption layer are expected to be bound less strongly to the drop surface, because they are not in direct contact with the oil−water interface. Therefore, one may expect that the protein adsorption in the second layer could be reversible and that the respective protein molecules could desorb upon rinsing with electrolyte solution.

To check this hypothesis, we rinsed with electrolyte solution the cream formed from the emulsion prepared with 0.1 wt % BLG solution (with Γ = 2.9 mg/m²). The rinsing of the cream was performed in the following way: the emulsion was first kept undistributed in a gravity field for 1 h to cream. Afterward, the serum was removed by using a syringe, and the same volume of 0.15 M NaCl

and 0.1 wt % $NaN_3$ solution was gently introduced below the cream. The sample was gently shaken by hand until the emulsion drops were completely dispersed and then left undistributed for 1 h. A sample from the rinsing solution that remained below the cream was taken, and the protein concentration was determined by the method of Bradford.[17] By using a mass balance of the protein, from the average drop size $R_{32}$ and from the protein concentrations in the initial solution, $C_{INI}$, in the serum, $C_{SER}$, and in the rinsing solution, $C_{RIN}$, we were able to calculate the protein adsorption before and after rinsing of the cream. We found that the initial adsorption of 2.9 mg/ $m^2$ decreased down to 1.6 mg/$m^2$, as a result of rinsing. Note that the latter value practically coincides with the adsorption of the first monolayer, $\Gamma_M$. This result strongly supports the idea that a second adsorption layer, of reversibly bound protein molecules, is built on the drop surfaces at a protein concentration above ca. 0.03 wt % of BLG.

**3.4. Effect of Protein Concentration and Adsorption on the Emulsion Stability Investigated by Centrifugation.** The stability of oil-in-water emulsions in the concentration range from 0.01 to 0.5 wt % BLG was investigated by centrifugation. The emulsions were stored for 30 min in a gravity field for accomplishment of the protein adsorption, before starting the centrifugation. The height of the layer of released bulk oil, $H_{REL}$ (see Figure 2C), was measured immediately after stopping the centrifuge.

The results for the critical osmotic pressure, $P_{OSM}^{CR}$, as a function of the protein concentration in the serum, $C_{SER}$, are shown in Figure 9A. It is seen that $P_{OSM}^{CR}$ increases significantly with the protein concentration—for an increase of $C_{SER}$ from 0.0017 to 0.5 wt %, the critical osmotic pressure increases by 2 orders of magnitude (from 360 to 36 000 Pa). As discussed in section 3.3, this increase is due exclusively to the change in the protein adsorption (the effect of the oil drop size is expected to be below 25%; see eq 6 and the comments below it).

It is much more instructive to plot $P_{OSM}^{CR}$ as a function of the protein adsorption on the drop surface, Γ (see Figure 9B). For comparison, we include in this graph also four points for $P_C^{CR}$ obtained from the FTT experiments (at $2 \times 10^{-4}$, $5 \times 10^{-4}$, $5 \times 10^{-3}$, and $10^{-2}$ wt % of BLG). For these points, we have used literature data for the BLG adsorption at an air—water interface, measured by ellipsometry 30 min after the beginning of the adsorption process.[11]

As one can see from Figure 9B, there is a large step in the emulsion stability at a protein adsorption $\Gamma^* \approx 1.55$ mg/$m^2$. The emulsion stability below this value is very low, and a gradual increase in stability is observed at $\Gamma \geq 1.8$ mg/$m^2$. The stability increase at $\Gamma^* \approx \Gamma_M$ means that almost a complete protein monolayer should be built on the drop surface.

The increase of emulsion stability at a higher protein adsorption, $\Gamma \geq 1.8$ mg/$m^2$, requires further explanations. As discussed above, these higher values of Γ most probably correspond to the formation of a second layer of protein molecules on the interface. Thus, the increased emulsion stability can be explained in two different ways: First, the second adsorption layer may play the role of a reservoir of molecules that can immediately fill up the gaps formed in the first adsorption layer when the oil—water interface is expanded (as a result of drop deformation or thermal fluctuations) (see Figure 10). This would prevent the formation of a bare oil—water interface and would stabilize the emulsion films between the oil drops. Second, the thicker adsorption layer (probably a bilayer) could lead to an additional steric repulsion between the surfaces of the emulsion films, leading to thicker and more stable films, respectively. We expect that both these effects play a role in the observed increase of emulsion stability at high protein concentrations. The first effect ("healing" of an expanding adsorption layer) probably plays a more important role under dynamic conditions, whereas the steric effect is probably more important under static or quasistatic conditions.

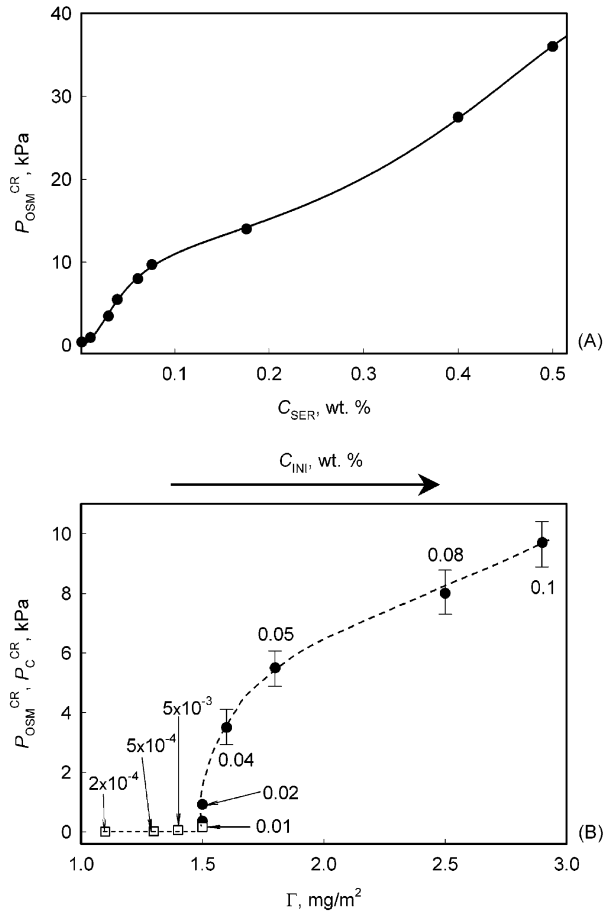


**Figure 9.** (A) Critical osmotic pressure, $P_{OSM}^{CR}$, measured by centrifugation as a function of the protein concentration in the serum. (B) Critical capillary pressure, $P_C^{CR}$ (measured by FTT, open squares) and critical osmotic pressure, $P_{OSM}^{CR}$, (measured by centrifugation, solid circles) as functions of BLG adsorption (natural pH, 0.15 M NaCl, 0.01 wt. % $NaN_3$). The numbers associated with the experimental points show the initial protein concentration ($C_{INI}$).

**3.5. Drop—Drop or Drop—Large Phase Coalescence?** When the emulsion is placed in a centrifugal field, a bulk oil layer is formed on top of the cream (above a given critical acceleration), as a result of drop coalescence. The latter can occur at least in two different modes: (1) at the interface between the cream and the already released bulk oil layer, that is, as a *drop-large phase coalescence*, and (2) through a *drop-drop coalescence* inside the cream, leading to the formation of larger drops, which are subsequently pushed by the centrifugal field toward the cream—oil interface, where they coalesce (we recall that the larger drops are less stable). Let us mention again (see section 2.5) that millimeter-sized oil lenses, present on top of the cream prior to centrifugation, could serve as nuclei for initiation of the bulk oil formation during centrifugation.

To check which one of these coalescence processes was more important in the studied systems, we compared the

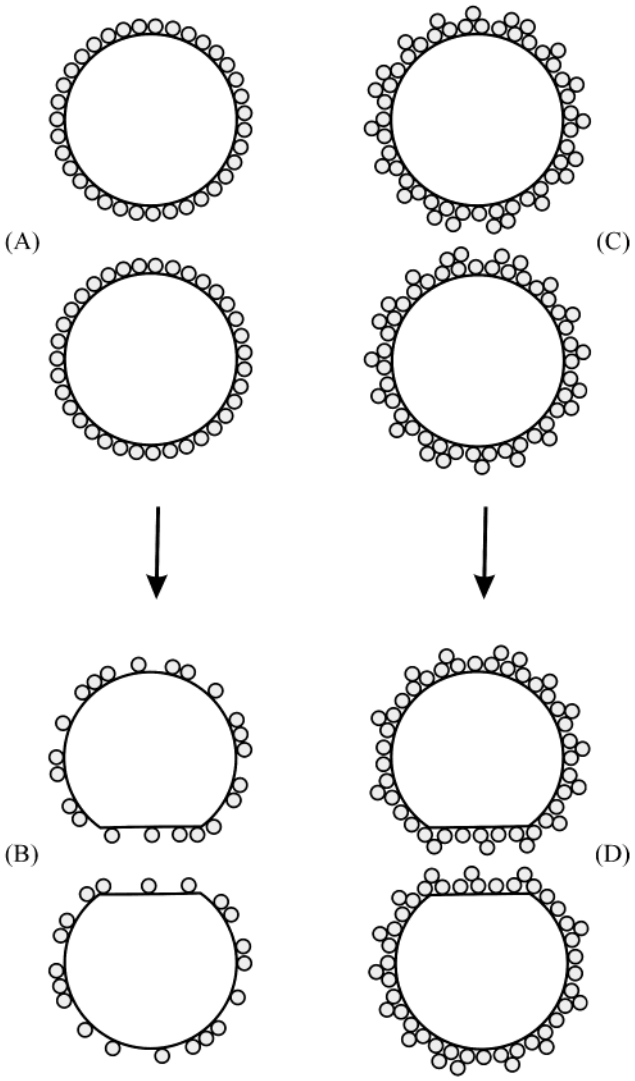


**Figure 10.** Schematic illustration of the possible stabilizing effect of the second adsorbed layer of protein on emulsion stability: (A, B) If only a monolayer of adsorbed protein is formed, the process of emulsion film formation and/or expansion would lead to occurrence of bare oil−water regions on the film surfaces. These regions are unstable and can induce film rupture and drop coalescence. (C, D) If a second adsorption layer of protein is present, the gaps formed as a result of the surface expansion can be filled ("healed") by molecules from the second layer. Another possible explanation is that the second adsorption layer leads to a steric repulsion between the film surfaces.

drop size distributions in emulsions, stabilized by 0.01, 0.02, and 0.1 wt % BLG before and after centrifugation for 3 h at an acceleration that was just below the critical value leading to release of bulk oil on top of the cream. The centrifuged emulsions were redispersed in 20 mM SDS solution, and the drop size distribution was measured as explained in section 2.3. The results showed that the size distribution remained exactly the same for the emulsion stabilized by 0.1 wt % BLG. At the medium BLG concentration, 0.02 wt %, we observed a slight increase of the mean drop size in the cream, from 21 to 26 $\mu$m. At the lowest BLG concentration, 0.01 wt %, the drop size increased very significantly, from 24 to 40 $\mu$m. These results suggest that for protein concentrations above 0.01 wt %, the prevailing coalescence process is that between the drops at the top of the cream and the bulk oil layer, whereas for 0.01 wt % both processes (drop−drop and drop−bulk oil) are important.

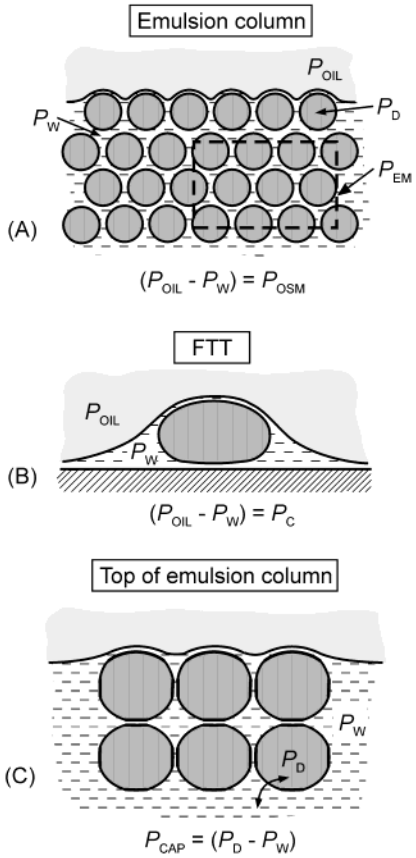


**Figure 11.** Comparison of the capillary pressures and emulsion films that appear: (A) at the boundary between a concentrated oil-in-water emulsion with bulk oil phase, (B) in the film trapping technique (FTT), and (C) between two drops in concentrated emulsion. Note that $P_{OIL} - P_W$ is equal to the osmotic pressure, $P_{OSM}$, at the top of the cream in part A, whereas $P_{OIL} - P_W$ is equal to the capillary pressure $P_C$ in part B. Note also that the diameter of the film formed between a drop and the oil macrophase is larger than the diameter of the film between two drops of the same size in part C.

**3.6. Relation between $P_C^{CR}$ and $P_{OSM}^{CR}$.** It is important to find the relation between the two quantities that we use as measures of the emulsion stability: the critical osmotic pressure, $P_{OSM}^{CR}$ (measured by centrifugation), and the critical capillary pressure, $P_C^{CR}$ (measured by FTT).

Let us prove first that

$$P_{OSM} = P_{OIL} - P_W \tag{7}$$

where $P_{OIL}$ is the pressure in the bulk oil layer above the cream, whereas $P_W$ is the pressure in the aqueous phase outside the emulsion films (in the Plateau channels) (see Figure 11). In equilibrium, $P_{OIL}$ must be exactly equal to the macroscopic (average) pressure in the emulsion, $P_{EM}$, for having a planar emulsion/bulk oil interface (Figure 11A). On the other hand, from the definition of the osmotic pressure, $P_{OSM} = P_{EM} - P_W$ (see section 2.5), one can deduce that the pressure in the aqueous phase between the emulsion drops is equal to

$$P_W = P_{EM} - P_{OSM} = P_{OIL} - P_{OSM} \tag{8}$$

which directly leads to the sought for relation, eq 7.

Equation 7 shows that $P_{OSM}$ can be used as a measure for the pressure that squeezes the aqueous film formed between an oil drop in the uppermost layer of the cream and the bulk oil phase. In this aspect, there is complete

analogy with the capillary pressure in the FTT experiments, $P_C$: both quantities characterize the pressure jump, $P_{OIL} - P_W$, at the meniscus around the compressed drops (cf. Figure 11A,B). It is worthwhile noting that $P_{OSM}^{CR}$ has the advantage to be easily calculated from eq 4, without the necessity to take into account details of the structure of the emulsion (drop size distribution, film size, etc.). In this aspect, $P_{OSM}^{CR}$ is much more convenient than other possible measures of the emulsion stability, which would require some assumptions about the detailed emulsion structure.

Let us note that if the drop−drop coalescence is prevailing, the adequate quantitative characteristic of the process should be another capillary pressure, $P_{CAP} = P_D - P_W$ (where $P_D$ is the pressure of the oil phase *inside the drops*), because this is the driving pressure that acts to squeeze the emulsion films formed between two drops of similar size (Figure 11C).[23,26−28] As shown theoretically by Princen,[23] $P_{CAP}$ is always larger than $P_{OSM}$, and the difference is at least several times for typical values of $\Phi_{OIL} < 95\%$. The values of $P_{CAP}$ and $P_{OSM}$ asymptotically approach each other only when the volume fraction of the oil, $\Phi_{OIL} \rightarrow 1$. On the other hand, the emulsion film between a drop and a bulk oil layer is about twice larger in area than the film between two drops of the same size when other conditions are equilavent (this can be shown by a force balance for a drop in mechanic equilibrium[26]). Therefore, one of the factors, $P_{CAP} > P_{OSM}$, favors the drop−drop coalescence, whereas the other factor (the film size) favors the drop−bulk oil coalescence. One cannot predict in advance which of the coalescence processes would prevail for a given system, without having detailed quantitative information about the effect of film size on its stability.

We were able to determine $P_C^{CR}$ by FTT and $P_{OSM}^{CR}$ by centrifugation for one and the same system only in the case of 0.01 wt % of BLG. However, the comparison of the values with respect to emulsion stability is not justified for this system—as explained in section 3.5, the drop−drop coalescence (instead of drop−bulk oil coalescence) was prevailing at this low BLG concentration, so no agreement between the values of $P_{OSM}^{CR}$ and $P_C^{CR}$ was expected. Another reason to refrain at this moment from direct comparison of the values of $P_C^{CR}$ and $P_{OSM}^{CR}$ for the studied protein emulsions is that such a comparison is sensible only for the same adsorption of the emulsifier on the drop surface. Due to the different procedures for preparation of samples for centrifugation and FTT, the protein adsorption during the experiments was expected to be different even at one and the same bulk BLG concentration. Future FTT and centrifugation experiments are planned with low-molecular mass surfactants (where the adsorption is expected to be the same at equivalent bulk concentration of the emulsifier, due to the complete reversibility of the adsorption process) to make a direct quantitative comparison of $P_C^{CR}$ and $P_{OSM}^{CR}$.

## 4. Conclusions

The effects of drop size and protein adsorption on the stability of BLG-containing emulsions were studied experimentally. The main results can be summarized as follows.

The critical capillary pressure for coalescence of emulsion drops with a large oil−water interface strongly decreases with the increase of the drop size (see eqs 6 and 6′) (Figure 5). A similar result is obtained for the critical osmotic pressure corresponding to the onset of coalescence in batch emulsions, stabilized by whey protein concentrate (Figure 6). These results suggest that one of the main reasons for the enhanced stability of batch emulsion at higher protein concentrations is that smaller oil drops are produced during emulsification, which are more stable against coalescence.

The BLG adsorption on the surface of oil drops in batch emulsions is determined (Figure 8). At a protein concentration between 0.001 and 0.03 wt % in the aqueous phase, the adsorption is close to the value $\Gamma_M \approx 1.65$ mg/m$^2$, which corresponds to a saturated adsorption monolayer at air−water interface.[8] At higher protein concentration, the adsorption increases up to 2.9 mg/m$^2$ (at 0.1 wt % BLG), possibly due to the formation of a second adsorption layer over the first one.

The emulsion stability gradually increases with the protein concentration (Figure 9A). In contrast, the emulsion stability increases in a stepwise manner, when plotted as a function of the protein adsorption (at almost constant drop size). The emulsions are relatively unstable at $\Gamma < \Gamma_M$, the stability steeply increases at $\Gamma \approx \Gamma_M$, and the further increase of $\Gamma$ leads to a much slower but steady increase of emulsion stability (Figure 9B). Possible explanations for the role of the second adsorption layer in the emulsion stability are discussed (Figure 10).

The experiments show that in most of the studied emulsions (except for the relatively low protein concentration of 0.01 wt % BLG) the coalescence of the oil drops with the uppermost oil layer is governing the final volume of released oil, while the process of drop−drop coalescence inside the cream is of secondary importance (section 3.5).

The analysis of the mechanical equilibrium at the top of the emulsion cream, during centrifugation, shows that one can use the critical osmotic pressure at the top of the cream, $P_{OSM}^{CR}$ (as defined in section 2.5), as a relevant and convenient quantitative characteristic of the coalescence process in the studied emulsions (Figure 11).

In conclusion, the used methods and procedures allowed us to separate from each other the effects of the drop size and of the protein adsorption on the emulsion stability. The obtained results have clear (though qualitative at that stage) physical interpretation. The same set of methods turned out to be very useful for investigation of the role of other important factors, such as the pH, the thermal treatment, and the time of shelf-storage of the emulsion (manuscript in preparation).

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## Appendix: Tests of the Used Experimental Methods for Possible Artifacts

**A.1. Check of the Method for Determination of $P_{OSM}^{CR}$ by Centrifugation.** Several tests of the procedure for determination of $P_{OSM}^{CR}$ by centrifugation for possible artifacts were made.

*A.1.1. Rates of Acceleration and Deceleration of the Centrifuge.* To check whether the measured critical pressures for emulsion destabilization are affected by the rates of increase and decrease of the centrifugal acceleration, we performed a set of measurements of $P_{OSM}^{CR}$ by using different rates. The rates of accelerating and decelerating the centrifuge rotor were varied from 167 rpm/s (fastest possible), through 16.7 rpm/s (used in all

(28) van Aken, G. A. Flow-induced coalescence in protein-stabilized highly concentrated emulsions. *Langmuir* **2002**, *18*, 2549.

**Table 2. Critical Osmotic Pressure, $P_{OSM}^{CR}$, Determined for Different Protein Concentrations and Centrifugal Accelerations, $k = g_k/g$[a]**

| $C_{INI}$, wt % | k | $H_{REL}$, mm | $P_{OSM}^{CR}$, Pa: homogeneous field, eq 4′ | $P_{OSM}^{CR}$, Pa: nonhomogeneous field, eq 9 |
|---|---|---|---|---|
| 0.01 | 20.1 | 0.1 | 346 | 345 |
| | 22.8 | 0.5 | 385 | 384 |
| 0.02 | 50.0 | 0.1 | 860 | 852 |
| | 60.7 | 1.0 | 1000 | 996 |
| 0.04 | 200 | 0.1 | 3437 | 3385 |
| | 226 | 1.0 | 3725 | 3688 |
| 0.1 | 503 | 0.7 | 8408 | 8299 |
| | 603 | 1.9 | 9512 | 9458 |
| | 754 | 3.0 | 11243 | 11235 |

[a] $H_{REL}$ is the height of the released oil at the top of the cream after finishing the centrifugation. The centrifugation duration is 2 h and $H_{OIL}$ = 22 mm in all experiments (see section A.1 for additional explanations).

other experiments), down to 4.2 rpm/s (slowest possible). Emulsions prepared with 0.01, 0.02, and 0.1 wt % BLG were studied in this series of experiments. We found that the obtained values of $P_{OSM}^{CR}$ were not affected by the rate of rotor acceleration or deceleration. One can conclude from these measurements that the emulsion destabilization in our centrifugation experiments took place under quasistatic conditions.

*A.1.2. Effect of the Centrifugal Acceleration on the Calculated Value of $P_{OSM}^{CR}$.* Equation 4′ implies that $P_{OSM}^{CR}$ for a given emulsion can be determined from a range of centrifugal accelerations, $g_k$, which are larger than a certain critical value. In the ideal case, the product $g_k H_k \Phi$ and the respective calculated value of $P_{OSM}^{CR}$ should not depend on the applied overcritical acceleration, $k$ (see eq 4′). To check how close we are to this ideal situation, we determined $P_{OSM}^{CR}$ for emulsions stabilized by 0.01, 0.02, 0.04, and 0.1 wt % BLG at several different accelerations (see Table 2). We observed a slight tendency for increase of the calculated value of $P_{OSM}^{CR}$ with the raise of the centrifugal acceleration (i.e., with the volume of released oil on top of the cream at the end of the centrifugation cycle). Two explanations can be given for this tendency:

(1) The coalescence of the first drops should lead to an accumulation of protein in the top layer of the cream, because the oil−water interface disappears and the adsorbed protein is released. Therefore, the local protein concentration at the top of the cream increases in the course of the coalescence process, which could lead to an increase of the critical coalescence pressure.

(2) The largest drops coalesce at lower pressure, as shown in section 3.2. Therefore, one may expect that the largest drops in the upper layer of the cream will first coalesce. The coalescence of the remaining smaller drops should take place at higher values of $P_{OSM}^{CR}$.

Nevertheless, as seen from Table 2, the observed slight increase of $P_{OSM}^{CR}$ with the rise of the centrifugal acceleration is within the limits of our accuracy (±5%) and is much smaller than the studied effects of protein concentration and adsorption. To diminish as much as possible the role of the centrifugal acceleration on the final results, and to make them comparable, we have always used the values of $P_{OSM}^{CR}$ that are obtained at the lowest possible accelerations, when the height of the released oil is less than 1 mm. This corresponds to an uncertainty in the calculated value of $P_{OSM}^{CR}$ of about 10%.

*A.1.3. Estimate of the Error Caused by the Homogeneous Field Approximation.* One assumption made in the derivation of eq 4′ is that the centrifugal field is homogeneous along the emulsion column. This assumption is not strictly valid, because the acceleration depends on the distance to the axis of rotation, ζ, and this distance changes along the cream (Figure 2C). To estimate how large the error from the homogeneous field approximation could be, we calculated $P_{OSM}^{CR}$ for the systems shown in Table 2 by using a more rigorous (and more complex) equation, which follows from the approach of Princen.[23,24] In this approach, eq 4′ for determination of $P_{OSM}^{CR}$ is replaced by

$$P_{OSM}(\Phi_{OIL}) = \int_0^z \Delta\rho\omega^2\zeta\Phi_{OIL}(z)\,dz = \Delta\rho\omega^2\int_0^z \Phi_{OIL}(z)(\zeta_2 - z)\,dz = \Delta\rho\omega^2[\zeta_2 H_{OIL} - \int_0^z \Phi_{OIL}(z)z\,dz] \quad (9)$$

which explicitly takes into account the variation of the acceleration along the cream (see Figure 2C for the used notation). This more detailed approach requires one to have an explicit expression for the function $\Phi_{OIL}(z)$, which accounts for the variation of the volume fraction of oil with the coordinate $z$. In our calculations, we used the function proposed by Princen (eq 14 in ref 24). As seen from Table 2, the values of $P_{OSM}^{CR}$ calculated by these two approaches practically coincide. The relative difference is about 1%, which is well below the experimental accuracy of our measurements (±10%). Therefore, we can use with sufficient accuracy the simpler approach, eq 4′, for calculation of $P_{OSM}^{CR}$.

**A.2. Checks of the Method for Determination of Γ in Emulsions.** *A.2.1. Effect of Protein Aggregates on the Results of the Bradford Method.* During the preparation of the emulsions we apply an intensive stirring of the system by an Ultra Turrax homogenizer. This stirring could change the aggregation state of the protein in the solution (e.g., to disrupt large aggregates and/or to induce aggregation of the single molecules).[29] If the reaction between the protein and the Bradford reagent depends on the aggregation state of the protein (a possibility that cannot be ruled out a priori), the determination of the protein concentration in the serum would be affected by the stirring, and the results about the protein adsorption would become erroneous, because the calibration curves plotted in Figure 1 are obtained with nonstirred protein solutions. To check whether this effect is detectable, we compared the light absorption, after preparing the protein−dye complex as described in section 2.4, for protein solutions before and after stirring them by the Ultra Turrax homogenizer (under conditions equivalent to those during emulsification). The light absorption was practically the same for the stirred and nonstirred solutions, which showed that the effect of stirring on the protein concentration determination was negligible.

*A.2.2. Check for a Possible Entrapment of Large Protein Aggregates in the Cream.* Another problem in the determination of Γ can appear if some large protein aggregates (possibly present in the solutions) are trapped in the cream during its formation, so that the serum remains depleted of these aggregates. If such an effect were present, it would lead to an overestimate of the protein adsorption. This possibility was ruled out by the following experiment. A protein solution was filtered through a standard 0.22 μm filter (Millipore) to potentially remove any large protein aggregates, and the protein concentration in the filtered

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solution was determined by the method of Bradford and compared to that before filtering. The two results (before and after filtration) practically coincided, which showed that the quantity of large protein aggregates in the used solutions was below 5%. Note that protein aggregates of size below 0.22 $\mu$m are not expected to be trapped in the channels of the cream, because the typical drop radius is on the order of 3−20 $\mu$m, which means that the cross section of the interdroplet voids in the cream is about 0.15 $R_D \approx 0.5-3\,\mu$m.[30] Therefore, all entities of size below 0.5 $\mu$m are expected to flow freely through the network of plateau channels (before centrifugation, when the emulsion drops are only slightly deformed).

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