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PAPER

Unique emulsions based on biotechnically produced hydrophobins

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The emphasis of this manuscript is on emulsions with gel-like properties based on biotechnically produced hydrophobins. These emulsions are compared to emulsions based on surfactants. Even though the preparation conditions for both emulsion types were the same, the structure and the properties were very different. Homogeneous, gel-like emulsions could be obtained with a protein concentration between 0.02 and 1 wt% and an oil mass fraction of more than 0.65. The gelified state is formed because the protein-covered droplets behave like sticky spheres even when the globules are ionically charged and the long range interaction is repulsive. Conductivity and microscopy measurements showed that the emulsions were of the oil in water (o/w) type. The size of the emulsion droplets depends on the mixing apparatus. With a vortex shaker oil droplets of up to 100 μm diameter were obtained indicating some protein remained in the bulk aqueous phase. With a high pressure homogenizer the emulsion droplets got much smaller and the protein was completely adsorbed at the droplet interface. Interestingly the emulsions aged with time without changing their structure. The aging was a result of the increase of the storage modulus G'. In the case of surfactants no homogeneous stable emulsions could be obtained under the same conditions.

Introduction

Due to their building blocks proteins are amphiphilic compounds. They are surface active and therefore lower the surface tension of aqueous solutions.[1–3] Proteins bind to hydrophobic surfaces[4–6] and make, for example, beer foam.[7,8] Some proteins, such as β-casein, self-aggregate into micelles.[9,10] Thus proteins have many properties in common with surfactants, but the structures of the two compounds are quite different. Surfactants consist of a hydrophobic part and a polar group. The amphiphilic properties are a result of these two competing properties.[11]

For proteins, the situation is different and more complicated. The long amino acid chain of the molecule is usually folded and many hydrogen and often disulfide bonds are involved in the folded state.[12,13] The molecule folds itself in such a way that an energy minimum results. The molecule can exist in other states which might have local energy minimum which is somewhat higher than the lowest energy minimum.[14] Many proteins are soluble in water and are of globular shape, such as β-lactoglobulin or bovine serum albumin (BSA). Their amphiphilic properties are a result of the hydrophobic and polar groups which are on the surface of the folded molecule. The reason for the surface activity of the proteins is the presence of some hydrophobic groups that lie on the surface of the molecules when the molecules are in the energy minimum in the folded state.[15] If such a molecule binds at a water/oil interface some of the hydrophobic groups lose their hostile environment. But it is also clear that the hydrophilic groups on the other side of the molecule remain exposed to water. Whereas when a surfactant molecule adsorbs on the same interface, the whole hydrophobic group is in contact with the oil and the polar group remains in water.[16]

It is obvious that the energy minimum of the protein in the folded state in water might probably not be the lowest energy minimum as in the adsorbed state. The molecule might therefore rearrange to a new conformation upon adsorbing to a solid or liquid interface.[17–20] Therefore emulsions which are prepared from surfactants or from proteins should have different properties.

Different natural proteins have already been used for the preparation of emulsions.[21,22] The emphasis of the investigations usually was on the stability of the produced emulsion,[23] on the size distribution of the emulsion droplets,[24] on the coalescence of droplets[25] and on the up-creaming of oils.[26] The present investigation will focus on the rheological properties of the emulsions, because it is likely that the differences in the interaction between two droplets which are covered either by surfactants or by proteins will be reflected in the storage moduli G'. While it has already been discussed that the aqueous film between two droplets can be in the state of a Newton black film (NBF) or a common black film (CBF),[27] the consequences for the storage moduli of the bulk emulsion have not been discussed. If the interaction between droplets of emulsions is similar to the

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interaction between micelles or swollen micelles in ringing gels the storage modulus should be given by thermodynamic parameters between the droplets like in ringing gels. In these phases the storage modulus is given by the number density of the droplets and the structure factor of the phase. If, on the other hand, the proteins in the adsorbed state form a cross-linked film and the films of two neighbouring droplets are also cross-linked, the storage modulus should be given by the mechanical strength of the resulting three dimensional network. The storage modulus of the emulsion could be much higher than for the previously discussed case.

The emulsions were formed with recombinantly produced hydrophobins, called H Star Proteins®.[28] They are produced as fusion proteins harbouring the hydrophobin protein of the fungi *Aspergillus nidulans*. Hydrophobins act as highly surface active proteins[29,30] and are well known for their strong tendency to self-aggregate.[31,32] These properties combined with the now obtained high availability due to genetic engineering make the H Star Proteins® interesting for industrial applications. The aim of this article is to investigate the differences of emulsions which are prepared in the same way, with the same mass fraction of oil and water but with surfactants or with H Star Proteins® as emulsifying agents.

Materials and methods

H Star Proteins® A and B, from now abbreviated as HPA (46 kDa; IEP: 5.65) and HPB (19 kDa; IEP: 6.15), are recombinant hydrophobins and were a gift from BASF, Ludwigshafen. HPA and HPB consist of the class I hydrophobin DewA from the fungi *Aspergillus nidulans* and the *Bacillus subtilis* protein yaaD, respectively, a truncated form of yaaD. For more detailed information about the H Star Proteins® please refer to ref. 28. The cationic surfactant cetyltrimethylammonium bromide (CTAB) was obtained from Merck, Darmstadt, whereas the non-ionic surfactant isotridecyloctaethyleneglycolether (product name Marlipal O13/80; abbreviated in the text as $C_{13}E_8$) was purchased from Sasol, Hamburg. The used bidistilled 99.5% w/v glycerol was received from VWR, Briare. Calcium chloride ($CaCl_2 \cdot 2H_2O$) was acquired from Grüssing, Filsum. Fluka, Buchs, supplied the oil dodecane, whereas polydimethylsiloxane (PDMS) was purchased from Shinetsu Kagaku, Tokyo. It has the following general formulation: $(CH_3)_3SiO[(CH_3)_2SiO]_nSi(CH_3)_3$. The polymerization degree η ranges from 5 to 19 (>98%) and the viscosity is approximately 6 mPa s. Other chemicals not specified in the text were of analytical grade or equivalent.

Surface and interface tension (against decane) were measured with the volume-drop tensiometer TVT1 from Lauda, Königshofen, at a constant drop-formation speed of 3 μl s^{-1}. The dynamic mode allowed surface tension measurement with dependence on the drop formation speed in the range of 3–43 s μl^{-1}.

Cryo-TEM specimens were arranged in a controlled environment vitrification system (CEVS) and thrown into liquid ethane at its freezing point. The specimens, kept below −178 °C, were studied by an FEI TI2 G^2 transmission electron microscope, operating at 120 kV, using a Gatan 626 Cryo holder system. Using the Digital Micrograph software package the images were documented in the minimal electron dose mode by a Gatan US1000 high-resolution CCD camera.

All emulsions were prepared from aqueous solutions of the desired emulsifier. Additionally all protein emulsions contained 0.5 wt% phenoxyethanol as an antimicrobial agent. As one step oil addition to the aqueous phase led to the breakdown of protein emulsion abilities, it was only possible to produce high oil content emulsions with stepwise addition of oil. Emulsions were prepared with different devices. Samples emulsified with a vortex shaker (IKA Genius 3, Staufen) were treated for 0.5 h with the maximum power, while samples prepared with a Homo Disper (Tokushu Kika, Osaka) underwent revolutions per minute (rpm) between 100 and 9000 for 120 s. Using the High Pressure Emulsifier (APV 1000, Albertslund) required pre-emulsification of the sample using the Homo Disper at low values of around 100 rpm. Afterwards the sample was emulsified three times at the desired pressure (100–1000 bar).

Computer tomography (CT) measurements were performed with the Fraunhofer homemade device called HR-CT 150/3. The distance between the detector and the sample was 0.15 m, while the minimal focus was 3 μm.

For conductivity measurements, the Microprocessor Conductivity Meter LF2000 from the WTW Co., Weilheim, was used.

The rheology of the emulsion layers was measured with the cone-plate rheometer RheoStress 600 from Haake Thermo Scientific, Karlsruhe. The experimental data were analysed with the Haake RheoWin Data Manager, Version 3.3.

For scanning electron microscopy (SEM) the emulsion sample was one day stored at room temperature and finally incubated in a cabinet dryer at 60 °C for two weeks. The dried sample was investigated at a Zeiss 1530 Scanning Electron Microscope with a field emission cathode.

Experimental results and discussion

Properties of the protein solutions

Both biotechnical hydrophobins HPA and HPB are soluble in water up to a concentration of 5 g per 100 ml. The solutions have a pH of 7.95 (HPA) and 7.54 (HPB). Both hydrophobins are surface active and lower the surface and the interfacial tension between oil and water. Surface and interface tension values were obtained with the drop volume technique. The results are shown in Fig. 1.

Both values decrease continuously with increasing protein concentration up to their solubility limit. The continuous decrease of the values is a sign that the proteins do not form micelles in the aqueous solution. In the concentration range where the proteins start to lower the surface tension, the obtained values depend on the drop time. This feature is a typical sign that slow reactions follow the adsorption of the protein.[35] The surface tension profiles of HPA and HPB for very short and very long drop formation time are shown in Fig. 2. It is conceivable that the decrease of the surface tension is due to the formation of a thin film of the molecules in the adsorbed state.[31]

In Fig. 3, the pH of a 1% HPA solution is plotted against the added HCl concentration. With lowering of the pH the protein flocculates in the pH range between 5.73 and 3.12 and at even

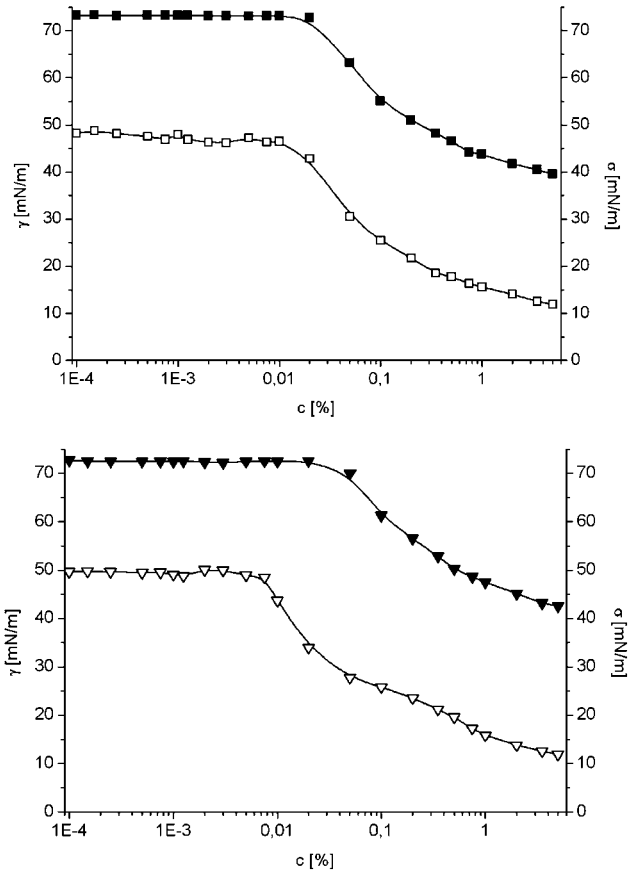

Fig. 1 Surface and interface tension against decane for increasing concentrations of HPA (squares) and HPB (triangles) determined by the drop volume technique. Filled symbols: surface tension γ; open symbols: interface tension σ.

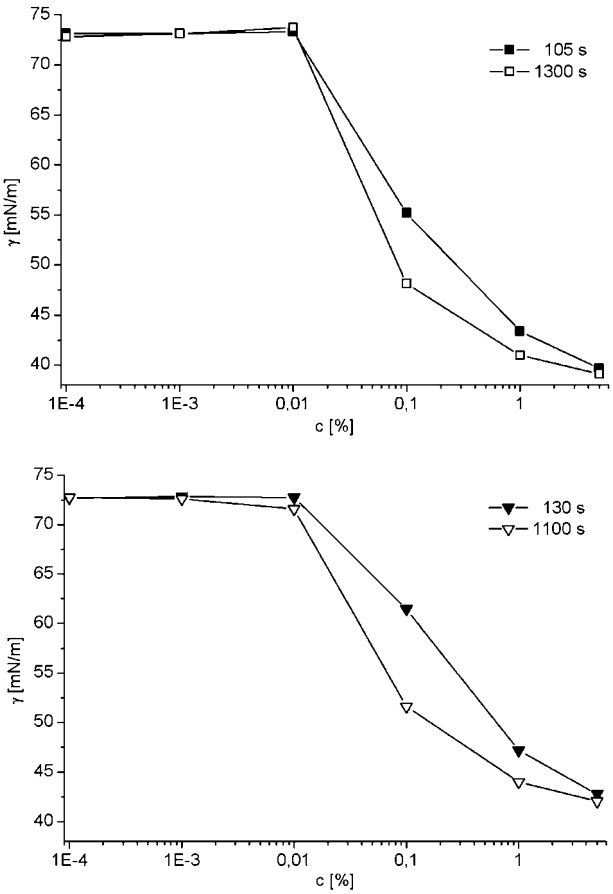

Fig. 2 Time-dependent surface tension profile for HPA (squares) and HPB (triangles). Plotted are the surface tensions γ for very short drop formation times (filled symbols: 1 s μl^{-1}) as well as for very long drop formation times (open symbols: 43 s μl^{-1}).

lower pH values it becomes soluble again. Obviously the proteins are negatively charged. On neutralisation of the molecules, they show maximum attraction to each other and flocculate. On reversing the charge by protonation the molecules become soluble again. The change of the pH of the protein solution is accompanied by a change of the surface tension (Fig. 3).

As one can clearly see in Fig. 3 the surface tension of the supernatant decreases with the increasing amount of HCl ($\leq$7 mM), indicating the protein becomes more hydrophobic due to a lower total intrinsic charge. In the range between 9 and 11 mM HCl the protein solution shows strong flocculation. Nevertheless, as the supernatant's surface tension increases again, it is obvious that not all protein is flocculated. According to Fig. 1 a surface tension value γ of 46 mN m^{-1} corresponds to a free HPA concentration of 0.4%, indicating that not all protein are in the flocculated state. Therefore the crossover from negative–neutral–positive protein charge seems to be very sharp. For HCl concentration higher than 11 mM more and more of the flocculated protein fraction becomes soluble again resulting in lower surface tension values.

One can conclude that pH tuning strongly affects the net charge and the interactions of proteins. Lutz *et al.* showed the strong correlation between pH and stability of emulsions prepared by pectin and whey protein.[36]

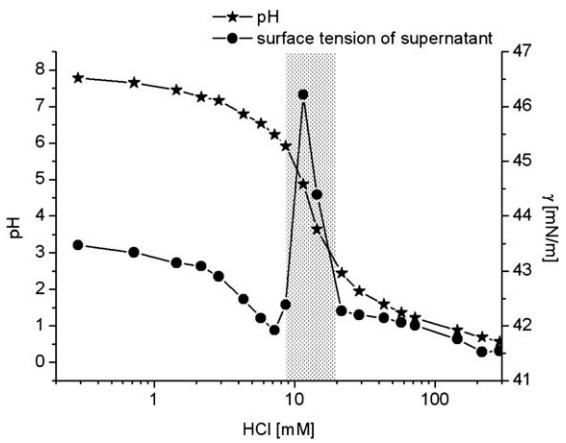

Fig. 3 Plot of pH (stars) and surface tension γ (circles) of 1% HPA (IEP: 5.65) solutions against HCl concentration (mM). The shaded area indicates the HCl concentrations where HPA shows strong flocculation.

It is also well known that proteins can interact with surfactants[37] and ions.[38] Flocculation of the negatively charged hydrophobins can not only be reached by a change of the pH but also by binding of cationic surfactants, such as CTAB, or by

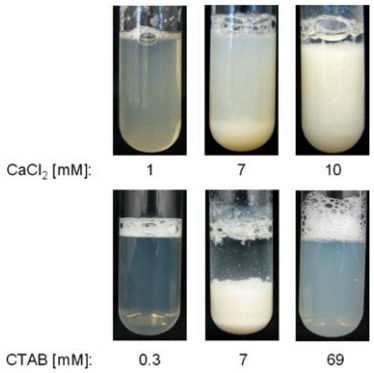

Fig. 4 Solutions of 1% HPB with increasing amounts of $CaCl_2$ and cationic surfactant CTAB. Excessive protein flocculation takes place at 10 mM $CaCl_2$ and 7 mM CTAB, respectively. Adding excess CTAB leads to HPB resolubilisation, whereas the flocculated state remains even at higher $CaCl_2$ concentrations.

binding of Ca^{2+} ions. Results of such titrations are shown in Fig. 4.

The binding of the cationic surfactant CTAB leads first to flocculation and then to resolubilisation. In this process the proteins are completely saturated with the surfactant molecules. During the titration of the proteins with CTAB the surface tension reaches first a minimum and then passes through a maximum. Finally the surface tension of the pure CTAB solution is reached when the free monomer solution of CTAB reaches the critical micellar concentration (cmc) (Fig. 5).

Obviously the protein solutions foam when they are freshly prepared. The foam stability depends very much on the pH and the charging degree of the proteins. Interestingly the samples shortly before and after protein flocculation have best foaming properties.

A Cryo-TEM micrograph is shown in Fig. 6. The protein molecules with a molecular weight of 46 kDa (HPA) and 19 kDa (HPB) are in the size range ~5 nm) in which they should be.

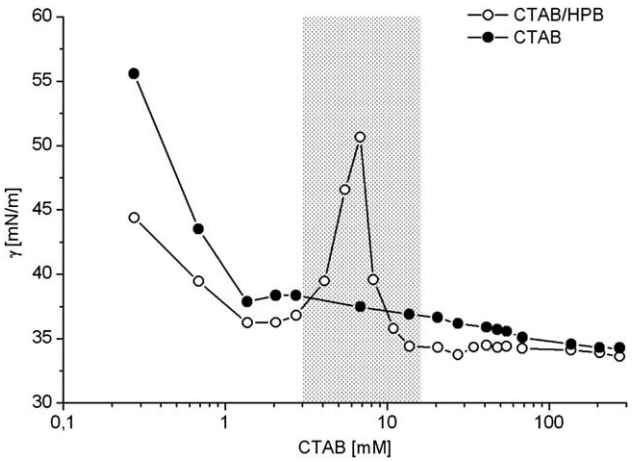

Fig. 5 Surface tension γ profile of the supernatants of mixtures from 1% HPB and increasing amount of CTAB (open circles) in comparison to the surface tension of a pure CTAB solution (closed circles). The shaded area indicates the CTAB concentration range where HPB is in the flocculated state.

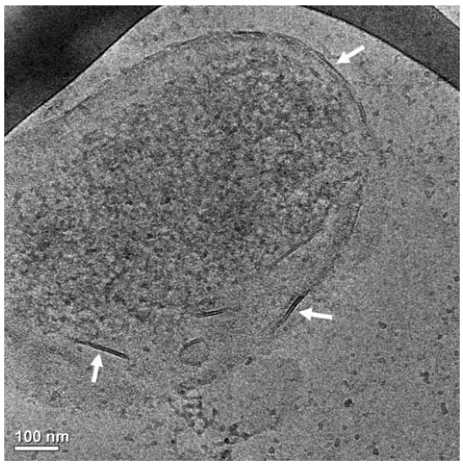

Fig. 6 Cryo-TEM micrograph of a 0.1% HPA solution. White arrows show membrane fragments formed by big protein aggregates.

Moreover the micrograph shows pieces of thin films (marked with white arrows in Fig. 6) that are formed by interpenetrating protein aggregates. This experiment confirms the strong tendency of self-aggregation at the air/water interface even for the technical hydrophobins as it was recently observed by Kisko *et al.* for natural hydrophobins.[32] Most likely those films were formed at the air/water interface as the local concentration of the surface active H Star Proteins® compared to the bulk solution is much higher. The film formation could be a result of the time dependence of the surface tension.

We also looked for larger molecular aggregates with the electric birefringence technique.[39] Large signals were observed which increased in amplitude and time constant with time. These signals disappear when the hydrophobin solution is filtered through micropore filters. Small signals appear again after several days. Obviously, the proteins form aggregates with time in an irreversible process.

Protein *vs.* surfactant as emulsifier

Four samples which were prepared from aqueous solutions of proteins HPA and HPB, of the non-ionic surfactant $C_{13}E_8$, the cationic surfactant CTAB and 20 wt% dodecane were compared. All the samples are separated into two phases: a lower phase and a milky upper phase. The volume of the upper phase is only slightly larger than that of the pure oil phase before the emulsification process. The upper phases from the protein samples have increased considerably with respect to the oil phase. Without having other information it can be assumed that the upper phases are w/o emulsions in which a small fraction of the aqueous phase is dispersed in the oil phase. However this is not the case, as can be concluded from conductivity measurements and the rheological properties of the phases.

In Fig. 7 rheograms of the upper phases measured 1 day after emulsification are shown. The protein emulsions behave like weak gels. The storage modulus G' is only weakly frequency dependent and is much larger than the loss modulus G''. These are typical signs of a gel.

The emulsions in the upper phases that were produced with surfactants can also not be w/o emulsions with low water

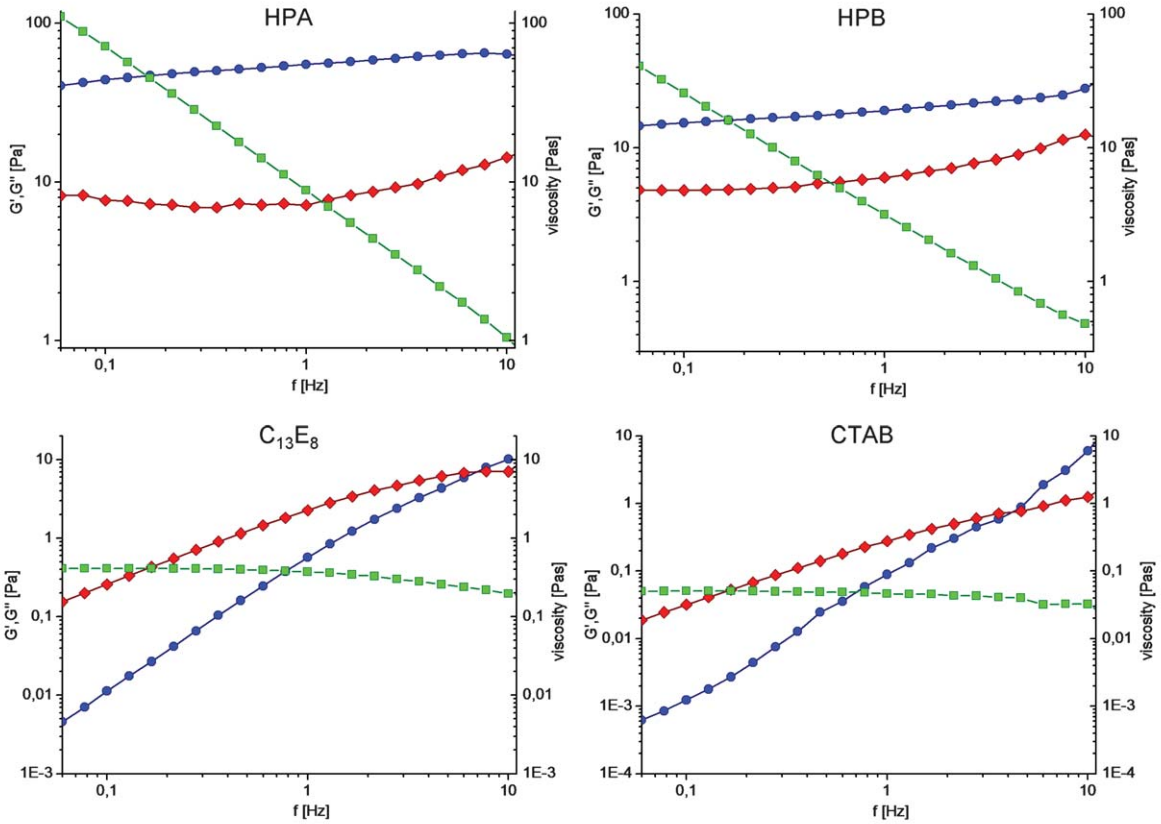

Fig. 7 Rheograms of the emulsion layers containing 1 wt% emulsifier and $\Phi = 0.2$ dodecane measured at $\tau = 0.5$ Pa one day after emulsification. Blue: storage modulus G' (Pa), red: loss modulus G'' (Pa) and green: viscosity η (Pa s).

fractions. In this situation the viscosities should only be somewhat increased with respect to the viscosity of dodecane. The viscosities, however, are very much increased and the phases show non-Newtonian behaviour. Furthermore, the emulsions have a conductivity that is much higher than the conductivity an oil phase can have. These properties, the conductivity and the rheological properties, prove that the emulsions must contain a network of an aqueous phase. It is likely that the network is an aqueous foam that contains dodecane. Investigations of the phases with optical microscopy indeed show that the upper emulsions which were prepared with surfactants are high internal phase emulsions (HIPE). In spite of the appearance indicating the phases to be w/o emulsions they are o/w emulsions. The oil is encased in a foam structure.

Such structures have been described in the literature.[40,41] The phases are usually prepared in a complicated multi-step process. It is therefore surprising that the HIPE phases can also be formed by a simple emulsification process. Not all of the amphiphilic compounds are adsorbed in the network. Surface tension measurements show that some of the surfactants are left in the lower aqueous phase. Obviously not enough surface was produced in the emulsification process which could accommodate all the amphiphilic compounds on the surface.

As is obvious from the volumes of the protein emulsion, these phases contain more water than the emulsions from the surfactants. It is likely therefore that their structure is different. Light micrography of the phase proved them to be normal o/w emulsions with a high polydispersity (5–90 μm) of the oil droplets. As it is obvious from the gel-like behaviour of the phase, the oil droplets with the adsorbed protein film must stick together and form a three dimensional network. All emulsion droplets observed with light microscopy had bridging points with each other indicating that they are truly forming a protein network with the droplets incorporated.

The described results make it clear that the hydrophobin proteins and surfactants form emulsions with different properties. It is likely that this behaviour of the proteins is due to the fact that the surface of the protein molecule keeps its amphiphilic nature and can form sticky contacts when it comes into contact with other such surfaces. Protein–protein interaction and entanglement in the emulsion layer are also supported by previous findings. Globular protein molecules at the interface can no longer rotate freely but are fixed in the protein monolayer in a well defined conformation and aligned position.[19] The molecules probably form a film in which the adsorbed molecules are connected with each other through physical bonds. Evidence for such films has been reported from rheological measurements on interfacial films.[42]

In the following sections we study the properties of protein emulsions, when parameters of the systems are changed.

From the described results and the proposed explanation it is already clear that the rheological properties of the emulsions are

not determined by the volume fraction of the droplets and the size distribution, but by the properties of the three dimensional protein network that is formed in the emulsion.

The influence of glycerol on the emulsions

Many cosmetic products contain glycerol for different reasons. Glycerol lowers the freezing point of water and the samples can be exposed to lower temperatures without losing their homogeneity. Glycerol also gives the samples a softer touch and keeps the water for longer times. A high glycerol concentration also increases the cmc of surfactants.[43] Even more important for the appearance of the samples is the fact that glycerol increases the refractive index of the aqueous phase[44] and can reduce the refractive index contrast between the water phase and the oil. Emulsions become therefore more transparent with the increasing glycerol content. Glycerol at the same time changes the interaction between the oil droplets because the Hamaker constant depends on the refractive index of both the solvent and the oil and with the decrease of the refractive index contrast the attraction between the droplets is lowered.

This effect has been used to prepare stable and transparent high internal phase o/w emulsions.[40] Contrast matching of the refractive index can also be used in two phase samples of L_1/L_α to increase the interlamellar distance in the L_α-phase to transform the system into a transparent single L_α-phase.[45,46]

Emulsion prepared with 1% HPB protein and 0–60% glycerol in the aqueous phase and oil mass fraction $\Phi = 0.2$ dodecane proved that glycerol has little influence on the visual appearance of the samples up to 40% glycerol. However a strong change in the transparency of the emulsion phase takes place between 40% and 60% glycerol. This effect is obviously due to the refractive index matching. The emulsion phases do not flow when the samples are turned upside down. Interestingly the upper emulsion phase for the sample without glycerol is about twice as large as the amount of dodecane ($\Phi = 0.2$) that was used for the sample preparation. The emulsion must therefore contain about equal volumes of oil and water. However when the glycerol concentration increases up to 60% the volume fraction of the emulsion layers stays more or less constant in spite of changing the density of the solvent and the Hamaker constant for the droplet interaction. Because of the Hamaker constant reduction the attraction between the emulsion droplets becomes smaller and the structure factor S should increase. This has obvious consequences on the storage moduli of the emulsion phases as shown in eqn (1):

$$G' = \frac{\nu kT}{S} \quad (1)$$

The structure factor S is >1 for attractive particle interaction and <1 but >0 for repulsive interaction.[33] In this simple model in which the modulus is determined by the osmotic interaction of the particles in the system, the storage modulus of dense emulsions should be 10^6 times smaller than the modulus of ringing gels.[34]

In the case of the emulsions containing increasing amounts of glycerol, the structure factor S decreased from a value much larger than 1 to smaller values, but still larger than 1 resulting in larger G' values (Fig. 8).

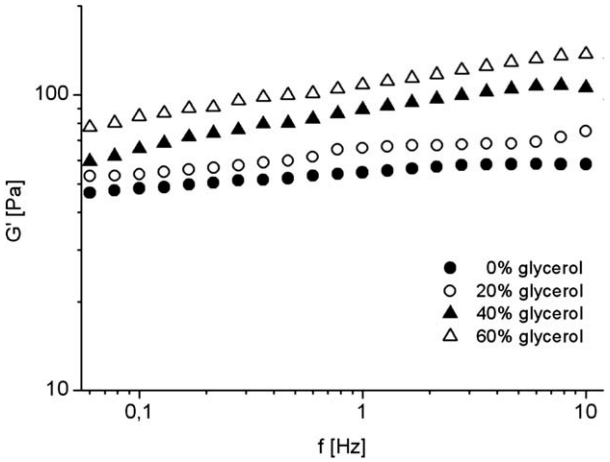

Fig. 8 Storage moduli G' (Pa) against frequency (Hz) measured at $\tau = 0.05$ Pa for emulsions prepared with various amounts of glycerol after 1 day. Sample composition: aqueous phase: 1% HPB and 0–60% glycerol; oil $\Phi = 0.2$ dodecane.

Computer tomography of emulsions

The structure of emulsions can be made visible by Computer Tomography (CT). Obviously, the contrast in electron density for water and dodecane is large enough for the oil structures to be seen. Fig. 9 shows a micrograph of a transparent, homogeneous emulsion containing 0.5% HPB and 60% glycerol in the aqueous phase, pH 6, and an oil mass fraction $\Phi = 0.6$ dodecane. The emulsion was prepared with the vortex shaker. The smallest droplets which can be resolved have a diameter of about 50 μm.

Light microscopy proved that the diameters of the oil droplets are in the range of 50 μm. The more interesting information of the CT micrograph is, however, that the small droplets form aggregates with a typical size of 200 μm. It is obvious that the size of these clusters is given by the vortexing method. It is

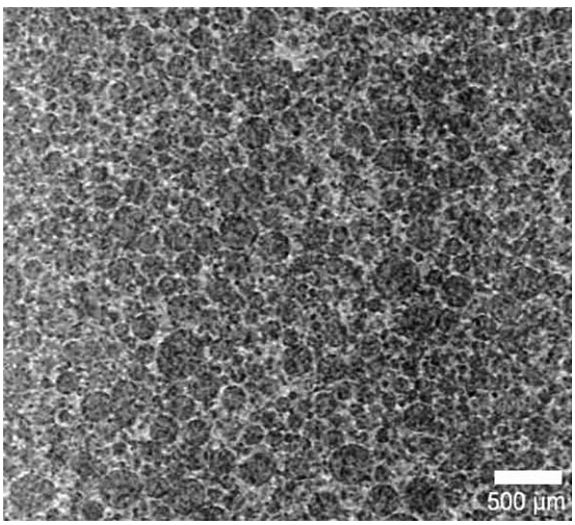

Fig. 9 Computer tomography of a homogeneous protein emulsion. The emulsion contained 0.5% HPB and 60% glycerol in the aqueous phase and $\Phi = 0.6$ dodecane, pH 6. The average droplet diameter is 50 μm.

conceivable that these larger objects rotate as whole units in the shear flow.

Emulsion with flocculated protein

In the discussion about the protein solutions it was mentioned that the protein could be flocculated by changing the pH, by adding $CaCl_2$ or CTAB. The three different procedures have in common that the ionic charge of the protein particles is compensated and the particles attract each other. We have used such flocculated protein dispersions for the preparation of emulsions. The samples prepared from the flocculated protein state using HCl and $CaCl_2$ look like the sample without flocculation agents, but the flocculation with CTAB led to a dramatic decrease in the emulsfying ability of HPB.

The storage moduli of the samples after 1 day incubation at room temperature are compared in Fig. 10. It is interesting to note that G' for the emulsion with the unmodified proteins is similar to the storage moduli of the flocculated systems. It is, however, much larger than the storage modulus in the emulsion layer that had been produced with 20 wt% dodecane (Fig. 7).

The excess concentration of protein in the lower phase did have an influence on the modulus of the upper phase. It is conceivable that the two phase system was affected by depletion flocculation and that the concentrations of protein in the upper emulsion phases and in the lower aqueous phases were not the same and as a consequence the storage modulus in the 20 wt% emulsion was lower than in the single phase emulsion with an oil mass fraction Φ of 0.65 (Fig. 10).

The most startling result is, however, the storage modulus of the sample with added CTAB (Fig. 10). It has been noted in the literature for protein emulsions that the most stable emulsions were obtained with a flocculated emulsifier.[47] In the present system this is obviously not the case, even when only very little CTAB was added to compensate the ionic charge of the protein and not as much to saturate the protein with a surfactant and reverse the charge on the protein.

The sample with CTAB shows that the upper emulsion layer is no longer a homogeneous layer but the emulsion has become unstable and has separated into oil and emulsion. Obviously coalescence between the droplets has occurred which resulted in an excess oil phase. It is then likely that the small amount of the added CTAB did not only compensate the charge on the protein but also effected the protein conformation. The surfactant can possibly do this by binding to the hydrophobic part of the protein molecule. By doing this the protein surfactant complex can no longer act as a sticky protein molecule but it acts more as a normal surfactant molecule with one hydrophilic and one hydrophobic part.

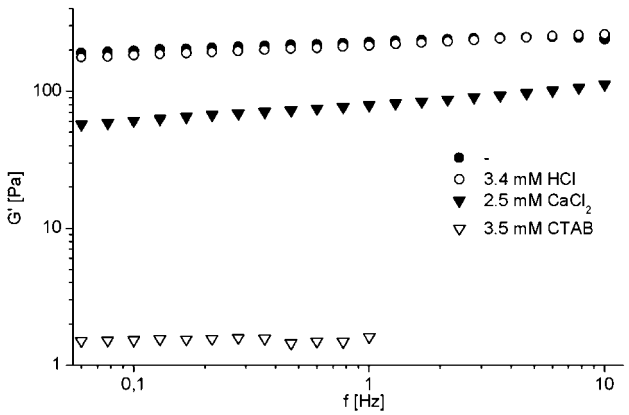

Fig. 10 Storage moduli G' ($\tau = 0.5$ Pa) of emulsions prepared from flocculated protein. Final concentrations: 0.5% HPB without and with flocculation agent (3.4 mM HCl, 2.5 mM $CaCl_2$ and 3.5 mM CTAB) and a mass ratio Φ of 0.65 dodecane.

The influence of heating on protein emulsions

It is known that the properties of many proteins are heat sensitive. The best known example is egg protein. Many other proteins are known to flocculate when they are heated. The transition of a dissolved protein from the liquid state to the flocculated state should be independent of whether the protein is in the three dimensional bulk state or in the adsorbed monomolecular film of the emulsion. To find out about the heat sensitivity of the emulsions, we measured the rheological properties of a freshly prepared emulsion and of an emulsion which was heat treated for a short time period. The results are shown in Fig. 11 for the emulsion containing 0.5% HPB and $\Phi = 0.65$ dodecane.

The storage modulus of the emulsion in the heat treated state is twice as high as that of the unheated emulsion. This is a clear indication that the stiffness of the protein film in the monolayer has become much larger during the short time heat treatment.

It is furthermore noteworthy that the properties of the heat treated emulsions no longer change with time as opposed to the unheated emulsion. This is an indication that the heat treated state of the protein is a very stable state and can no longer change its configuration. Similar results with emulsions stabilized by proteins, like β-casein, have shown that emulsions are usually more resistant to droplet aggregation during heating if the protein configuration does not change completely upon heat treatment.[48]

Shear-rate influence on properties of protein emulsions

The emulsion droplets in the samples are produced by shear stresses that act on the bulk oil phases. In such situations higher

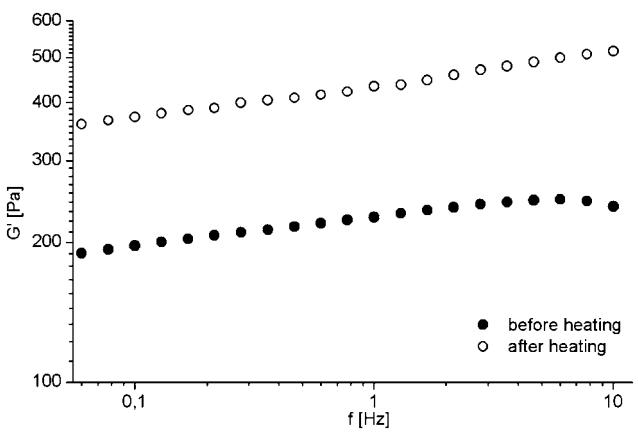

Fig. 11 Storage moduli G' ($\tau = 0.5$ Pa) of an emulsion with 0.5% HPB and $\Phi = 0.65$ dodecane before and after heating for 5 min at 92 °C.

shear stresses should produce smaller droplets. Different shear stresses should therefore result in emulsions with droplets of different dimensions and different properties.

In order to investigate the influence of shear time on the emulsion properties, the storage modulus G' was determined for emulsions prepared with a constant shear rate (5000 rpm), but different shear times. The moduli were measured at a small shear stress ($\tau = 0.05$ Pa) to avoid disruption of the disordered, fresh droplet structure. It turned out that with increasing shear time (0–120 s) the storage modulus G' of the emulsion was also becoming higher. For shear times higher than 120 s, the corresponding emulsion modulus did not change significantly any more.

Emulsions were prepared which have the same composition (1% HPB and $\Phi = 0.65$ dodecane) but have been emulsified with different mixing aids. One emulsion was prepared with a vortex shaker while other samples were prepared with a Homo Disper with revolutions per minute (rpm) of 1000, 5000 and 9000 with a shear time of 120 s. All samples look alike and are homogeneous emulsions. However, their rheological properties are different. All samples have gel-like properties which is evident from the result that the storage modulus is independent of frequency and larger than the loss modulus. The storage modulus that is the stiffness of the samples is increasing with the shear stress that is produced in the techniques (Fig. 12).

These results are an indication that the dimension of the droplets is decreasing while the storage moduli increase. This is indeed the case as it is shown in Table 1.

The dimensions of the droplets which have been prepared with the vortex shaker are considerably larger than the droplets prepared with the high pressure emulsifier. With an average droplet size of 9 μm at the highest rpm stage the droplets have reached a dimension which is not close to the values that can be calculated with the theoretical core shell model (eqn (2)).

$$\frac{r}{3d} = R \tag{2}$$

where d is the thickness of the adsorbed layer and R is the mass ratio of oil to amphiphile. From the two parameters the radius of the emulsion droplets r can be calculated.

Table 1 Comparison of the droplet size (μm) of emulsions prepared at different mixing rates. Emulsion concentrations: 1% HPB and $\Phi = 0.65$ dodecane

	Vortex	1000 rpm	5000 rpm	9000 rpm
Droplet size/μm	60 ± 34	41 ± 18	17 ± 8	9 ± 4

It is therefore likely that the used emulsification devices are not suited to produce smaller oil droplets in order to completely use up the protein for the emulsion preparation. The samples should still contain proteins in the aqueous phase.

The aging of the emulsions with time

Homogeneous emulsions that do not seem to change with time can easily be prepared from the proteins when the protein concentration is in the range between 0.02% and 1% and the oil mass fraction Φ is larger than 0.65. The samples did not phase separate with time and their appearance did not change. However when rheological measurements are made after different times it turns out that the elastic properties increase with time but approach a constant value with time. Fig. 13 contains the storage modulus with time of an emulsion containing 1% HPB and $\Phi = 0.65$ dodecane prepared with the Homo Disper at a shear rate of 9000 rpm.

It is noteworthy that the storage modulus more than doubles with time. During this time the structure of the emulsion as observed under the microscope does not seem to change. It is likely therefore that the increase of the storage modulus is given by the increase of the stiffness of the network structure. In the literature, partial entanglement of the adsorbed protein molecules is declared to be the reason for aging for β-casein and BSA films.[49] Other rheolgical measurements showed that not only the storage modulus changes with time, but also the deformation of the emulsion phase before the storage modulus breaks down increased with time. This means that the protein network has become more elastic.

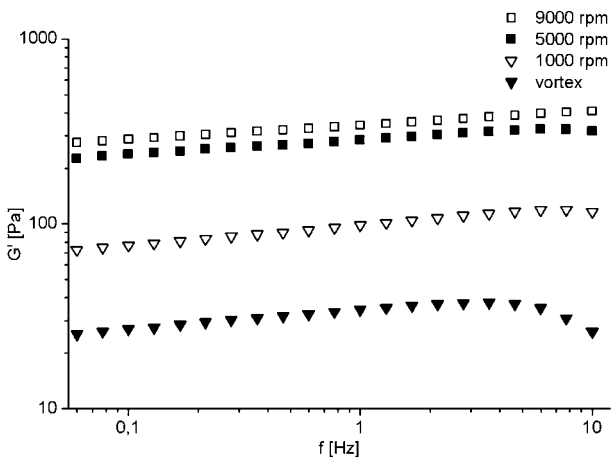

Fig. 12 Storage modulus G' ($\tau = 0.5$ Pa) of emulsions prepared with different mixing aids. Final concentrations: 1 wt% HPB and $\Phi = 0.65$ dodecane.

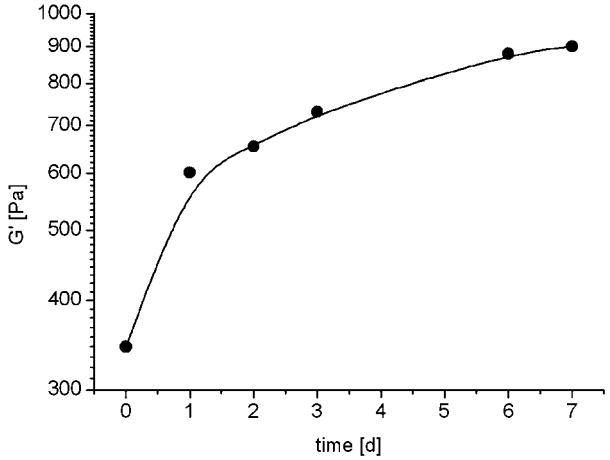

Fig. 13 G' (Pa) at $\tau = 0.5$ Pa and $f = 1$ Hz measured at different time points. Sample composition: 1% HPB and $\Phi = 0.65$ dodecane, prepared with the Homo Disper at 9000 rpm.

Evidence of film formation in the adsorbed monolayer

The described experiments have indicated that biotechnically H Star Proteins® in the adsorbed monolayer in the emulsions might form thin films, which means that the individual molecules crosslink irreversibly with each other. The surface tension measurements showed signals of irreversible adsorption, the Cryo-TEM micrographs showed pieces of thin films, the electric birefringence measurements could be explained by the growth of large aggregates and finally the large storage moduli of the emulsions were indications that a strong three dimensional network was formed in the emulsions.

In order to demonstrate the formed three dimensional network, we designed an experiment to prove the existence of this network. An emulsion was prepared containing 1% HPB and a mass fraction Φ of 0.65 dodecane, prepared with the Homo Disper at 9000 rpm. The emulsion was dried in a cabinet dryer at 60 °C for two weeks. Large pieces of a little light material were obtained. A REM-micrograph (Fig. 14) of the material showed that the emulsion droplet size was identical to the one observed with the light microscopy (Table 1). Obviously the structure had not collapsed during the removal of the oil and water. This seems to have been only possible if the individual films were cross-linked to a supermolecular structure.

Emulsions from silicon oil and hydrophobin

Gel-like emulsions can not only be prepared from dodecane but also from other oils. Emulsion layers with a high internal content of polydimethylsiloxane (PDMS) and 0.5% HPB have also been prepared. One sample was prepared with the vortex shaker while the other samples were prepared with a high-pressure emulsifier at pressures of 100 bar, 300 bar and 1000 bar. The vortex sample and the sample prepared at 1000 bar separated into two phases: an upper emulsion and a lower aqueous phase. It is surprising that the sample which had been produced with the highest pressure is not stable. Such situations have also been described in the literature.[22] It is usually assumed that there is not enough emulsifier in the sample that covers the droplets completely with a monolayer. This would also be the situation in the shown sample. The dimension of the droplet decreased as the pressure was increased as is shown in Table 2.

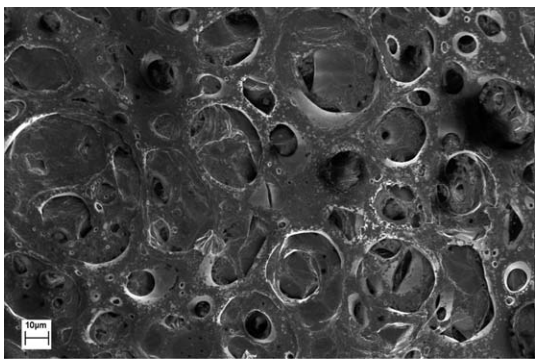

Fig. 14 REM micrograph of the drying residue of an emulsion containing 1% HPB and $\Phi = 0.65$ dodecane, prepared with the Homo Disper at 9000 rpm.

Table 2 Droplet size (µm) of emulsions prepared with a vortex shaker and a high pressure emulsifier at different pressures. Final concentrations: 0.5% HPB and $\Phi = 0.65$ PDMS

	Vortex	100 bar	300 bar	1000 bar
Droplet size/µm	100 ± 61	4.2 ± 0.7	3.9 ± 1.0	3.1 ± 0.9

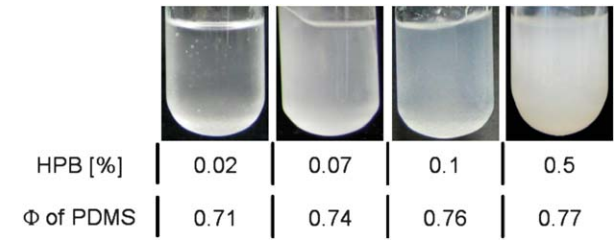

Fig. 15 Determination of the maximum oil content for homogeneous emulsions depending on the used protein concentration. Aqueous phase contained 60% glycerol.

With 1000 bar, a droplet diameter of about 3 µm is reached. With the simple theoretical core shell model (eqn (2)), one obtains a diameter of 1 µm when a thickness of the protein layer of 3 nm is assumed. The viscoelastic properties of the sample increase with increasing pressure in the emulsifier. It is interesting to note that the storage modulus G' of samples with the same composition can be changed from 1 Pascal to more than 100 Pascal. When the concentration of hydrophobin is doubled in the sample, the emulsions are also stable at the highest pressure used for emulsification. This experiment shows that the interpretation for the two phase formation is probably correct.

More transparent and single phase emulsions are obtained when part of the water is replaced by glycerol as is shown in Fig. 15. These samples were prepared with the vortex shaker. The HPB concentration was varied in the samples. The results show that homogeneous, gel-like emulsions can already be obtained with a protein concentration as low as 0.02%.

Conclusions

The investigations on the presented systems have shown that emulsions from hydrophilic surfactants are low viscous solutions without a yield stress. The H Star Proteins®, in contrast, form emulsions with gel-like properties with a yield stress. The gel-like properties are formed because the protein covered oil droplets are sticky particles. The stickiness of the particles is due to the fact that the amphiphilic properties of the protein particles are distributed over their whole surfaces. This property controls also the solubility of the proteins in water. The amphiphilic properties do not disappear when proteins bind to oil droplets. On binding the proteins to an oil droplet, the local environment on part of the molecule is changed. As a consequence the protein molecule has to change its folded structure. It is conceivable that as a result of the change of the conformation, neighbouring protein molecules interpenetrate with each other and form a thin protein film around the oil droplets. This process could be the reason for the aging of the emulsion and the increase of the shear modulus of the emulsion with time.

Under high shear conditions emulsions are obtained in which nearly all the protein is adsorbed at the interface of the droplets. The dimensions of the droplets are then given by the oil/protein ratio. The size of the droplets in the emulsion is determined by the existing shear rates in the emulsifier as long as enough protein is available to cover the entire formed oil/water interface. While normal emulsions can be theoretically treated as a dispersion of repulsive droplets as it is the case for ringing gels or cubic phases for which systems the rheological properties are due to the number density of the particles and their interfacial tension the emulsions from proteins have to be looked at differently.

The properties indicate that the storage modulus of the protein emulsions is determined by the elastic three dimensional network that surrounds the droplets and connects the droplets. Otherwise the high storage moduli of the emulsions could not be understood. The elastic film around the droplets is probably the reason for the high stability of the emulsions. The protein covered droplets are present in a flocculated state with direct contact between the droplets. In spite of this situation, the droplets do not coalesce and form an excess oil phase.

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