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Globular proteins as emulsion stabilizers - similarities and differences with surfactants and solid particles

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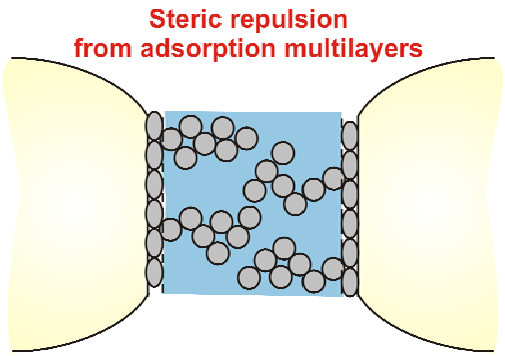


ABSTRACT

Proteins are widely used as emulsion stabilizers in food, beverage, pharmaceutical, cosmetic and other industries. Despite the continuous and focused efforts by many research groups, the basic mechanisms of emulsion stabilization by protein molecules are still poorly understood. Among the main reasons for the lack of definite understanding of these mechanisms are (1) the complex structure of the protein adsorption layers formed on drop surfaces, and (2) the complex evolution of the protein molecules (unfolding, changing of secondary structure, bond-formation) after adsorption, upon heating and during shelf-storage of the emulsions. This complexity poses the question whether one could apply to protein-stabilized emulsions some of the general concepts, originally developed to explain the properties of emulsions stabilized by low-molecular weight (LMW) surfactants and solid particles.

To deepen our understanding of the mechanisms of emulsion stabilization by globular proteins, we performed series of related studies of oil-in-water emulsions, stabilized by the globular protein beta-lactoglobulin and its technical grade analog, whey protein concentrate [1]. The current presentation summarizes the main conclusions from these studies with

emphasis on the similarities and differences between the globular proteins and the other major types of emulsifiers, such as the LMW surfactants and solid particles.

First, a series of experiments were performed to clarify the role of several factors on the mean size of the oil drops, formed during emulsification in turbulent flow. These factors include the protein and electrolyte concentrations, pH, intensity of turbulent stirring, interfacial tension, and oil viscosity. The results show that the mean drop size in protein-stabilized emulsions could be explained essentially by the same basic principles, which are used to explain the emulsification in the presence of surfactants and particles. The main specific features of the proteins in the emulsification context are the slower adsorption (as compared to LMW surfactants) and the possible formation of protein adsorption multilayers. These features can be incorporated in a straightforward manner into the governing equations, which describe rather well the effects of all factors studied.

Next, the coalescence stability of the formed emulsions was characterized by centrifugation and the effect of the above and some additional factors (such as emulsion heating, and shelf-storage) was evaluated. To explain the observed non-trivial results, we measured the amount of reversibly and irreversibly adsorbed protein on the surface of the emulsified drops, recorded the FTIR spectra of the protein in the emulsions, and modeled theoretically the interactions in the films between two neighboring drops. The experimental results and their interpretation revealed three qualitatively different cases of emulsion stabilization by globular proteins: (1) via electrostatic barrier; (2) via steric barrier, created by protein adsorption multilayers; (3) via steric repulsion, created by protein monolayers. The protein stabilization of type (1) is similar to the one observed with ionic LMW surfactants and can be described reasonably well by the DLVO theory. We found that the protein stabilization of type (2) could be described well by the model of Dolan-Edwards, which was originally developed for polymer-stabilized liquid films. The stabilization of type (3) is the least understood and might be governed by the rheological properties of the protein monolayers - hypothesis, which is often discussed in the literature but remains unproven. The effects of heating and aging of the emulsions on their coalescence and flocculation stability are controlled by the interactions between the adsorbed protein molecules, such as the formation of disulfide, hydrogen and hydrophobic bonds, and thus are strongly specific for the proteins.

1/ Introduction

Emulsions are thermodynamically unstable and various processes, such as drop-drop coalescence, flocculation, creaming, and Ostwald ripening lead to changes in the drop size-distribution and/or emulsion structure. The main focus of the current review is the coalescence stability of oil-in-water emulsions. The emulsifiers (low-molecular weight surfactants, solid particles, proteins, or synthetic polymers) suppress the drop-drop coalescence, mainly by stabilizing against rupture the emulsion films, formed between neighboring drops.

Depending on the type of emulsifier used, different colloidal forces govern the stability of emulsion films. In the presence of ionic low-molecular weight surfactants (LMW surfactants), the film stability is often described reasonably well by the DLVO theory, which accounts for the long-ranged electrostatic repulsion and van der Waals attraction (1-3), Figure 1A. In the presence of nonionic surfactants and polymers, the film stability is usually explained by steric repulsion created by the overlapping hydrophilic heads of the surfactant/polymer molecules, adsorbed on the two opposite film surfaces, Figure 1B (4-6). The stabilization of emulsion films by solid particles is explained by capillary forces, which appear when the menisci of the oil-water interface bend around the particles trapped in the emulsion films, Figure 1C (7-8).

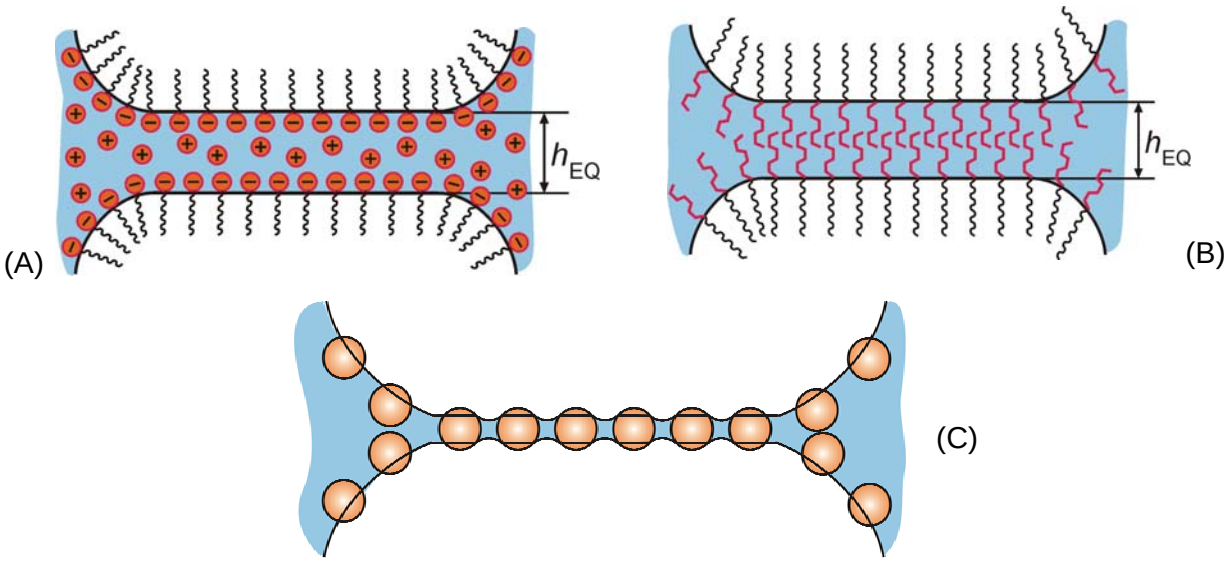


Figure 1. Schematic presentation of the structure of the adsorption layers and the respective mode of emulsion film stabilization by (A) ionic surfactants, (B) nonionic surfactants, (C) solid particles.

Despite the continuous and focused efforts by many research groups, the basic mechanisms of emulsion stabilization by protein molecules are still poorly understood. Among the main reasons for the lack of definite understanding of these mechanisms are (1) the complex structure of the protein adsorption layers formed on drop surfaces, and (2) the complex evolution of the protein molecules (unfolding, changing of secondary structure, bond-formation) after adsorption, and emulsion upon heating and shelf-storage. This complexity poses the question whether one could apply to protein-stabilized emulsions some of the general concepts, originally developed and proven to be useful in explaining the properties of emulsions, stabilized by low-molecular weight (LMW) surfactants, polymers, and solid particles, see Figure 1.

To deepen our understanding of the mechanisms of emulsion stabilization by globular proteins, we performed series of related studies of oil-in-water emulsions, stabilized by the globular protein beta-lactoglobulin and its technical-grade analog, whey protein concentrate (9-17). The effects of various factors (protein concentration and adsorption, drop size, electrolyte concentration, pH, thermal treatment and time of shelf-storage) on the protein adsorption and emulsion coalescence stability were determined in these studies. The major aim of the current review is to compare the main results from these experiments with results obtained in the presence of LMW surfactants and solid particles, and on this basis to outline some similarities and differences between the globular proteins and the other types of emulsifiers. This comparison helped us to formulate possible molecular mechanisms of emulsion stabilization by globular proteins, under the various experimental conditions encountered in practice.

The review is structured as follows: In Section 2 we describe the materials and methods used. In Section 3 we present some results and compare the different types of emulsifiers with respect to their ability to facilitate drop breakage and to prevent drop-drop coalescence during emulsification. In Section 4 we compare the different modes of emulsion stabilization by proteins under various conditions (different protein and electrolyte concentrations, pH close to and away from the isoelectric point of the protein, after shelf-storage and heating) with the modes of stabilization by LMW surfactants and particles. The conclusions are summarized in Section 5.

2/ Materials and methods.

2/1 Materials. As globular milk protein we studied β-Lactoglobulin (BLG) from bovine milk, as received from Sigma. Whey protein concentrate (WPC) of technical-grade (trade name AMP 8000; product of Proliant) was used as emulsifier, which contains 72 wt % globular proteins (44 % of which is β-lactoglobulin as the main component). The protein solutions were prepared with deionized water, purified by Milli-Q Organex system (Millipore), and contained 0.01 wt % of the antibacterial agent NaN_3 (Riedel-de Haën). The ionic strength was adjusted between 1.5 mM (only NaN_3) and 1 M, by using NaCl.

The LMW surfactants studied are the anionic surfactant sodium dodecyl sulfate (SDS; product of Acros) and the nonionic hexadecylpolyoxyethylene-20 (Brij 58; product of Sigma). Soybean oil (SBO) was used as oil phase, which was purified from polar contaminants by multiple passes through a glass column, filled with Florisil® adsorbent (Sigma).

2/2 Emulsion preparation.

Two emulsification procedures were used, depending on the specific aim:

(1) In the experiments aimed to clarify the effects of various factors on the mean drop size during emulsification, we used a narrow-gap homogenizer and two-step procedure, as described in Refs. (10,11). First, an oil-in-water premix was prepared by hand-shaking a vessel containing desired amounts of oil and aqueous phases. In the second step, this premix was circulated for 10 min in a closed loop through the narrow-gap homogenizer to achieve steady-state drop-size distribution.

(2) In the experiments aimed to study the dependence of emulsion coalescence stability on the factors related to system composition (such as protein and electrolyte concentrations, pH, aging time and heating), the emulsions were prepared by an Ultra-Turrax T25 rotor-stator homogenizer (Janke & Kunkel GmbH & Co, IKA-Labortechnik, Germany). The emulsification procedure consisted of intense stirring of 35 mL protein solution and 15 mL soybean oil for 3 min at 13 500 rpm, so that 30 vol. % oil-in-water emulsion with mean volume-surface drop radius $R_{32} \approx 20$ μm was formed.

2/3 Determination of mean drop size.

The drop-size distribution in the studied emulsions was determined by video-enhanced optical microscopy (18). The oil drops were observed with an optical microscope, connected to a CCD camera and a video-recorder. The drop diameters were measured from the recorded video-frames, using custom-made image analysis software. The mean volume-surface diameter, d_{32}, was calculated from the relation:

$$d_{32} = \frac{\sum_i N_i d_i^3}{\sum_i N_i d_i^2} \tag{1}$$

where N_i is the measured number of drops with diameter d_i. Another characteristic diameter, d_{V95}, is used as an experimentally accessible measure of the maximum drop size, which is compared in Section 3 with the respective theoretical predictions of the drop-breakup models. d_{V95} is defined as the diameter, for which 95 % by volume of the dispersed oil is contained in drops with $d \le d_{V95}$.

2/4 Determination of protein adsorption.

The protein adsorption on the surface of the emulsion drops, Γ, was determined from the decrease of protein concentration in the aqueous phase, $\Delta C = (C_{INI} - C_{SER})$, as a result of the emulsification process (9,10,13,14). Here C_{INI} is the initial protein concentration in the aqueous solution before emulsification, while C_{SER} is the concentration of the protein remaining in the aqueous phase after emulsification (in the serum). The following mass balance relating the adsorption, Γ, with ΔC and the specific surface area of the drops, S (m^2 of oil-water interface per 1 m^3 emulsion) was used to determine the protein adsorption:

$$\Gamma = \frac{V_C}{S V_{OIL}} \Delta C = \frac{(1-\Phi) d_{32}}{6\Phi} \Delta C \tag{2}$$

where V_C and V_{OIL} are the volumes of the aqueous and oil phases in the emulsion, and Φ is the respective oil volume fraction. C_{SER} was determined by the method of Bradford (19) or by the BCA-method (for the detailed procedures see Ref. (14)).

2/5 Characterization of emulsion stability.

For quantitative characterization of the coalescence stability of emulsions, we used a specially developed centrifugation procedure – see Ref. (9) for its detailed description and verification. Briefly, the studied emulsions are tested by centrifugation to determine the acceleration, g_K (m/s^2), at which a thin continuous oil layer is released on top of the emulsion cream, as a result of drop coalescence and emulsion decay. As a quantitative measure of emulsion coalescence stability we use the critical osmotic pressure of the emulsion, P_{CR}, at which this continuous oil layer is released. This pressure is easily calculated from the experimental data through the relation (9):

$$P_{CR} = \Delta\rho\, g_k \left(V_{OIL} - V_{REL}\right) / A_{TT} \tag{3}$$

Here $\Delta\rho$ is the difference in the mass densities of the oil and water phases; g_k is the centrifugal acceleration; V_{OIL} is the total volume of oil in the emulsion; V_{REL} is the volume of oil released on top of the emulsion cream after centrifugation; A_{TT} is the cross-sectional area of the centrifugation test tube.

3/ Results - emulsification in turbulent flow

In most practical systems, the emulsification is performed in a turbulent hydrodynamic flow, generated in the emulsification device (stirrer, high-pressure homogenizer, narrow-gap homogenizer, static mixer, etc.). The evolution of the drop-size during emulsification is governed by the competition of two opposite processes - drop breakage and drop-drop coalescence (20-22). At high emulsifier concentration and/or low oil volume fraction, the drop-drop coalescence is negligible and the evolution of the drop-size distribution in the formed emulsions is governed by the process of drop breakage only. According to the classical studies by Kolmogorov (23) and Hinze (24), the maximal diameter, d_K, of the drops formed inside turbulent flow, can be estimated by comparing the capillary pressure of the drops, P_{CAP}, with the fluctuations of the hydrodynamic pressure, P_T. The following expression for the maximal drop diameter was derived theoretically (23,24) and confirmed experimentally (25) for emulsions prepared with pure oil and water phases (without emulsifier):

$$d_K = A_1 \varepsilon^{-2/5} \sigma_{OW}^{3/5} \rho_C^{-3/5} = A_1 d_{KI} \tag{4}$$

where A_1 is numerical constant, σ_{OW} is interfacial tension, ρ_C is mass density of the continuous phase, and ε is rate of energy dissipation per unit mass of the fluid (J/kg.s), which characterizes the intensity of fluid stirring in the turbulent flow.

The Kolmogorov-Hinze approach was further developed for viscous dispersed phase by Davies (26) and by Calabrese et al. (27-29). These authors included the viscous stress inside the deforming drop into the total stress balance in the process of drop breakage and obtained the following expression for the maximum stable diameter of drops with viscosity η_D:

$$d_D = A_1 \left(1 + A_2 \frac{\eta_D \varepsilon^{1/3} d^{1/3}}{\sigma} \right)^{3/5} d_{KI} \qquad (\eta_D > \eta_C) \tag{5}$$

where A_1 and A_2 are numerical constants and η_C is viscosity of the continuous phase. The second term in the right-hand-side of Eq. (5) expresses the relative contribution of the energy of viscous dissipation in the drops during their deformation and breakage, normalized by the drop surface energy. At low viscosity of the dispersed phase, the viscous contribution is negligible and Eq. (5) simplifies to Eq. (4). Systematic series of experiments, aimed to quantify the effects of drop viscosity and interfacial tension on the maximum drop diameter, was presented in the papers by Calabrese et al. (27-29) (with pure phases without surfactants), and a good agreement with the theoretical expressions was observed. However, the numerical values of A_1 and A_2 found by Davies to describe the experimental data, differed by an order of magnitude from those determined by Calabrese, which has been a puzzling discrepancy for many years already (see below for possible explanation).

The effect of emulsifiers on the size of the drops formed in turbulent flow, is a matter of intensive discussion and investigations in the literature. Several experimental studies have shown that for all types of emulsifiers, one could distinguish two regimes of emulsification, depending on the emulsifier concentration (10,11,30). At low emulsifier concentration (in the so-called “emulsifier-poor” regime), the mean drop size rapidly decreases with the increase of the initial emulsifier concentration. In contrast, at high emulsifier concentrations (called “emulsifier-rich” regime), the mean drop size is almost independent of emulsifier concentration. Example for these two regimes is presented in Figure 2 and similar trend is reported in the literature for various proteins, LMW surfactants, and solid particles.

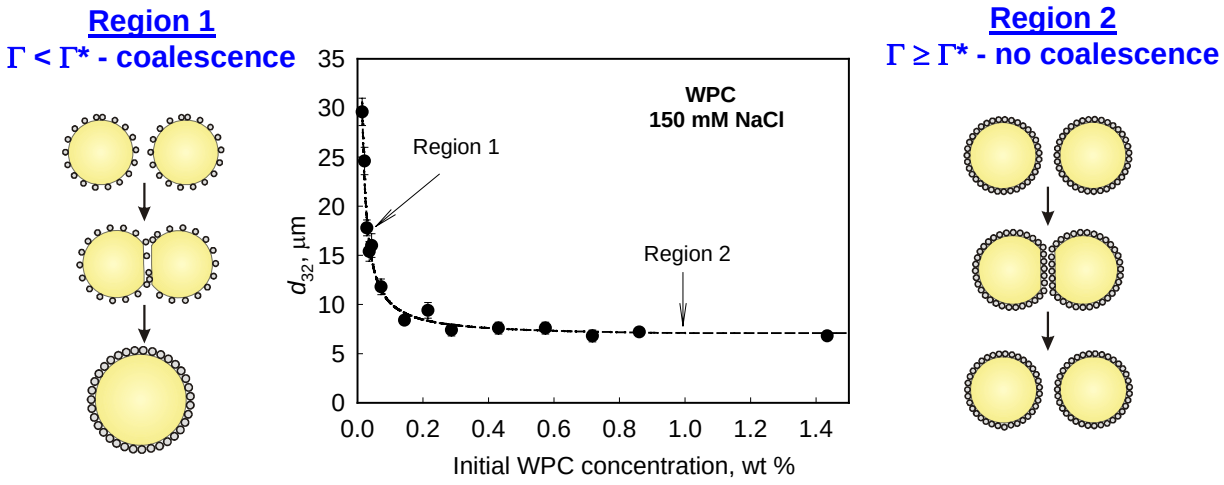


Figure 2. Mean volume-surface diameter, d_{32}, as a function of the initial emulsifier concentration, C_{INI}, for soybean oil-in-water emulsions stabilized by WPC. In Region 1, the mean drop size, d_{32}, is affected strongly by drop-drop coalescence during emulsification, whereas d_{32} in Region 2 is determined mainly by the drop breakage process. Similar trends have been reported for emulsions stabilized by LMW surfactants and solid particles.

In the emulsifier-rich regime (denoted as Region 2 in Figure 2), recent experiments showed that the data for d_{32} could be described rather well by the theory of emulsification in turbulent flow, for all types of emulsifiers studied (10,11,17,31). Direct comparison of the experimental results with the predictions of Eq. (5) showed a very good agreement between the theoretical prediction for the maximum drop diameter, d_D, and its experimentally measured counterpart, d_{V95}, for emulsions, stabilized by WPC, Na caseinate, Brij 58, SDS, or solid particles, see for example Figure 3 (17). One particular feature of the proteins and solid particles in this context was that we should use the dynamic interfacial tension of the respective systems to describe the experimental data (while the equilibrium interfacial tension was used for LMW surfactants), which emphasizes the effect of the much slower kinetics of protein/particle adsorption, as compared to LMW surfactants. In conclusion, in the emulsifier-rich regime, the mean drop size in the emulsions depends on the used emulsifier exclusively through the interfacial tension, and the main difference of the proteins and particles from LMW surfactants is the faster adsorption kinetics of the latter.

Besides, our experimental data for the maximum drop size were described very well by Eq. (5), with values of the numerical constant $A_1 = 0.86$ and $A_2 = 0.37$ (Ref. 17), which are very close to the values proposed by Davies (26) ($A_1 \approx 1$ and $A_2 = 0.35$) and significantly differ from those used by Calabrese et al. (27-29) to describe their data ($A_1 \approx 4.1$ and $A_2 = 0.054$). In Ref. (17) we analyzed the possible reasons for these discrepancies and found that the most probable one is related to the different definitions of the density of energy dissipation, ε, used in the various studies to characterize the turbulent flow. When we reinterpreted the experimental data reported by Calabrese et al. (27-29), with a definition of ε closer to the one in our studies, we were able to describe the Calabrese's data with numerical constants much closer to ours.

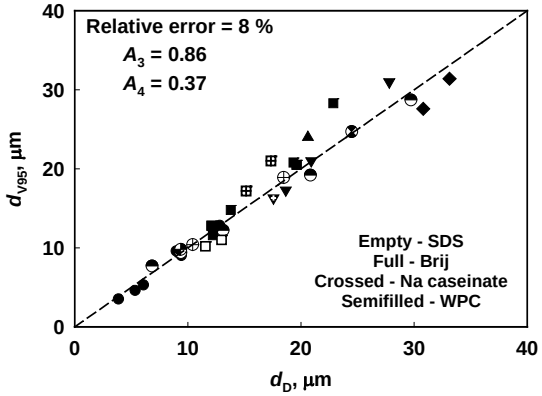


Figure 3. Correlation plot for the theoretically predicted values of the maximum drop diameter, d_D, (see Eq. 5) and the corresponding experimental values, d_{V95} (Ref. 17).

When the emulsifier is not very efficient or its concentration is low, the drop-drop coalescence becomes significant and the mean drop size during emulsification becomes much larger than the one predicted by Eqs. (4) and (5). Here the practical question for appropriate choice of emulsifier and its concentration (choice that depends very much on the specific conditions) becomes crucial for efficient emulsification. Detailed studies with various types of emulsifiers showed (10,11) that in the emulsifier-poor regime, in which the drop coalescence is important, one should distinguish two qualitatively different cases:

(A) At suppressed electrostatic repulsion between the drops (e.g. proteins and nonionic surfactants at high electrolyte concentrations, *ca.* C_{EL} > 100 mM NaCl), simple model was found to describe the experimental data for d_{32}. The main assumption in this model is that the drops coalesce during emulsification until the emulsifier adsorption on the drop surface reaches a certain threshold value, Γ*, which is independent of the oil volume fraction and intensity of stirring. The same phenomenon was termed "partial coalescence" in the studies with particle-stabilized Pickering emulsions (32). Assuming that most of the used emulsifier adsorbs on drop surfaces in the course of emulsification (which is often the case), one derives the following simple mass-balance, which expresses the relation between the emulsifier concentration and the mean drop diameter d_{32} (10,11)

$$d_{32} \approx \frac{6\Phi}{(1-\Phi)} \frac{\Gamma^*}{C_{INI}} \qquad C_{SER} \approx C_{INI} \qquad (6)$$

where C_{INI} is the initial emulsifier concentration in the aqueous phase, C_{SER} is the concentration of the emulsifier left in the aqueous phase after emulsification, and Φ is the oil volume fraction.

From the slope of the best linear fit of the dependence $d_{32}(1-\Phi)/\Phi$ vs. $1/C_{INI}$, one can determine the limiting adsorption needed to stabilize the drops, Γ*. An example is presented in Figure 4 for emulsions stabilized by WPC – from the linear fit we determined Γ* = 1.9 mg/m^2, which is very close to the protein adsorption in a dense monolayer, $\Gamma_M \approx 2$ mg/m^2, determined from the WPC adsorption isotherm (10). The same procedure, applied to emulsions prepared with Brij 58 + 150 mM NaCl solutions, gave Γ* ≈ 1.4 mg/m^2 (11). A counterpart of Eq. (6) for water-in-oil emulsions was proposed in the paper by Golemanov et al. (31) and was found to describe well the experimental data for water-in-hexadecane emulsions, stabilized by latex particles. For particle-stabilized emulsions, the limiting adsorption, Γ*, determined by this procedure, corresponded either to a dense monolayer of

particles (for non-aggregated particles) or to higher surface coverage (for aggregated particles) (8,32).

In conclusion, at relatively low emulsifier concentration and suppressed electrostatic repulsion, dense adsorption layers should be formed for suppressing the drop-drop coalescence during emulsification in the systems stabilized by non-ionic LMW surfactants, proteins and solid particles. In such systems, the drops coalesce during emulsification until the emulsifier adsorption becomes equal to Γ*, so that sufficiently dense adsorption layer is formed, which ensures strong steric repulsion and stabilizes the drops against further coalescence. During subsequent shelf-storage, these emulsions remain stable, i.e. Γ* is sufficiently high to ensure long-term emulsion stability (11).

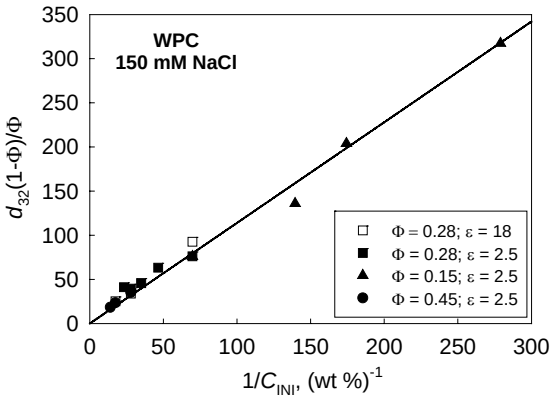


Figure 4. Normalized volume-surface diameter, $d_{32}(1-\Phi)/\Phi$, as a function of the inverse initial protein concentration, C_{INI}, for emulsions prepared under different emulsification conditions (Φ is oil volume fraction and ε is given in 10^5 J/kg.s). The line is a linear fit according to Eq. (6) (Ref. 11).

(B) Significant electrostatic repulsion. The experiments show that when ionic surfactants or charged protein molecules are used as emulsifiers at low and moderate electrolyte concentrations (*ca.* C_{EL} < 100 mM NaCl), the mean drop size in the formed emulsions falls in the range bounded by the Kolmogorov's Eq. (4) (derived under the assumption of negligible drop-drop coalescence) and Eq. (6) (derived under the assumption that the drop coalescence had ensured a complete adsorption monolayer Γ*). These experimental data were explained by comparing the theoretically estimated electrostatic barrier between the surfaces of the colliding drops and the turbulent force pushing the drops against each other in the flow - see Ref. (11) for a set of experimental data and their interpretation. Thus, in the surfactant-poor regime and significant electrostatic repulsion, the effect of the charged protein molecules on drop-drop coalescence is similar to that of the LMW ionic surfactants.

An important particular feature of the electrostatically-stabilized emulsions in the emulsifier-poor regime is that the drop-drop coalescence is incomplete during emulsification. The drops formed are rather small and, hence, the created oil-water interface has too large area to be covered by a dense protective layer of the available surfactant/protein molecules or solid particles. As a result, after stopping the homogenization, the oil drops continue to coalesce, so that much larger drops and/or bulk oil layer is formed on top of the emulsion cream, upon shelf- storage of the formed emulsions (for all types of emulsifier).

It is worthwhile mentioning that the solid particles exhibit one particular feature in this regime, which has no direct analog in protein- and surfactant-stabilized emulsions - the

electrostatic repulsion between the (usually) charged solid particles and the charged oil-water interface is often high (due to the relatively large particle size, as compared to the molecule size of proteins and LMW surfactants) and could not be easily overcome by the solid particles. As a result, the adsorption of the solid particles is suppressed, the drop surface is not well protected, and the drops intensively coalesce during emulsification, even if sufficiently high concentration of particles is used. This problem could be overcome by neutralizing the surface charge of the particles through addition of oppositely charged surfactants or multivalent counterions, and through screening of the electrostatic repulsion with electrolytes (8,31). Note that these additives suppress also the electrostatic repulsion between the particles themselves and, as a result, the particles could flocculate under these conditions.

4/ Coalescence stability during shelf-storage

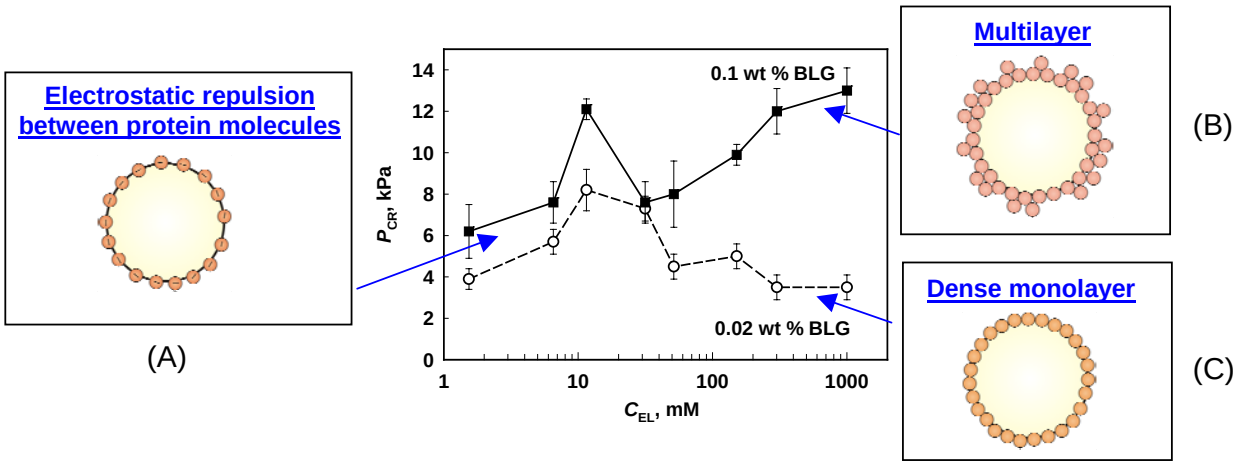
A large set of experiments, performed with emulsions stabilized by the globular protein BLG, revealed how the emulsion stability and protein adsorption depend on protein and electrolyte concentrations, pH, storage time and emulsion heating (9,12-14,16). The obtained results allowed us to distinguish three types of emulsion stabilization by globular proteins (see Figure 5), which could be compared also with the modes of emulsion stabilization by LMW surfactants and solid particles:

(A) Electrostatically stabilized emulsions. At $C_{EL} \leq 50$ mM and pH > 6, the protein molecules are charged, which leads to significant electrostatic repulsion between the neighboring protein molecules inside the adsorption layers, as well as between the adsorption layers on two neighboring emulsion drops. The results showed that the emulsion coalescence stability under these conditions is governed by long-ranged electrostatic and van der Waals forces, which could be described reasonably well by the DLVO theory. For example, the comparison of the experimentally obtained and the theoretically calculated electrostatic barriers revealed that the observed maximum in emulsion stability at $C_{EL} \approx 10$ mM (see Figure 6) corresponds to a maximum in the interdroplet electrostatic repulsion, as predicted by the DLVO theory.

Therefore, under these conditions (charged protein molecules and low or moderate electrolyte concentration) the protein molecules behave similarly to the molecules of ionic LMW surfactants. The most important factors affecting emulsion stability are the electrolyte concentration and pH, which govern the electrostatic repulsion between the adsorbed protein molecules and between the drops. Under these conditions, the structure of the protein adsorption layers and the emulsion stability do not change significantly after heating and with storage time (14).

The outlined mechanism of emulsion film stabilization by charged protein molecules, suggests the following detailed scenario of film rupture, which combines several steps considered in the literature as typical for LMW surfactants or solid particles, see Figure 7. When the drop surfaces are pushed against each other, the electrostatic repulsion between the adsorption layers creates electrostatic barrier, which resists the thinning of the emulsion film. When the compressing pressure becomes higher than the electrostatic barrier, the latter is overcome and the film spontaneously thins down, until the adsorption layers on the two opposite film surfaces come in contact with each other. Since the electrostatic repulsion is relatively “soft”, the protein molecules can rearrange and form a bridging monolayer between the two surfaces of the emulsion film, see Figure 7B (such a process was observed by optical microscopy in the experiments with solid particles (33,34)). Furthermore, the bridging monolayer of separated, uniformly spaced molecules depicted in Figure 7B, is inherently unstable, due to attractive capillary forces between the neighboring protein molecules in the film (33-35). Therefore, a spot deprived of protein molecules could spontaneously form and

expand with time in the emulsion film, leading to direct contact of the two opposite oil-water interfaces, with a subsequent film rupture and drop-drop coalescence.



$1 < C_{EL} \leq 50$ mM	Electrostatic repulsion (A)	(similar to ionic surfactants)
$C_{EL} > 100$ mM	Steric repulsion from adsorption multilayers (similar to aggregated solid particles) (B) 0.1 wt % BLG	Steric repulsion from adsorption monolayers (similar to nonionic surfactants and solid particles) (C) 0.02 wt % BLG

Figure 5. Critical osmotic pressure for drop coalescence, P_{CR}, as a function of electrolyte concentration, C_{EL}, in emulsions stabilized by the globular protein BLG at pH = 6.2. The structure of the protein adsorption layer and the mode of emulsion film stabilization in the various regions are schematically shown, for emulsions stabilized by: (A) electrostatic forces, (B) steric repulsion by adsorption multilayers, (C) steric repulsion by adsorption monolayers.

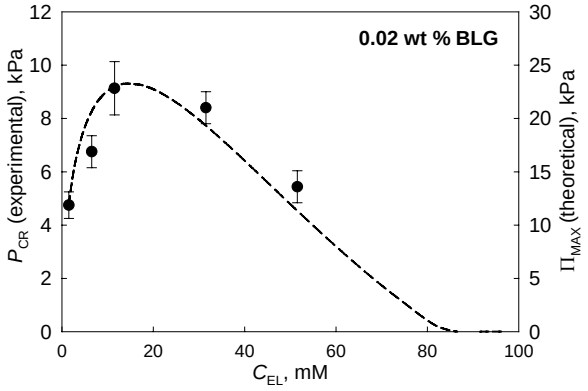


Figure 6. Comparison of the experimentally determined dependence of the barrier to drop coalescence, P_{CR} on C_{EL} (the circles), with the theoretical dependence Π_{MAX} on C_{EL} from the DLVO theory (dashed curve), for emulsions stabilized by 0.02 wt % BLG. The experimental points are associated with the left-hand-side ordinate, whereas the theoretical curve is associated with the right-hand-side ordinate.

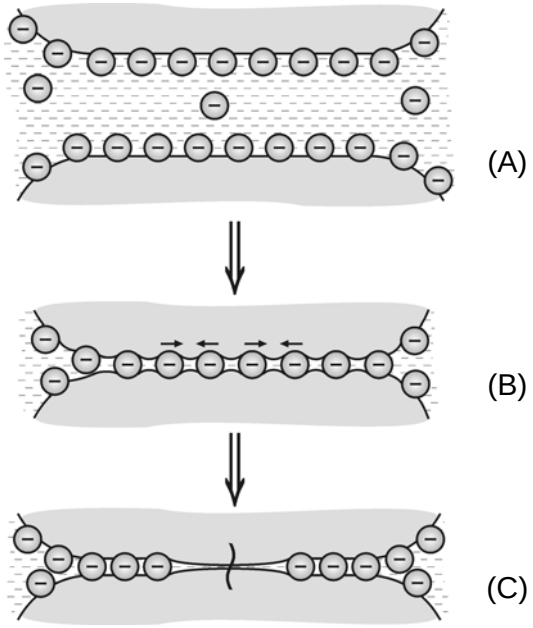


Figure 7. Schematic presentation of the proposed mechanism of emulsion film rupture in emulsions stabilized by charged protein molecules, at low and moderate electrolyte concentrations: (A,B) After the electrostatic barrier is overcome, the two film surfaces spontaneously thin down and a bridging monolayer of protein molecules is formed. (B,C) This monolayer is unstable, because capillary attractive forces between the protein molecules (indicated by arrows in B) act, so as to create bare thin spots in the film, unprotected by protein molecules. The film rupture is schematically shown in (C) by vertical wavy line. For clarity, the positively charged counterions are not shown.

(B) Emulsions stabilized by steric repulsion between adsorption multilayers

For the emulsions prepared with 0.1 wt % BLG solutions, we observed significant increase in emulsion stability at C_{EL} > 50 mM, which was due to formation of protein adsorption multilayer, stabilizing the emulsion films by steric repulsion, see Figure 5B. The stability of these emulsions was described (13) by a simple model accounting for the steric + DLVO interactions, see Figure 8. Based on the entire set of experimental results, the following molecular mechanism of emulsion film rupture was proposed for the systems stabilized by BLG-adsorption multilayers (13). The emulsion films are primarily stabilized through the steric repulsion, created by the overlapping adsorption multilayers. As seen from Figure 5, the steric stabilization by protein monolayers (obtained at the same pH and ionic strength, but at lower protein concentration), provides a lower barrier to coalescence in comparison with the barrier created by multilayers. Therefore, once the steric barrier created by the multilayers is overcome due to drop-drop compression or emulsion shear, one may expect an almost immediate film collapse and drop coalescence, because the secondary barrier created by the adsorption monolayers is lower and cannot ensure film stability under these conditions.

Stabilization of emulsions by aggregated solid particles (resembling in some aspect the structure of protein multilayers) have also been reported (8), but the capillary interactions responsible for the emulsion film stability in the particle-stabilized systems seem conceptually different from the steric stabilization by protein multilayers. No direct analog of this type of stabilization could be envisaged for the typical LMW surfactants.

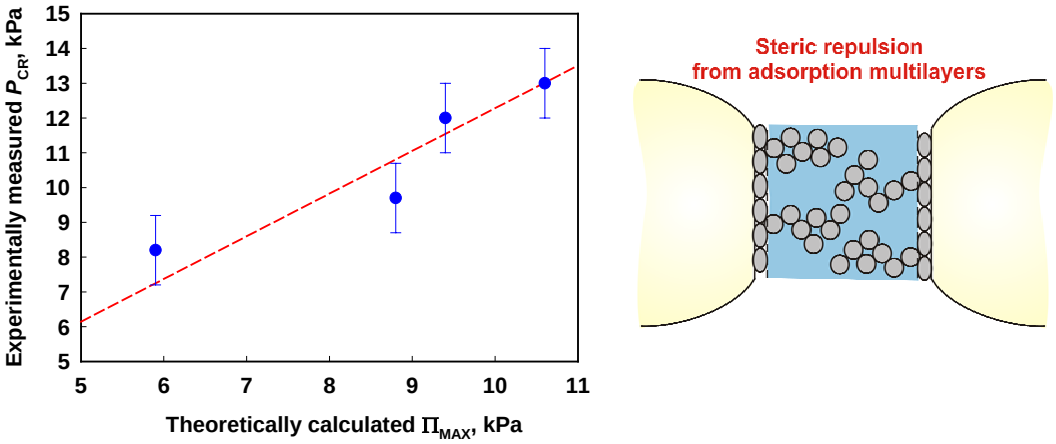


Figure 8. Correlation plot of the theoretically calculated values of the maximal disjoining pressure, Π_{MAX}, accounting van der Waals+electrostatic+steric repulsion from multilayers with experimentally determined values of P_{CR}, at different protein and electrolyte concentrations and at natural pH = 6.2 (Ref. 13).

Effects of emulsion heating and shelf-storage. A remarkable feature of the protein-stabilized emulsions is the observed strong effects of heating (above the denaturing temperature of the protein) and shelf-storage on emulsion coalescence stability, which was particularly pronounced for the emulsions stabilized by protein adsorption multilayers. The experiments showed that, upon shelf-storage of the emulsions, the adsorbed BLG molecules undergo conformational changes, accompanied with formation of predominantly non-covalent intermolecular bonds, which lead to a gradual but significant decrease of emulsion stability with time (aging effect of storage). In contrast, the emulsion heating leads to formation of covalent S-S bonds between the adsorbed molecules, thus, reinforcing the adsorption layers

and strongly increasing emulsion stability. Furthermore, the emulsion heating preserves the initial conformation of the adsorbed protein molecules and the aging effect disappears.

Although such type of stability changes (due to storage, heating, or other external stimuli) are not very typical for emulsions stabilized by LMW surfactants and solid particles, one may expect that such a root for design of "smart" emulsions will be intensively studied in the near future. For example, melting of adsorbed polymer particles or polymerization of adsorbed functional surfactant molecules on drop surface, are only two of the many possible ways for controlled change of emulsion stability after external stimulus and for creating new materials, such as core-shell particles and other more complex structures.

(C) Emulsions stabilized by steric repulsion created by adsorption monolayers.

The stability of emulsions prepared with C_{BLG} = 0.02 wt %, at C_{EL} > 50 mM, is governed by a steric repulsion between adsorption monolayers on the drop surfaces, see Figure 5C. Such type of emulsion stabilization occurs also at arbitrary electrolyte concentration, if pH is close to the protein isoelectric point (IEP) and the protein concentration is low. Under these conditions, the electrostatic repulsion is negligible, due to the small net charge of the protein molecules and/or to the screening of the electrostatic fields by the electrolytes. The stability of such emulsions depends mainly on pH (which governs the conformation of the adsorbed molecules) and on the protein concentration (which governs the amount of adsorbed protein). The storage time and heating have intermediate effects, in comparison with cases (A) and (B) considered above, whereas the electrolyte concentration has small effect in these systems.

One may expect that in these emulsions, the film rupture and drop coalescence occur after expansion of the drop surface (as a result of drop deformation or thermal fluctuations of the film surface) and/or upon application of tangential stress to the film surface (e.g., in sheared emulsions), which break the continuous adsorption layers and create "weak" spots deprived of protein molecules. The stability of such emulsions could be related to the rheological properties of the adsorption layers, such as yield stress or yield strain, mechanical elasticity, etc., which depend mostly on the conformational state of the adsorbed protein molecules and on the intermolecular bonds formed between them (36-38). Some similarities between these protein-stabilized emulsions, and the emulsions stabilized by adsorption monolayers of nonionic surfactants and solid particles could be envisaged. However, the differences between the structures of the respective adsorption layers and between the forces responsible for the emulsion stability are so significant in these systems, that it is difficult to transfer directly a knowledge from one type of system to another.

5/ Conclusions.

The obtained results allowed us to distinguish three different modes of emulsion stabilization by globular proteins, see Figure 5:

(a) At low electrolyte concentration (C_{EL} < 50 mM) and pH away from IEP, the charged protein molecules are separated from each other in the adsorption monolayer, and ensure film stability by creating electrostatic barrier between the surfaces of the emulsion films. The emulsion stability could be reasonably well described by the DLVO theory both during emulsification and upon subsequent shelf-storage. No significant effect of heating and aging is observed for these systems. Under these conditions, the proteins resemble closely the LMW ionic surfactants.

(B) At high electrolyte and protein concentrations, multilayers are formed on the drop surface, which ensure steric stabilization of the emulsions. During emulsification, an important difference between the proteins and particles, on one hand, and the LMW surfactants, on the other hand, is the much slower kinetics of protein/particle adsorption. If this difference is properly accounted for (e.g., by using the dynamic instead of the equilibrium

interfacial tension), the mean drop size in the protein- and particle-stabilized emulsions can be described by the same concepts and theoretical expressions, as those used for surfactant-stabilized emulsions. In contrast, during storage and heating, the adsorbed protein molecules form non-covalent and covalent bonds, which affect significantly the emulsion stability - phenomena that have no direct analogs in the typical surfactant or particle stabilized emulsions.

(C) At high electrolyte and low protein concentrations, the emulsions are stabilized by steric repulsion of adsorbed monolayers. During emulsification, the globular proteins behave similarly to the nonionic surfactants and solid particles. In contrast, during shelf-storage and upon heating, the protein molecules could change their conformation and form intermolecular bonds, thus differing qualitatively from the other types of emulsifiers.

6/ References.

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