Unique Emulsions based on recombinant Hydrophobins

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„Ideen, wie absolute Gewissheit, absolute Genauigkeit, endgültige Wahrheit und so fort, sind Erfindungen der Einbildung und haben in der Wissenschaft nichts zu suchen...“

Max Born

„Am Ende gilt doch nur, was wir getan und gelebt – und nicht was wir ersehnt haben“

Arthur Schnitzler

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Zusammenfassung

Hydrophobine sind Pilzproteine aus etwa 100 Aminosäuren. Hydrophobine sind die am stärksten oberflächenaktiven, natürlichen Proteine mit ausgesprochener Tendenz zur Selbstaggregation. Aufgrund ihrer vielseitigen Eigenschaften wirken Hydrophobine in vielen Strukturen des Pilzes, unter anderem als Mantelsubstanz der Hyphen. Anwendungen der Hydrophobine sind im großen Maßstab bisher an den Kosten und dem Aufwand der natürlichen Hydrophobin Reinigung gescheitert. Dies änderte sich grundlegend durch den Einsatz weißer Biotechnologie grundlegend, welche rekombinante Hydrophobine heute im großen Maßstab zugänglich macht.

In dieser Arbeit werden zwei rekombinante Hydrophobine beispielhaft für diese Proteinklasse auf Ihre Fähigkeit zur Emulsionsstabilisierung hin untersucht: H Star Protein ® A und B. Die physikalisch-chemische Charakterisierung der Hydrophobine zeigt, dass sich diese rekombinanten Proteine in ihrer Oberflächenaktivität tatsächlich wie die natürlichen Hydrophobine verhalten.

Der Einsatz rekombinanter Hydrophobine als Emulgator führt zur Ausbildung gelartiger Öl-in-Wasser Emulsionen, die sich nicht mehr auftrennen, also weder Aufrahmen noch Koaleszenz zeigen. Die Emulsionen werden sogar zeitabhängig, in Stunden oder Tagen, noch wesentlich stabiler. Der Grund ist der kinetisch kontrollierte Aufbau eines räumlichen Netzwerkes aus Hydrophobin, das die Öltröpfchen umgibt. Diese Art der Emulsionsstabilisierung ist neuartig auf dem Feld der Emulsionstechnologie.

Es wurde gefunden, dass Hydrophobine in der Lage sind, an Clays, also scheibchenartige Schichtsilikatpartikel, teilweise oder vollständig zu adsorbieren. Hieraus ergibt sich die Möglichkeit, die Eigenschaften der Systeme Wasser, Öl und Schichtsilikat kontrolliert zu verändern. Es wurde gezeigt, wie durch synergistische Effekte homogene und höchst stabile Pickering Emulsionen hergestellt werden können, die sich durch hohen Ölgehalt bei extrem geringem Emulgatoranteil auszeichnen. Die planaren Schichtsilikate können auch durch stäbchenförmige Teilchen ersetzt werden. Boehmitenadeln bilden ebenfalls in Verbindung mit Hydrophobin Pickering Emulsionen.

Abschließend wird gezeigt, wie Hydrophobin in der Kombination mit Schichtsilikat auch durch andere Proteine, Amphiphile oder Tenside ersetzt werden kann. Durch Einstellen der Präparationsbedingungen, der Emulgatorenkonzentration oder des Ölmassenbruchs besitzt man nun ein neues, universelles Werkzeug, um Pickering Emulsionen mit gewünschten Eigenschaften herzustellen.

Summary

Hydrophobins are very interesting proteins of fungal origin. Beside their relatively small size of around 100 amino acids, they are well known to be the most surface active, natural proteins that have a strong tendency for self-assembly. Due to their versatile properties hydrophobins are present in different fungal structures, like as coaters of hyphae. These diversified properties of hydrophobins raised great interest among scientists. Possible applications in surface modification or emulsion industry were always restricted by the cost and effort of natural hydrophobin purification. This changed dramatically by the use of white biotechnology resulting in the availability of high amounts of recombinant hydrophobins nowadays.

This study started with the physicochemical characterization of two recombinant hydrophobins, called H Star Proteins ® A and B. Both show a remarkable, time-dependent surface activity as well as a distinct aggregation behaviour indicating them to have the typical properties of natural hydrophobins. The use of the recombinant hydrophobins as emulsifier resulted in the formation of gel-like oil in water emulsions. Interestingly, without the occurrence of typical emulsion instability processes like creaming or coalescence, these emulsions showed significant aging effects. We conclude them to be the consequence of the time-dependent formation and progression of a self-supporting, three-dimensional protein network that evolves in the emulsion. The self-assemble tendency of recombinant hydrophobins is clearly not limited by adsorption to the oil-water interface. Obviously the long term stability of the emulsion is determined by the sticky character of the hydrophobin coated oil droplets that attract each other in the short range distance. This type of emulsion stabilization mechanism is absolutely novel in the field of emulsion technology.

Moreover we used the hydrophobins' ability of surface modification in order to coat disk-like clay particles. These clay-hydrophobin sandwiches were used for the formation of Pickering Emulsions. It turned out that the synergistic use of clay and hydrophobin resulted in homogenous, long-term stable and tooth-paste like emulsions. The clay particles improved strikingly the rigidity and elasticity of the self-supporting hydrophobin network. Substitution of the clay particles by boehmite needles resulted in similar Pickering emulsions.

Finally, we report that it is possible to replace hydrophobin in combination with clay by other proteins, amphiphiles or surfactants. By adjusting the preparation conditions, the emulsifier concentration or the oil mass fraction one has a versatile tool to obtain Pickering emulsions with the desired properties. A new stabilization mechanism in emulsion science is introduced, supported and confirmed by our results.

1 Introduction

1.1 Hydrophobins

Fungi play an important role in the digestion of dead organic leftovers like leaves, woods or dead insects. For this purpose it is obvious that a huge amount of metabolizing enzymes, like cellulases, have to be produced and secreted by fungi. At the same time it is indispensable that fungi can effectively infiltrate the material to be recycled. Therefore the fungi form apically growing hyphae within the substrate [1]. By branching of the hyphae a wide mycelium can be established. Colonies of the fungus *Armillaria bulbosa* for example are known to form a several hectares big mycelium [2]. In order to fulfill reproduction purposes conidiophores that are derived from aerial hyphae are built up. At all stages of fungal life cycle one special protein group, called hydrophobins, is involved. Hydrophobins have been identified to be part of the aerial hyphae and fruit bodies [3] and consequently as modules of the spore wall [4]. In order to achieve an efficient diffuse of the fungal reproduction structures their water-based dispersal is mediated by hydrophobins [5]. Even fungal attachment to hydrophobic surfaces is realized by hydrophobins [6]. Recently it was shown that hydrophobins also mask the recognition of airborne fungal spores by the immune system [7]. Hydrophobins therefore seem to take over the functions of surfactants in biological systems. The question that immediately arises is what special features make this class of proteins so multivalent and unique?

Hydrophobins are relatively small proteins of about 100 amino acids and were first discovered in the fungi *Schizophyllum commune* in the early eighties of the last century [8]. Studies about their amino acid composition showed that hydrophobins characteristically contain a remarkable amount of hydrophobic amino acids as well as eight cysteine residues [9]. An early amino acid sequence comparison of different hydrophobins led to the classification of class I and class II hydrophobins [10]. Moreover both types differ in the solubility of their aggregates, the so called rodlets [11]. Class I hydrophobin rodlets are much easier to dissolve than those formed by class II hydrophobins. Solvents like trifluoracetic acid are needed in order to dissolve them [12].

Interestingly, beside the obvious differences in the hydropathy patterns and the consequent solubility behaviour, the eight cysteine residues of all hydrophobins are aligned in the same, symmetric way. Only the second and third as well as the sixth and seventh cysteine residue track each other directly in the amino acid sequence. The remaining cysteines are much more delocalized from each other and therefore more isolated according to the primary protein

structure. The consensus sequence of the conserved cysteine pattern is schematically shown in fig 1.1.

$$X_{2\text{-}38}\text{-}\mathbf{C}\text{-}X_{5\text{-}9}\text{-}\mathbf{C}\text{-}\mathbf{C}\text{-}X_{11\text{-}44}\text{-}\mathbf{C}\text{-}X_{8\text{-}23}\text{-}\mathbf{C}\text{-}X_{5\text{-}9}\text{-}\mathbf{C}\text{-}\mathbf{C}\text{-}X_{6\text{-}18}\text{-}\mathbf{C}\text{-}X_{2\text{-}14}$$

Fig. 1.1 *Consensus amino acid sequence of the preserved cysteine pattern. Cysteine residues are abbreviated as C and are separated from each other by a variable number of amino acids indicated by X. Only the cysteine residues two and three as well as six and seven are paired. Figure modified from [13].*

More clarity towards understanding its amphiphilic function was supplied by the first atomic resolution structure of a hydrophobin published in 2004. The crystal structure of the hydrophobin HFBII from *Trichoderma reesei* was resolved with 1.0 Å [14]. The schematic topology (A) as well as the structure (B) are shown in fig. 1.2.

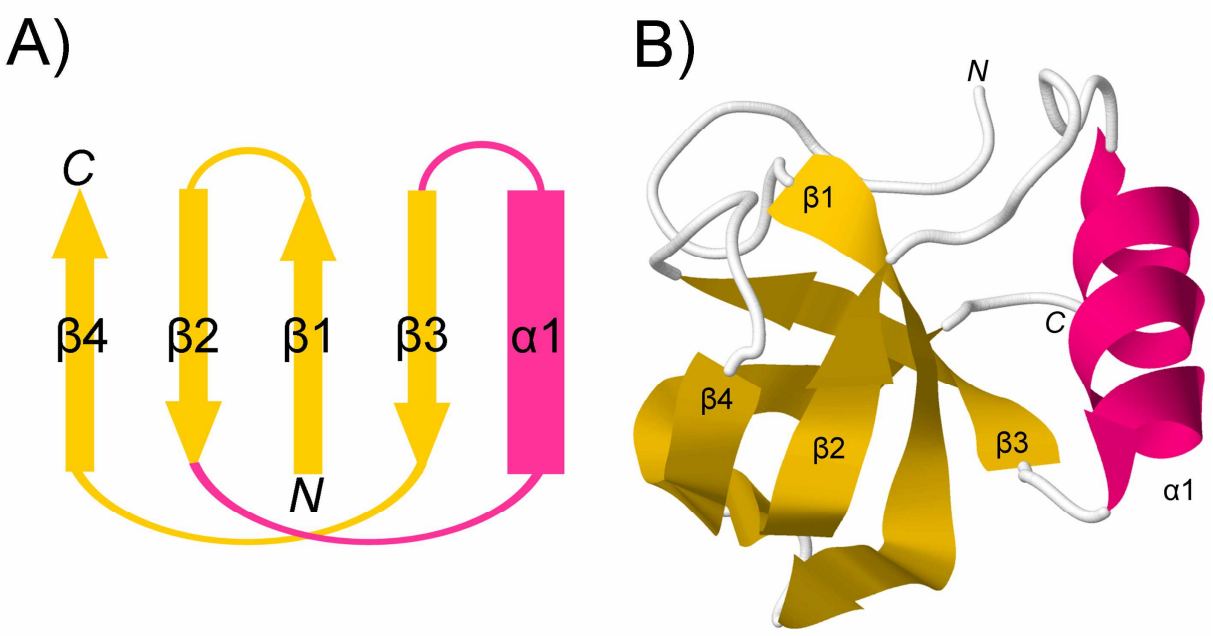

Fig. 1.2 *Topology (A) and structure (B) of the hydrophobin HFBII of* Trichoderma reesei. *Characteristic features of the single domain protein are the four β-sheets (indicated as β1-4) forming a barrel-like structure as well as one α-helix (α1). The topology structure was modified from ref. [14], whereas the tertiary structure was drawn with the software JMol; pdb number of HFBII: 2B97.*

The single domain hydrophobin is of globular shape with a diameter of around 3 nm. The four β-sheets are orientated antiparallel to each other forming a barrel-like structure. A deeper investigation of the tertiary structure of HFB II also pointed out that the rigidity of the globular hydrophobins is provided by the existence of four disulfide bridges. Due to the protein folding even the locally distinct cysteine residues (fig. 1.1) come close to each other in

the three-dimensional state and are now able to form chemical bonds, respectively disulphide bridges. These make the hydrophobins extremely resistant against thermal heating. No sign of denaturation is observed after incubation for 15 min at 90°C [15]. Beside the conserved cysteine residues the primary sequence comparison detected also several preserved hydrophobic amino acids, like valine or leucine residues. In the tertiary protein structure, they built up a characteristic, flat hydrophobic surface patch that makes at least 12% of the total protein surface area [14]. Due to these unique protein structural features, it is obvious that hydrophobins can be considered as rigid bio-surfactants as they provide distinct hydrophobic and hydrophilic properties (fig. 1.3).

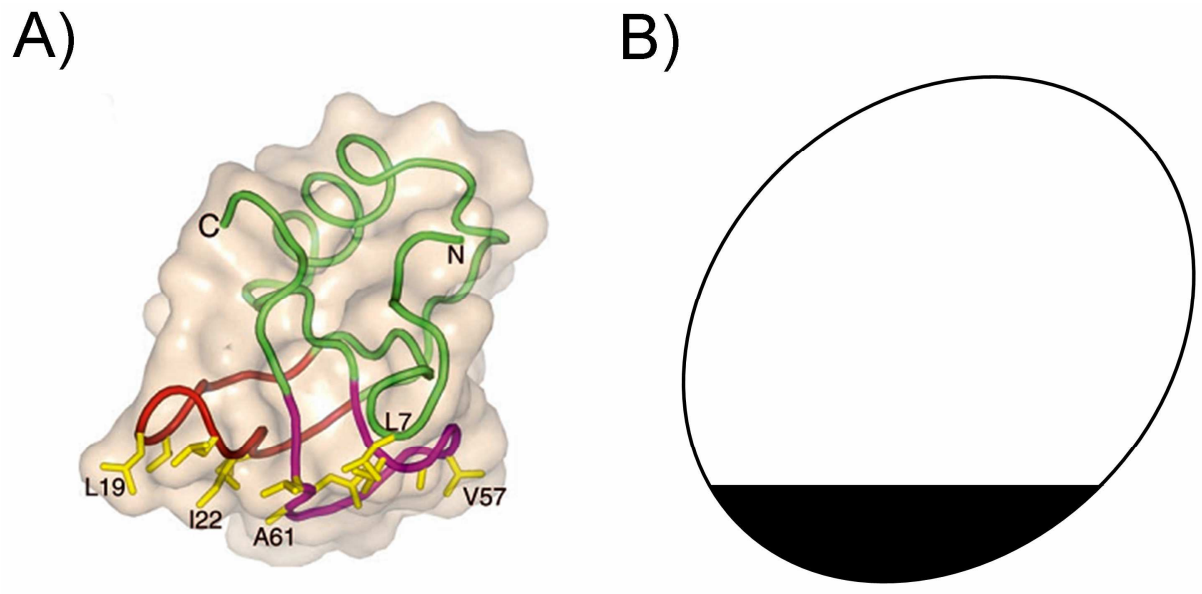

Fig. 1.3 *Illustration of the position of the hydrophobic surface patch in the tertiary structure of HFBII from* Trichoderma reesei *(A). The amino acid residues that are part of the hydrophobic patch are shown in yellow. An abstract figure (B) was drawn in order to point out more clearly the distinct amphiphilic character of hydrophobin. The hydrophobic patch is drawn in black. The tertiary structure (A) was taken from ref. [14].*

Simple surfactants, like Cetyltrimethylammoniumbromide (CTAB), consist of a hydrophilic head group and a lipophilic tail. Due to their amphiphilic nature surfactants are surface active and lower the surface tension of water. Keeping in mind the described, exclusive architecture of hydrophobins, it is evident that even they were discovered early on as surface active agents. In literature hydrophobins are referred to be the most surface active proteins [16]. Furthermore their strong tendency of self-assembly [17], even at interfaces [18], is characteristic for hydrophobins.

Similar to the publication of our first results about the unique properties of emulsions based on hydrophobins (Publication A), the group of A. Lips described analogous findings, that proved our conclusions to be correct. Two outstanding properties of hydrophobins that clearly separate them from any other, classic amphiphilic protein were mentioned by Lips *et al*. Hydrophobins form characteristic self-assembled-bilayers due to the existence of significant attraction energies between the hydrophilic parts of hydrophobin molecules. Interestingly it turned out, that the hydrophobic interaction is more important in the short range distance than the electrostatic interactions [19]. The observation of non-spherical bubbles was correlated with the formation of hydrophobin adsorption monolayers that provide an extraordinary mechanical rigidity [20]. The high shear elasticity of the formed hydrophobin monolayer was explained by its individual construction. Six hydrophobins build up the unit cell [21], very similar to the assembly concept of rigid graphene sheets [22].

Hydrophobins are versatile amphiphilic proteins with very unique properties that offer great potential for industrial purposes, like as emulsifying agent. The use of hydrophobins, however, was always restricted by its low availability. The complex purification from mushrooms is very expensive, because a poor yield of only milligram amounts of hydrophobin can be achieved. Due to the use of white biotechnology, things have changed now. The BASF SE established two recombinantly produced hydrophobins on the market in 2009 [23]. They are called H Star Protein ® A (HPA) and B (HPB). For large scale biotechnically production the construction plan shown in fig. 1.4 proved to be advantageous.

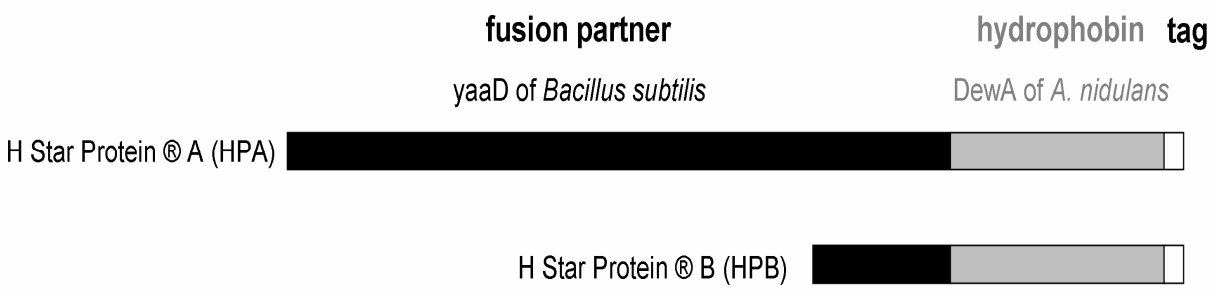

Fig. 1.4 *Schematic design of H Star Protein ® A and B, as well as genomic origin and local arrangement of the used amino acid sequences. H Star Proteins ® A and B differ remarkable in the length of the fusion partner.*

As one can see in fig. 1.4, H Star Proteins ® are a combination of two different genes and a coding sequence for a short histidin purification-tag. The gene *DewA* is the genomic sequence for the hydrophobin from the filamentous fungi *Aspergillus nidulans*. As a fusion partner the coding sequence of a highly expressed protein yaaD from *Bacillus subtilis* was chosen. Therefore H Star Protein ® A and B differ significantly in the length of the *yaaD* gene. This

influences the molecular weight strongly. HPA has a molecular weight of 46 kDa and a charge density of 0.16 e/nm^2, whereas HPB has only 19 kDa and a charge density of 0.10 e/nm^2. Due to the reduced length of the fusion partner, one can conclude that HPB is closer to the natural hydrophobin DewA than HPA is.

1.2 General Remarks on Emulsions

Emulsions are defined as fine dispersions of two immiscible liquids, typically oil and water. Depending on the fluid that makes the continuous phase, emulsions are classified into oil in water (o/w) or water in oil (w/o) emulsions. Emulsions are kinetic but not thermodynamic stable systems. The thermodynamic instability of emulsions is understandable by comparing the free energy ΔG before (G^S) and after the emulsification process (G^E) [24]. The free energies of the water and oil phase are unchanged for G^S and G^E. However the free energy of the emulsion system ΔG increases with the new interfacial area ΔA and is reduced by a much smaller entropic term which origins from the configurational entropic win ($-T\Delta S_E$). This relation can be summarized in equation (1).

$$\Delta G = \gamma \cdot \Delta A - T\Delta S_E \qquad (1)$$

Consequently the most stable system is formed when the water oil interface is as small as possible. This corresponds to the situation where all emulsion droplets have disappeared and both phases, respectively oil and water, are completely separated again. When the interfacial tension γ becomes extremely small, thermodynamically stable systems, known as microemulsions are formed [25]. The phase behavior of microemulsions is controlled by the laws of thermodynamics.

Accordingly the emulsification process for ordinary emulsions is non-spontaneously and the produced emulsions will tend to typical emulsion instability mechanisms. Nevertheless most emulsions for daily life, like skin care products, are stable for weeks, months or years. Kinetic stable emulsions are stabilized by an energy barrier ΔG^*. Similar to an activation energy, ΔG^* has to be sufficiently large in order to prevent the emulsion from achieving the thermodynamic stable state for a reasonable time, which corresponds to a complete phase separation into oil and water again (fig. 1.5).

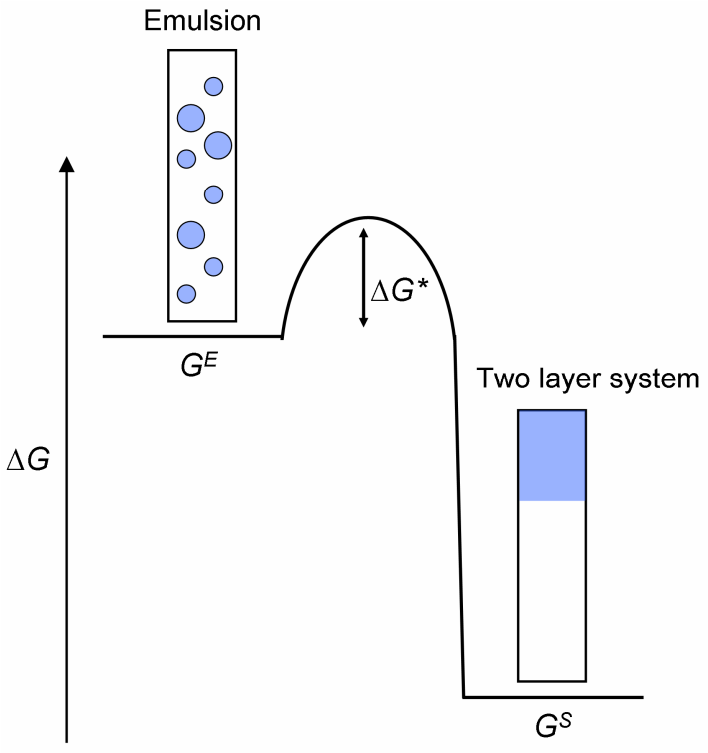

Fig 1.5 *Schematic representation of the kinetic emulsion stability. The free energy of an emulsion* G^E *is higher than the one of a separated, two layer system* G^S. *The existence of a sufficient high enough energy barrier* G^* *is indispensable for emulsion stabilization. Graphic modified from [26].*

Collision events between the emulsion droplets are likely to happen, because they are in constant Brownian motion. The well known DLVO-theory explains the kinetic stability of colloidal systems with an activation barrier. Without an energy barrier ΔG^*, van der Waals attraction leads to irreversible coagulation of particles or droplet coalescence, when droplets or particles come into direct contact with each other. So the life time of an emulsion system increases with the height and the relative thickness d of the barrier ΔG^*, which reduces the probability of direct contact by a factor *f* (2):

$$f = d \cdot \exp(-\frac{\Delta G^*}{RT}) \qquad (2)$$

The emulsion lifetime is proportional to *1/f* and so it is obvious that emulsions may exist with life times of hundred of years. In typical emulsions the activation barrier is a consequence of electrostatic and steric repulsion between charged particles. In many systems this electrostatic repulsion is hindered by high salt or ionic concentrations in the solution.

Here, stabilization of the oil droplets by natural polymers and especially by changing the rheological parameters of the solvent is used. Sufficient yield stress values avoid Brownian contact and therefore coagulation. Nevertheless emulsions may separate even when sufficient high barriers prevent coalescence or flocculation. The most regular forms of emulsion instability are shown schematically in fig. 1.6.

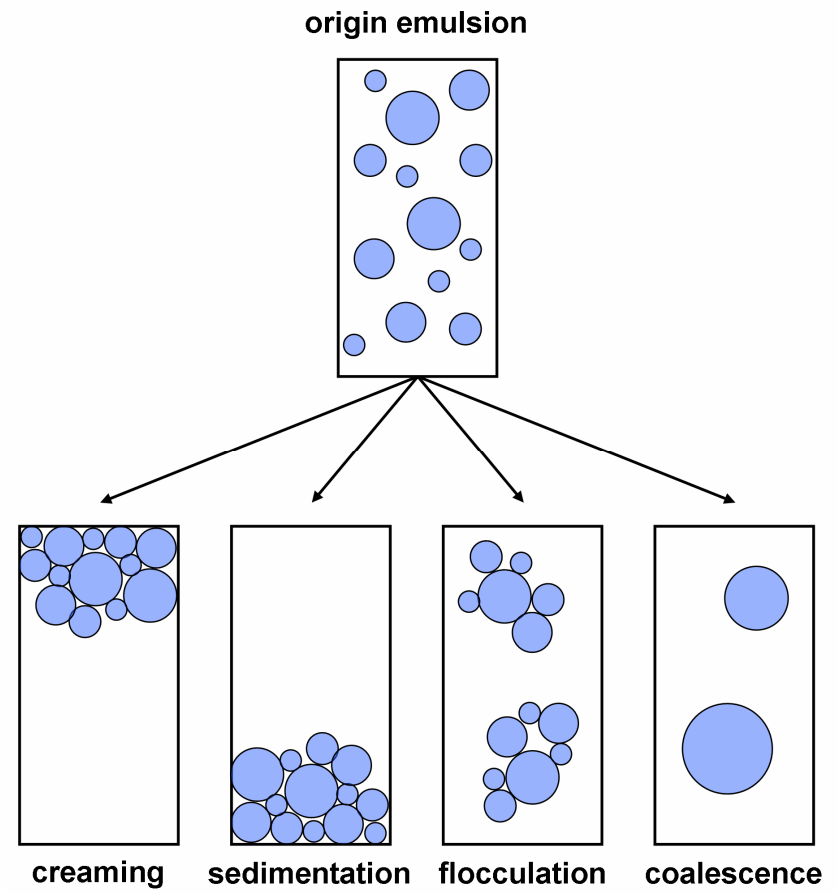

Fig 1.6 *Schematic overview of common emulsion instability mechanisms. A variety of physical mechanism including creaming, sedimentation, flocculation and coalescence can be responsible for a decreasing emulsion stability. Figure modified after [26].*

Stable emulsions neither show measurable changes in their emulsion droplet size or their polydispersity nor in the state of droplet aggregation during the observation or storage period [27]. Creaming and sedimentation are types of gravitational separation that is the result of differences in the density between the emulsion droplets and the continuous phase. Typically, o/w emulsions tend to creaming, whereas w/o emulsions undergo sedimentation. This is because most industrially used oils have a lower density than water. Both, creaming and sedimentation are not accompanied by a change in the emulsion droplet size distribution [28]. Nevertheless most customers do not tolerate the change of commercial products from a homogenous emulsion to a two phase system. Another emulsion instability mechanism is

known as flocculation that means the assembly of emulsion droplets. Obviously between the emulsion droplets may act attractive forces that are responsible for agglomeration. Flocculated systems are obviously more likely to undergo enhanced gravitational separation. No changes in the emulsion droplet size are induced by flocculation, but the visual appearance of the emulsion will probably change. Moreover coalescence is the result of the unification of two or more emulsion droplets to a big one. An increase in the emulsion droplet size in dependence of the incubation time is a clear sign of coalescence. Creaming, sedimentation and flocculation can be supportive to coalescence events, because the droplets are packed more densely. The final step of coalescence is the complete phase separation of an emulsion into its thermodynamical favourable state. An alternative way of emulsion instability, called Ostwald Ripening, occurs in emulsions with big and small emulsion droplets. In those polydisperse emulsions larger droplets form at the expense of the smaller ones, because these larger particles are more thermodynamically stable than smaller particles [29]. Larger droplets however tend to increased gravitational separation again. As one can clearly see, all emulsion instability mechanisms are somehow linked with each other and therefore may occur simultaneously or successively.

Gravitational separation could theoretically easy be prevented by the use of oils that have a density that is like water. In practical applications, however, density matching is not workable or at least extremely restricted by sticking to low levels of adequate oils. Moreover the production of small emulsion droplets is useful in order to reduce creaming and sedimentation, because both are directly proportional to the second power of the emulsion droplet radius. The achievement of a small polydispersity among the emulsion droplets is also very beneficial in order to suppress Ostwald Ripening.

However, without the use of an effective emulsifying agent, all strategies mentioned before are insufficient at all. Homogenized pure oil and water will quickly segregate as the energy barrier ΔG^* is too low. The use of amphiphilic agents, called emulsifiers, leads to an increase of ΔG^* by inducing repulsion forces between the droplets by electrostatic or steric means. During the emulsification process an effective emulsifier has to adsorb rapidly to the new created oil-water interface and prevent the freshly produced droplets from immediate coalescence. A very successful method for emulsion stabilization is the introduction of a yield stress into the system in order to decelerate the movement of the droplets. A decreased emulsion droplet motion can be related to an increased emulsion stability, because the rate of

droplet-droplet collisions will be reduced. Emulsion instability mechanisms (fig. 1.6) are less likely to happen. The repertory of potential emulsion stabilizing ingredients is inexhaustible and complex. Amphiphilic surfactants [30], surface-active polymers [31], colloidal particles [32] or combinations of them [33] are already in use for long times.

Proteins are emulsion stabilizers of special interest. Due to their building blocks, respectively a set of different hydrophilic and hydrophobic amino acids, proteins are amphiphilic compounds. Proteins are surface active agents and lower the surface tension of water [34]. However, in contrast to surfactants proteins, beside ß-casein, do not have a critical micelle concentration (cmc), from which the surface tension stays constant while the surfactant concentration is still increasing.

Anyhow, proteins have a high tendency to adsorb at oil-water interfaces. The thermodynamic driving force for protein interface adsorption is determined by the removal of hydrophobic amino acid residues from the aqueous milieu to the oil-water interface and the synchronous displacement of water molecules from the apolar environment of the oil-water interface [35]. Conformational changes upon interface adsorption are likely to happen as proteins usually mask their hydrophobic regions in a polar medium within the molecule. The final protein conformation at the interface is determined by the balance of various forces including van der Waals attraction and hydrophobic effects [36]. These conformational changes of proteins upon interface adsorption are known in the literature as “surface denaturation” [37]. The extent of these conformational rearrangements is of course protein dependable.
In emulsion science this “surface denaturation” of protein has another consequence that is known as aging [38]. It is conceivable that as a result of the conformational changes, neighboring protein molecules can interact with each other by cross-linking or interpenetration by offering new or alternative contact amino acids. As a consequence a protein film evolves around the emulsion droplets. Accordingly, in contrast to surfactants proteins offer beside an electrostatic stabilization of the emulsion droplets also a mechanical film that can be considered as a steric barrier. These two mechanisms in combination provide an enhanced stability against typical instability mechanisms already discussed before and are also able to introduce yield stress stabilization.

Many proteins, such as milk proteins, ß-caseins or bovine serum albumins (BSA), are known for many decades as emulsifiers and enormous research work about the protein properties under different conditions using a broad range of methods is still carried out. Nowadays the customer demands the replacement of surfactants to more natural emulsifiers. Therefore proteins are suitable candidates. Unfortunately a lot of emulsions based on proteins offer technical disadvantages, like the need of high protein concentrations, a fast phase separation of emulsions or an ultra-sensitivity against extrinsic factors, like pH, temperature or salt.

In summary, the perfect protein emulsifier should fulfill besides a pronounced costumer acceptance following requirements:

- stabilize homogenous emulsions at low concentrations
- providing an electrostatic and mechanical barrier
- might introduce an increased viscosity in the emulsion phase
- available in sufficient amounts and constant quality
- tolerant against storage effects, like temperature fluctuation

Solid particles, which are adsorbed at the droplet, are able to provide a form of stabilizing emulsions which is known as Pickering emulsions [39]. They have already been discovered 1903 by Ramsden [40]. In the last hundred years a broad range of solid materials has been investigated towards its stabilizing properties. Metal oxides, like TiO_2 or ZnO, silica or clays are just a short selection of the used solid particles [41-44]. The emulsion stabilization mechanism of these solid particles is almost identical with the one of amphiphilic molecules.

Of special interest are such Pickering emulsions with clays. Natural or synthetic clays are well structured, colloidal building blocks [45]. The disc-like clay particles have a thickness of only 1 nm. The negative excess charge is a consequence of special ion-substitutions, like Si by Al, Al by Mg and Mg by Li. Alkali-metal ions usually separate the clay building blocks. Solutions of exfoliated clays are low viscous and transparent. Interestingly, solutions of clay undergo an abrupt sol-gel transition with increasing clay concentration [46]. Due to their large surfaces of 1000 m^2/g clays are perfect adsorption substrates for the immobilization of dyes, multivalent cations or surfactants [47].

The advantages of using clay particles as emulsion stabilizing agents were summarized by *Lagaly et al.* [48]:

- relatively small size
- fractions of different particle size available
- able to increase the viscosity of the continuous phase
- clay surface can easily be modified by adsorption

The possibility of surfactants to adsorb on clay particles can also be used for preparing Pickering emulsions. Such emulsions from clay-non-ionic surfactants systems have already been proven to be quite stable [49]. Nevertheless, it seems that nobody tried to replace surfactants by proteins in combination with clays. Furthermore it is well known, that hydrophobin adsorbs to hydrophilic surfaces. In this thesis, therefore, it will be shown, that clay-hydrophobin systems are ideal colloidal stabilizers for the formation of long-term stable, homogenous and gel-like Pickering emulsions. Moreover other combinations of clay-protein as well as the replacement of clay to alumina powder, called boehmite, will be introduced and characterized to their potential as stabilizer of Pickering emulsions.

2 Motivation

Despite the technological considerations and importance of any potential emulsifier molecule, there has been developing an increasing consumer interest and pressure to use more non-toxic and biodegradable ingredients for formulations, like emulsions. Even cosmetic products contain nowadays more and more natural ingredients. As such products have a very high consumer acceptance and demand, companies consequently have a major interest to produce and establish innovative products containing natural compounds.

The aim of my PhD-thesis was to study the physicochemical properties of suitable, new and large-scale available emulsifiers with low toxicity, no skin irritation and enhanced biodegradability for cosmetic use. Of course, proteins are well suited to fulfill the mentioned requirements. Recently developed, in kilogram scale available recombinantly produced hydrophobins from fungal analogous, called H Star Protein ® A and B, aroused our interest.

The first part of my PhD-thesis is about the physicochemical characterization of the newly developed H Star Proteins ®. Thus, the basic analysis included among others the surface and interface behavior as well as their tendency of self-aggregation and film formation.

As the recombinant hydrophobins should be used in cosmetics, the second part concentrates on the emulsion performance of the H Star Proteins ®. Of special interest is the determination of the minimum needed emulsifier concentration that is required to obtain homogenous emulsions. Moreover the rheological properties of the emulsion are discussed.

Pickering emulsions are well known for many decades. However experiments about proteins in combination with clay acting as emulsifying agent have not been carried out at all. As hydrophobins are well known to adsorb to hydrophilic surfaces, like clays or boehmite particles, the resulting hydrophobin coated particles should be tested towards their emulsifying performance.

Finally, my PhD thesis closes with the section about the replacement of hydrophobin in combination with clay to other proteins, polymers or surfactants. The effects on emulsion stability, average droplets size and rheological properties will be discussed.

3 Synopsis

3.1 Physicochemical characterization of H Star Proteins ® (Publication A)

The biotechnically produced hydrophobins, called H Star Proteins ®, were received without any further available information about their properties. Therefore a basic physico-chemical characterization seemed to be indispensable.

H Star Proteins ® A and B, from now abbreviated as HPA (46 kDa; IEP: 6.15) and HPB (19 kDa, IEP: 6.15) are soluble to a concentration of 5 wt%. The pH was determined to 7.95 (HPA), respectively 7.54 (HPB) for 1 wt% solutions of each protein. Both biotechnical hydrophobins have been tested towards their surface and interface activity using the drop volume technique. Both values decrease constantly with increasing protein concentration up to their solubility limit (Publication A, fig. 1). Comparative study of the surface tension profiles for five different proteins confirmed the thesis that hydrophobins are among the most surface active proteins [16]. Hydrophobin HPB was in comparison to five other proteins, like BSA or soy protein, the most surface active one (Publication D, fig. 1). A very important property of the biotechnically hydrophobins is observed by monitoring their time-dependent surface tension behavior (fig. 3.1).

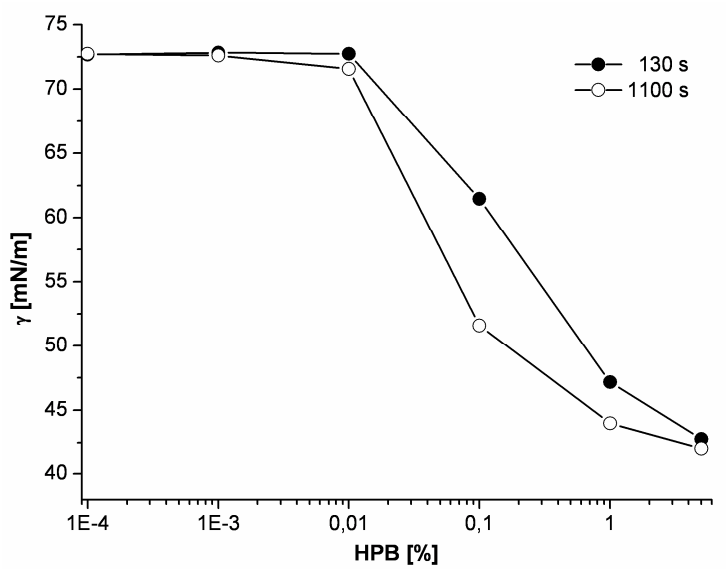

Fig. 3.1 *Time-dependent surface tension profile of HPB. Plotted are the surface tension γ for very short drop formation times (filled symbols: 1s/µl) as well as for very long drop formation times (open symbols: 43s/µl). Modified from Publication A, fig. 2.*

Applying a longer drop formation time obviously leads to a decreased surface tension of HPB (fig. 3.1). In this work, it is also shown that the decrease of the surface tension is a result of the formation of a thin hydrophobin film in the surface layer [17]. As the same time-

dependent effects have been observed for HPA (Publication A, fig. 2), the recombinantly H Star Proteins ® obviously offer the same properties as natural hydrophobins, respectively a distinct surface activity as well as a tendency for interface self-assembly [18]. These experimental results are not self-evident at all. Despite the fact that the fusion partner in the case of HPA is more than three times larger than the actual hydrophobin sequence (fig. 1.4), the natural hydrophobin properties are dominating and are not limited by the fusion partner.

Titration with HCl showed that both, HPA and HPB are negatively charged in aqueous solution. That is in agreement with the isoelectric point (IEP). Increasing the HCl concentration in a 1% HPA solution leads to flocculation around its IEP and to hydrophobin resolubilisation again. Moreover the charge density of the hydrophobins could be determined by titration to 0.16 e/nm^2 (HPA) and 0.10 e/nm^2 (HPB). By titrating the recombinantly hydrophobins with HCl, their charge can be tuned from negative over neutral to positive. This transition is accompanied by a change in the surface tension behavior of the hydrophobin (Publication A, fig. 3).

In general proteins can not only be flocculated by achieving their IEP due to acid-base titration, but also by interaction with surfactants [50] and ions [51]. Treating 1% HPB solutions with the cationic surfactant CTAB and the divalent ion Ca^{2+} led in both cases to flocculation (Publication A, fig. 4). In contrast to CTAB and pH treatment, adding excess Ca^{2+} induced no resolubilisation of the hydrophobin.

By using Cryo-TEM the size of the hydrophobins was resolved as well as explicit signs of membrane fragments were observed. In fig. 3.2 the Cryo-TEM micrograph of a 0.1% HPA solution is shown.

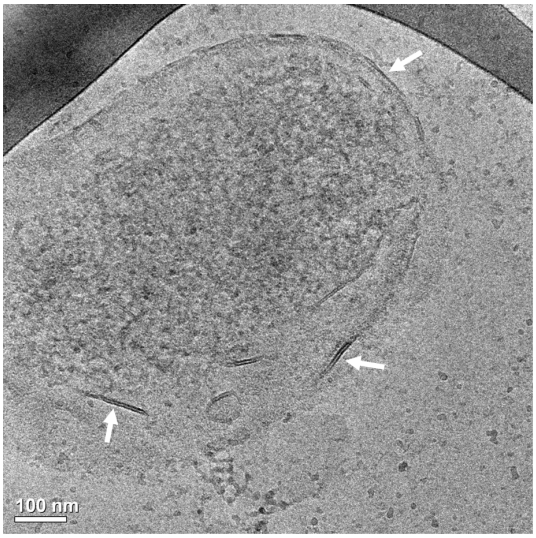

Fig. 3.2 *Cryo-TEM of a 0.1% HPA solution. The size of individual protein molecules is about 5 nm. White arrows indicate the membrane fragment formation built up by assembled hydrophobins (Publication A, fig. 6).*

The Cryo-TEM results also confirm the tendency of the biotechnical hydrophobins to self-assembly and film formation as it is already described for natural hydrophobins [21]. Large hydrophobin aggregates have also been observed with the electric-birefringence technique [52].

Using three different methods, respectively time-dependent surface tension, Cryo-TEM and electric birefringence identified the biotechnical hydrophobins to tend to self-aggregation and film-formation. Interestingly at the same time, *Lips et al.* cleared up the mechanisms of hydrophobin self-assembled bilayers and the nature of the adhesion energy between them [19-20]. These results also confirm our conclusions. The next chapter is about using these unique hydrophobin properties in emulsion science.

3.2 H Star Proteins ® as emulsifiers (Publication A)

The first step in characterization the H Star Proteins ® towards their emulsifying performance was a comparison with simple surfactants, like CTAB. The oil mass ratio was kept at 20 wt%. Both type of emulsifiers form o/w emulsions as determined by conductivity measurements. All samples phase separated, with an upper emulsion layer and a lower aqueous phase. Interestingly the rheology of the emulsion phases stabilized by hydrophobins indicated them to be weak gels, whereas the surfactant based ones were viscous (Publication A, fig. 7).

In order to use the emulsions for cosmetic formulations, the next objective was to obtain homogenous emulsions. Therefore the required minimum amount of oil and hydrophobin had to be determined. It turned out, that with increasing the oil mass fraction Φ to more than 0.65, homogenous emulsions could already be obtained with little as 0.02 wt% HPB (Publication A, fig. 15). Of special technical interest was the observation, that the use of biotechnical hydrophobins as emulsifying agents is not accompanied with the restriction to special oil types. Moreover a broad range of oils, starting from the apolar dodecane over the silicone oil polydimethylsiloxane (PDMS) to the polar oil octylmethoxycinnamate (OMC) could easily be emulsified. An exemplary rheogram of a homogenous emulsion containing 1% HPB and an oil mass fraction Φ of 0.65 dodecane is shown in fig. 3.3.

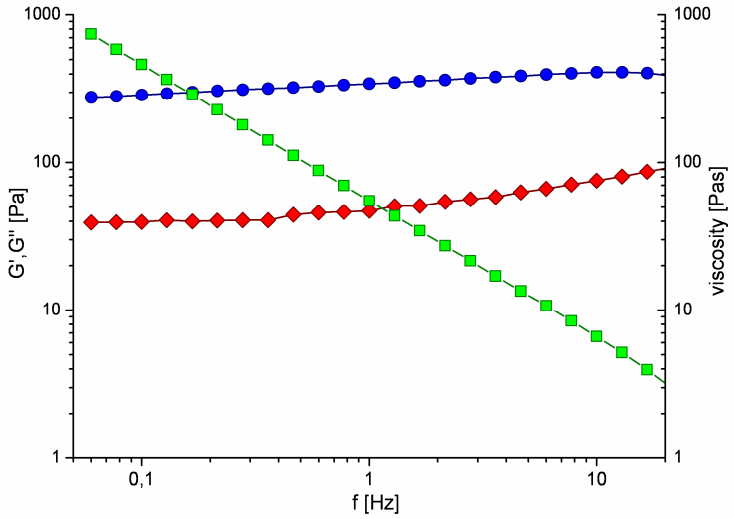

Fig. 3.3 *Rheogram (τ=0.5 Pa) of the emulsion containing 1% HPB and an oil mass fraction Φ of 0.65 dodecane measured directly after preparation. The emulsion was prepared at a shear rate of 9000 rpm. Blue: Storage modulus G' [Pa], red: Loss modulus G'' [Pa] and green: Viscosity η [Pas]. Data used in Publication A, fig. 12.*

The rheogram shown in fig. 3.3 obviously indicates the emulsion to be gel-like. Both, the storage modulus G' as well as the loss modulus G'' are frequency independent. The viscosity decreases linearly.

While the emulsions prepared under these conditions did not show any instability mechanisms, the elastic properties increased with further incubation at room temperature. A distinct ripening of the emulsion layer was observed, the storage modulus G' increased almost three times within seven days (Publication A, fig. 13). This effect can be explained by the time-dependent evolution of the three-dimensional hydrophobin network that forms around the emulsion droplets. The hydrophobin molecules do not loose their ability of self-assembly due to interface adsorption. As indicated already by time-dependent surface tension measurements (fig. 3.1), the hydrophobins undergo conformational rearrangements at the interface followed by partial entanglement of the adsorbed hydrophobin molecules. The network stiffens with time as proven by the increase of the storage modulus. The emulsion stability mechanism provided by hydrophobin is therefore quite unique and new. Obviously the hydrophobin coated emulsion droplets attract each other in the short range [20]. Actually attraction of emulsion droplets promotes typical emulsion instability mechanisms, like coalescence. This is evidently not the case here. A hydrophobin matrix evolves without changing the visual appearance of the emulsion.

An additional experiment was performed in order to confirm the described network formation. By long term cabinet drying of an emulsion based on hydrophobin, the oil and water was removed. Afterwards REM-microscopy of the obtained light material was performed (fig. 3.4).

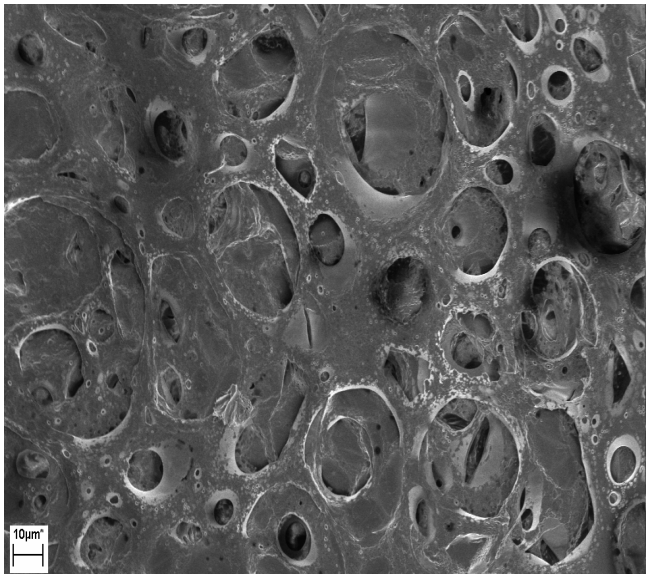

Fig. 3.4 *REM micrograph of the drying residue of an emulsion containing 1% HPB and an oil mass fraction Φ of 0.65 dodecane. The bar represents 10 µm (Publication A, fig. 14).*

Fig. 3.4 reminds of a sponge-like, three-dimensional structure. The hole size is identical with the determined emulsion droplet size before starting the drying process (Publication A, tab. 1).

An additional indication of a processed emulsion droplet aggregation was obtained by using computer tomography (CT). Aggregates with a typical size of 200 µm were resolved (Publication A, fig. 9). Emulsions based on the H Star Proteins ® are gel-like and have a yield-stress due to the existence of hydrophobin coated oil droplets. These sticky particles form a three-dimensional network within the emulsion. Finally the dilution of homogenous emulsions with water showed, that the emulsion phases contracts again. Obviously the emulsion droplets attract each other. Obtaining homogenous emulsions with less than 65 wt% oil could be realized by introducing a yield stress into the emulsions.

For a better scientific understanding as well as for industrial processing, the influence of extrinsic factors like heating, adding glycerole or applying different shear rates while homogenization is of big interest.

Heating a freshly prepared emulsion did neither change the visual appearance nor the emulsion droplet size, but led to a doubling of the storage modulus G' (Publication A, fig. 11). This effect can be explained by the accelerated stiffening of the hydrophobin film in the emulsion matrix. Consequently no aging effects could be detected for the heated emulsion. Moreover it can be concluded that hydrophobins emulsifying performance is not affected by heating, the emulsion remained in its homogenous state.

The emulsions prepared from hydrophobin and dodecane had a white visual appearance. Matching the refractive index by adding increasing amounts of glycerole is a well known method in order to get samples transparent [53]. A certain improvement of the emulsions transparency was obtained by adding 40-60 wt% glycerole, however the samples did not get completely transparent. This effect might also be related to the network formation, as big aggregates will scatter the light more effectively.

By applying a sufficient shear rate, the emulsion droplet size achieves values that are near to theoretical ones determined with the core shell model (Publication A, tab. 2, equation 2). Furthermore a decreased emulsion droplet size is related to an increased elasticity of the hydrophobin network (Publication A, fig. 12). A smaller average droplet size achieved at a constant oil mass ratio results in a higher amount of droplets. These droplets will have more connections and interactions with each other, what consequently results in higher storage moduli.

In summary, emulsions prepared from the H Star Proteins ® provide long-term emulsion stability by the formation of a three-dimensional network with the emulsion droplets trapped inside. As the HPB is more related to natural hydrophobins, we exclusively used it in the following studies.

3.3 H Star Protein ® B in combination with solids (Publication B & C)

Clays are used in cosmetics as rheological additives. In combination with non-ionic surfactant they have been used in order to prepare Pickering emulsions [49]. In this study, the adsorption of hydrophobin to clay is evaluated as well as both particles are tested towards their ability to stabilize Pickering emulsions.

Despite the fact that both, HPB and clay possess a negative excess charge, they obviously bind to each other. Mixtures of the two compounds are turbid (Publication B, fig. 1). As already mentioned, hydrophobins can bind to hydrophilic surfaces. The adsorption of HPB to clay, respectively a Laponite was evaluated qualitatively by Cryo-TEM (Publication B, fig. 4) as well as quantitatively by surface tension measurements (Publication B, fig. 2). 0.5 wt% clay can bind three times as much HPB (Publication B, fig. 3). This makes it conceivable that not all of the hydrophobin is arranged in monolayers at the two sides of the disc-like clay particles, but also in bi- or multilayers [19]. The hydrophobin coated clay particles can therefore be assumed as sticky or amphiphilic sandwiches.

Emulsions stabilized by HPB or clay alone and prepared with the high pressure emulsifier at 1000 bar are inhomogeneous systems. However, using both, clay and HPB synergistically as emulsifying agents resulted in Pickering emulsions with amazing tooth-paste, gel-like properties (fig. 3.5).

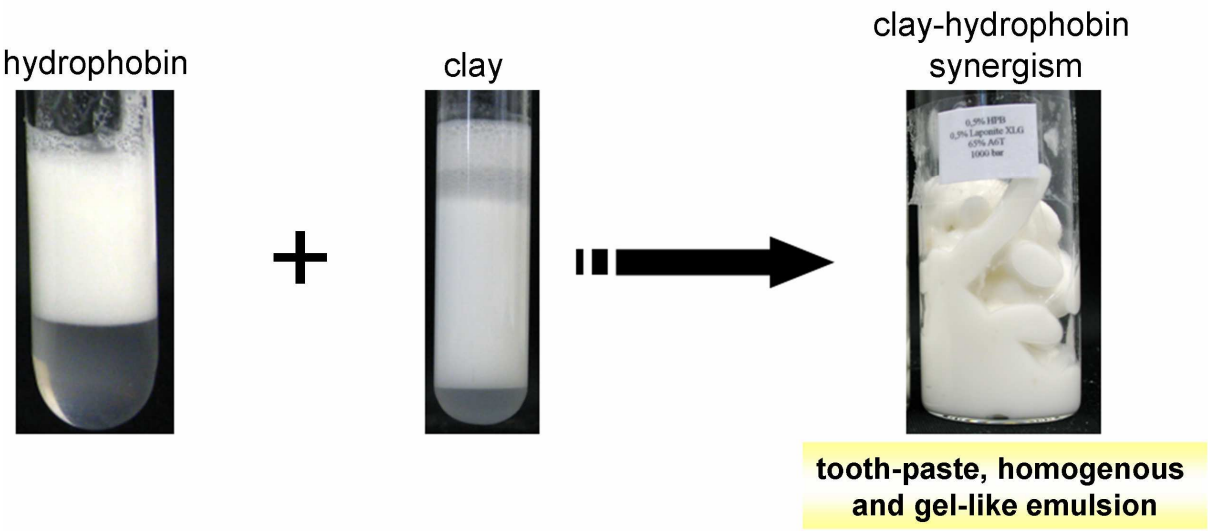

Fig. 3.5 *Synergistic emulsifying action of HPB and clay results in tooth-paste, homogenous and gel-like emulsions. The shown one day old emulsions contained 0.5 wt% of HPB or/and 0.5 wt% clay, the oil mass fraction Φ was fixed at 0.65 PDMS. Homogenization was carried out with the high pressure emulsifier (Publication B, modified graphical abstract).*

The emulsion prepared from 0.5 wt% hydrophobin is a two layer system (fig. 3.5, left). This may at first be counterinductively, but one has to be aware of the used high pressure of 1000 bar. The produced fresh oil interface is too large to be saturated completely by the existing hydrophobin particles. Coalescence events are likely to happen, the average droplet size increases until the oil droplets can be sufficient stabilized by hydrophobin. By using lower pressures or increasing the hydrophobin concentration homogenous emulsions could be obtained. Furthermore the emulsion stabilized by clay (fig. 3.5, middle) is a three layer system, with an upper oily phase, a lower aqueous phase and the emulsion phase trapped in the middle. Nevertheless, very stable, homogenous and gel-like emulsions could be obtained by the synergistic use of clay and hydrophobin (fig. 3.5, right). These emulsions provide long-term stability. In laboratory the hydrophobin-clay stabilized Pickering emulsions are already stable for more than nine months at room temperature without showing any signs of typical emulsion instabilities.

Using oscillating rheological measurements the properties of the Pickering emulsion stabilized by the hydrophobin clay synergism (fig. 3.5, right) were determined (fig. 3.6).

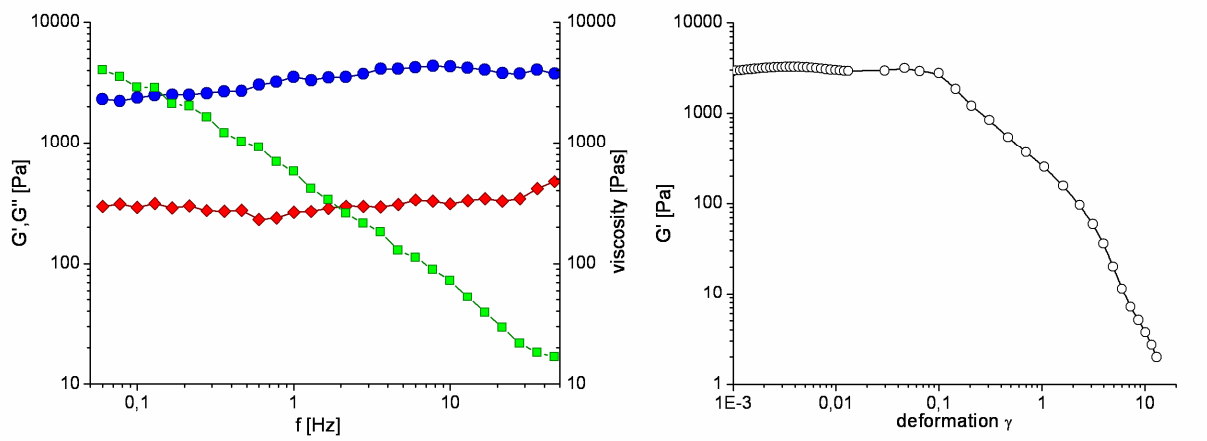

Fig. 3.6 *Rheological characterization of a Pickering emulsion based on the synergistic emulsifying action of 0.5 wt% hydrophobin and 0.5 wt% clay containing 65 wt% PDMS. Left side: Rheogram (τ = 0.05 Pa) with the colour code: blue: Storage modulus G' [Pa], red: Loss modulus G'' [Pa] and green: Viscosity η [Pas]. Right side: Storage modulus G' in dependency of the applied deformation γ is evaluated. The rheological properties were obtained one day after emulsification at 1000 bar. Modified from Publication B, fig. 6.*

Both the storage modulus G' as well as the loss modulus G'' are frequency independent (fig. 3.6, left). Moreover G' is an order of magnitude higher than G'' indicating the gel-like properties of the Pickering emulsions. Furthermore the storage modulus is with 2000 Pa much higher than it should be if it would be determined by the number density of the oil droplets

(Publication D, supplementary information). It is interesting to note that the network can be stretched to 10% before it breaks (fig. 3.6, right).

One could also argue that the gel-like properties are due to the sol-gel transition [46] of the clay in the aqueous phase. Anyway the used clay concentration is far away from its sol-gel transition. Consequently one can conclude that the storage modulus is determined by the elasticity of the hydrophobin network that is stiffened by the clay particles. Cryo-SEM investigations also confirmed that most of the clay particles are located at the interface of the emulsion droplets (Publication B, fig. 7). The self-supporting network was also investigated by SEM. Therefore a Pickering emulsion was freeze dried, the remaining network did not collapse (Publication B, fig. 9). A conceptual diagram of this self-supporting, three dimensional network between clay and proteins has been suggested (fig. 3.7).

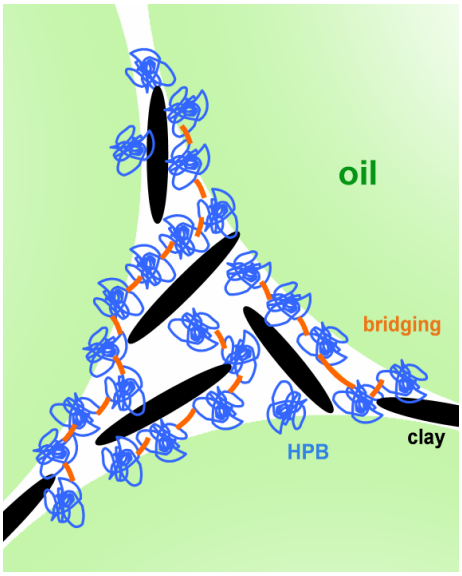

Fig. 3.7 *Conceptual diagram of the self-supporting, three dimensional network formed by the hydrophobin coated clay particles. The HPB molecules (blue) adsorbed on the clay (black) interact (orange) with each other (Publication B, fig. 10).*

The hydrophobin coated clay particles obviously act as stiffener of the self-supporting network formed by the hydrophobin molecules. The bridging shown in fig. 3.7 should indicate the self assemble tendency of hydrophobin.

Extrinsic factors like storage-time dependent aging (Publication B, fig. 8), shear influence (Publication B, tab. 1) and oil polarity did have similar effects as in the case of using hydrophobin exclusively as emulsifier (Chapter 3.2). This observation is not surprising as the hydrophobin is located at the oil-water interface and does obviously not loose its typical

emulsifying properties. Interestingly the needed oil mass fraction in order to obtain homogenous emulsions could be decreased from 0.65 to only 0.3 due to the combinatory use of clay and hydrophobin (Publication B, fig. 13).

By adding corresponding amounts of HCl to HPB, the charge of the protein can be reversed, respectively from negative to positive. In mixtures of clay and charge reversed HPB also precipitation was observed. In order to evaluate the differences of Pickering emulsions prepared with the same conditions, but using negatively or positively charged HPB, comparative rheological measurements have been performed. Interestingly the storage modulus G' in the case of charge reversed protein was considerably lower compared to the one obtained using negatively charged HPB (Publication B, fig. 14). This observation was explained by the stronger binding of the charge reversed HPB to the clay. As a consequence the hydrophobin-hydrophobin interactions that also determine the strength of the three-dimensional network became weaker.

A detailed comparison of the clay hydrophobin stabilized Pickering emulsion to others is given in Publication B. Unfortunately most available studies did not pay much attention to the rheological properties of the emulsions [49,54].

The described novel clay-hydrophobin synergism provides a fascinating, long-term emulsion stabilisation mechanism, although using low concentrations of emulsifier (≤1wt%). The emulsion droplets are prevented from typical emulsion instability mechanisms by being trapped in a self-supporting, three-dimensional hydrophobin network that is stiffed by clay particles.

A next step of the work presented in this PhD-thesis included the replacement of clay to boehmite particles. Boehmite is a γ-AlO(OH) and possesses in contrast to clay a cationic surface. Before using hydrophobin in combination with this alumina powder, a short basic characterization of it was performed. The anionic exchange capacity (aec) was determined to 50 meq/100g (Publication C, fig. 1). In contrast to clays, boehmite particles do not show a sol-gel transition in the concentration range 0.5 – 10 wt% (Publication C, tab. 1). Cryo-TEM observations revealed the structure of the boehmite particles as needle-like with a size distribution of 10-50 nm (Publication C, fig. 4). In contrast to clay, boehmite can bind not more than equal amounts of hydrophobin (Publication C, fig.3 and tab.2).

Beside the fact, that boehmite and clay particles have distinct different features, it was possible to obtain a similar synergism with the use of hydrophobin in combination with both solid particles (Publication C, fig. 5). The consequences of the synergistic use of hydrophobin and boehmite to the storage moduli of the emulsions are shown in fig. 3.8.

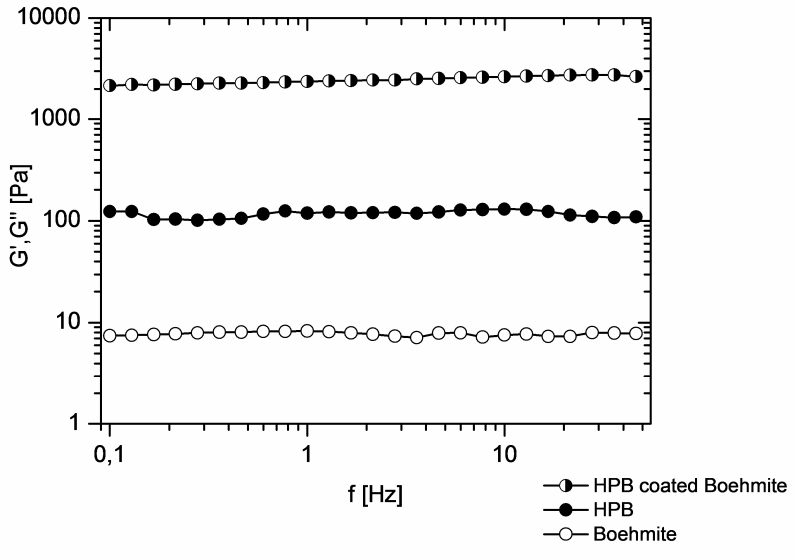

Fig. 3.8 *Trend of the storage moduli G' [Pa] of emulsions prepared from 0.5 wt% Boehmite (open circles), 0.5 wt% HPB (closed circles) and 0.5wt% HPB and 0.5 wt% Boehmite (half-closed circles) acting as emulsifying agents. The emulsions contained 65 wt% PDMS as an oil. The data was obtained one day after emulsification at a shear stress τ of 0.05 Pa (Publication C, fig. 6).*

The storage modulus G' for the synergistic use of boehmite and hydrophobin is 20 times higher than the one of HPB alone and even more than 200 times than the one of boehmite. Obvisously there is a huge synergistic effect. Similar effects have been observed in the case of using clay and hydrophobin (Publication B, fig. 6).

Cryo-SEM investigations of the Pickering emulsions stabilized by HPB coated boehmite showed that the needle-like structures are also located at the interface (Publication C, fig. 7). Moreover due to the high oil content of 65 wt%, the emulsion droplets are in close contact to each other. Entanglement of the hydrophobin coated droplets is also supported by these spatial limitations. Furthermore the Cryo-SEM pictures confirmed an average emulsion droplet size of 1 µm, the polydispersity was quite low. Interestingly the size of the emulsion droplets is half as big as the one obtained by the use of clay. This could due to the fact that the clay particles are disc-like particles and are not as flexible as the needle-like boehmite

particles. Moreover the non-adsorbed hydrophobin concentration is higher in the case of boehmite. Therefore more HPB molecules are available to stabilize smaller oil droplets.

As expected, the Pickering emulsions showed similar aging effects (Publication C, fig. 8), a comparable shear rate dependency (Publication C, tab. 3), independency towards the used oil type and an amazing long term stability (Publication C, tab. 4) as the ones prepared from clay and hydrophobin.

Preliminary, unpublished experiments using hydrophobin coated silica particles as stabilizers of Pickering emulsions also resulted in gel-like, homogenous properties. Therefore, we conclude that the synergistic use of hydrophobin and solid particles is a new way in providing long term-stability of Pickering emulsions using low emulsifier concentrations. Due to the formation of the self-supporting, three dimensional network that is stiffed by the solid particles, there is no need of the addition of ingredients, that increase the viscosity of the emulsions.

3.4 Replacement of H Star Proteins ® to other amphiphiles

Finally the consequences of replacing hydrophobin in combination with clay to other amphiphilic compounds have been investigated.

3.4.1 Proteins (Publication D)

Other commercially available proteins, respectively soy protein, bovine serum albumin (BSA), wheat protein (Plantasol W) and yeast extract, have been tested for their surface activity and emulsifying properties in comparison to hydrophobin.

All used proteins lowered the surface tension of water (Publication D, fig. 1). As expected, the biotechnically produced hydrophobin HPB turned out to be the most surface active protein used in our study. The surface tension lowering ability decreased from HPB over soy protein isolate, Plantasol W, BSA to yeast extract (Publication D, tab. 1).

In order to compare the emulsifying properties of the five proteins, emulsions were prepared under the same conditions, but varying the protein type. The obtained emulsions were analyzed for their visual appearance, stability, emulsion droplet size and rheological properties. All emulsions were of the o/w type. The most unstable emulsions were prepared from Plantasol W and yeast extract (Publication D, fig. 2), showing an immediate phase separation after preparation. BSA and HPB based emulsions did phase separate within three days. Additionally the BSA based emulsion showed signs of creaming. The only stable emulsion was prepared from soy protein isolate (fig. 3.9).

Fig. 3.9 *Emulsions containing 50 wt% PDMS stabilized by 0.5 wt% of different proteins. The upper row shows the emulsion directly after preparation, while the lower row demonstrates the situation after 3d incubation at room temperature. The dotted line in the Plantasol W based emulsion is as guide for the eye in order to demonstrate the phase boundary. The filling level is different for technical reason (Publication D, fig. 2).*

Similar trends as observed for the visual appearance have been detected in the time-dependent behaviour of the average emulsion droplet size (ads) (Publication D, tab. 2). The ads stayed constant in the case of using soy protein and HPB, what indicated the emulsions to be stable against coalescence. Similar trends were identified by evaluating the rheological properties of the emulsions (Publication D, fig. 3). Gel-like emulsions that undergo aging effects were obtained in the case of using soy protein or HPB. It has to be noted that the increase of the storage modulus in the case of BSA was a consequence of the oil squeezing (Publication D, fig. 4). In conclusion the soy protein isolate did have the best emulsifying abilities of all used proteins.

Accordingly all proteins have been used in combination with 0.5 wt% clay in order to stabilize Pickering emulsions. Due to the synergistic use of protein and clay, an enormous raise in the emulsification ability of yeast extract and Plantasol W could be observed (Publication D, fig. 5). Moreover the emulsion prepared from HPB and soy protein in combination with clay did not longer phase separate with increasing time. By raising the oil mass fraction Φ from 0.5 to 0.65, all protein-clay combinations resulted in homogenous emulsions (Publication D, fig. 7). The smallest average droplet size could be obtained with the combination of BSA and clay (Publication D, tab. 5), whereas the most stable, gel-like Pickering emulsions were produced from the combination HPB and clay (fig. 3.10).

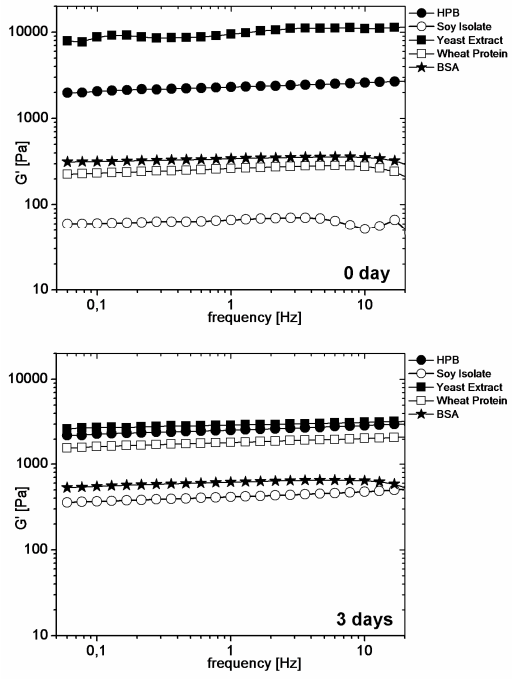

Fig. 3.10 *Trend of the storage moduli G' (τ=0.5Pa) evaluated in dependency of the storage time, respectively directly after preparation and after three days incubation at room temperature. All Pickering emulsions contained 0.5 wt% protein, 0.5 wt% Laponite XLG and an oil mass fraction Φ of 0.65 PDMS and were prepared at 300 bar (Publication D, fig. 8).*

As one can conclude from fig. 3.10, it is possible to obtain gel-like Pickering emulsions containing high amounts of oil by using 0.5 wt% protein and 0.5 wt% clay. All Pickering emulsions showed aging effects due to the time-dependent stiffening of the three dimensional protein films. The absolute value of G' is depending on the used protein. The most gel-like emulsion is prepared from clay and yeast extract (fig. 3.10, upper row). However, the emulsion prepared from yeast extract was extremely prone to microbial growth. This is also proven by the weakening of its storage moduli after three days (fig. 3.10, lower row).

Surely the most stable, homogenous Pickering emulsions can be prepared from the synergistic use of hydrophobin and clay, but nevertheless it is possible to replace it by other proteins. By varying the protein in combination with or without clay and by adjusting the oil mass fraction Φ, one has a very effective tool to fine-tune the desired emulsion properties.

3.4.2 Polymers and Surfactants (Publication E)

As the previous section was about replacing hydrophobin in combination with clay by other proteins, it is only logical to check the influence of replacing proteins by polymers and surfactants.

With the exception of anionic surfactants, like Sodium-Dodecylsulfate (SDS), all other types of surfactants or polymers bind to clay particles. The concentration-depending adsorption of surfactants to clay particles can be monitored by surface tension measurements. A schematic surface tension profile was recently suggested (Publication E, fig. 1). Even non-ionic surfactants bind to clays [55]. This adsorption has to be the consequence of hydrophobic interaction between both particles. Consequently clay particles have to be somehow hydrophobic. Nevertheless soluble clay particles are not surface active. This obvious antagonism can be explained by considering the low interface concentration of the clay particles. Consequently the observed surface pressure is too low for significantly influencing the surface tension (Publication E, theoretical part).

The used amphiphiles in this study were:

- cationic surfactant C16-Trimethylammoniumbromide (CTAB)
- non-ionic surfactant Isotridecyloctaethylenglycolether ($C_{13}O_8$)
- zwitterionic surfactant Tetradecyldimethylaminoxide (TDMAO)
- non-ionic triblock copolymer Pluronic F38
- polyvinylalcohol (PVA)

- polyvinylpyrrolidone (PVP)
- poly-diallyldimethylammoniumchloride (DADMAC)

Pickering emulsions containing 0.5 wt% amphiphile, 0.5 wt% clay and an oil mass fraction Φ of 0.5 PDMS have been prepared (fig. 3.11).

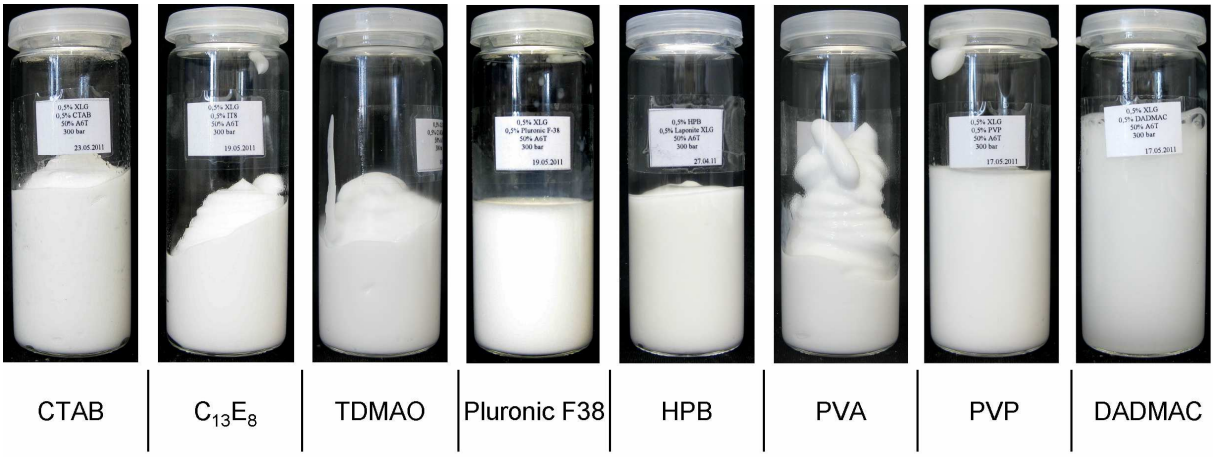

Fig. 3.11 *Pickering emulsions from different 1:1 mixtures of amphiphile and clay prepared at 300 bar. All samples contained 0.5 wt% amphiphile, 0.5 wt% Laponite XLG and 50 wt% polydimethylsiloxane (PDMS) and were photographed after 1 d (Publication E, fig. 2).*

Interestingly all combinations of clay and amphiphile, beside the one prepared from DADMAC, result in homogenous emulsions (fig. 3.11). Moreover the samples with CTAB, $C_{13}O_8$, TDMAO, HPB, PVA and PVP did not form a smooth, horizontal meniscus, indicating their gel-like properties. Especially the toothpaste like performance of the Pickering emulsion prepared from PVA can evidently be seen. A deeper comparison of these Pickering emulsions, containing the storage modulus G' (τ = 0.5 Pa and f = 1 Hz) and the needed shear stress τ [Pa] to break the elastic behaviour (if present) of the emulsion was performed (Publication E, tab. 3). After two month incubation at room temperature only the Pickering emulsions based on TDMAO and Pluronic F38 did phase separate, all other Pickering emulsions did not show any instability mechanisms.

The origin of the gel-like properties in the case of using hydrophobin and clay synergistically have been discussed already (Chapter 3.3). In the case of other polymers or surfactants it was shown that the surface coverage of the clay particles heavily influences the gel-like properties of the corresponding Pickering emulsions. While adding small amount of surfactant can boost the emulsifying properties of the solution, high amounts decrease them rapidly (Publication E, fig. 5 and 6).

In this section it was shown that almost all used amphiphilic compounds bind on negatively charged clay platelets. Stable Pickering emulsions can be prepared from samples with 50 % water and 50 % oil with as little as 0.5 % clay and low amounts of amphiphile. The Pickering emulsions are of the o/w-type and have gel-like properties. The shear modulus of these phases can be varied between a few Pascal and several thousand Pascal for small changes in the composition on the clay surface.

The synergistic use of clay and amphiphile is a novel emulsifying method that was introduced as a main part of this PhD-thesis. The gel-like properties were not a consequence of the sol-gel transition of the clay particles, as their concentration was quite far away from this point. Moreover the formation of a three-dimensional amphiphile network that is strengthened by the clay particles guarantees the long term stability of the obtained Pickering emulsions.

3.5 Perspectives

In summary the stability of Pickering emulsions is increased when proteins and clays are used synergistically. So the systems seem to be appropriate for various, different applications. However proteins are prone to microbial digestion. It is shown that this effect can be prevented by the replacement of proteins by surfactants or polymers. So a broad area for applications in various fields is now opened by our pioneer work.

4 Abbreviations and Symbols

ads	average droplet size
aec	anionic exchange capacity
BSA	Bovine Serum Albumin
cmc	critical micelle concentration
Cryo-TEM	cryogenic transmission electron microscopy
CT	computer tomography
CTAB	cetyltrimethylammoniumbromid
DADMAC	poly-diallyldimethylammoniumchloride
HPA	H Star Protein ® A
HPB	H Star Protein ® B
IEP	isoelectric point
OMC	octyl-methoxycinnamate
PDMS	polydimethylsiloxane
PVA	polyvinylalcohol
PVP	polyvinylpyrrolidone
rpm	revolutions per minute
SDS	sodium-dodecylsulfate
SEM	scanning electron microscopy
TDMAO	tetradecyldimethylaminoxide
TEM	transmission electron microscopy
Φ	oil mass fraction

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6 List of publications

All experiments of the manuscripts have been performed by me. My supervisor H. Hoffmann planned the experiments together with me. T. Sekine, T. Okamoto and K. Watanabe discussed the results and gave financial support.

Publication A

Martin Reger, Tomoko Sekine, Tohru Okamoto and Heinz Hoffmann, Unique emulsions based on biotechnically produced hydrophobins, *Soft Matter*, **2011**, 7, 8248-8257.

Publication B

Martin Reger, Tomoko Sekine, Tohru Okamoto, Kei Watanabe and Heinz Hoffmann, Pickering Emulsions stabilized by novel clay-hydrophobin synergism, *Soft Matter*, **2011**, 7, 11021-11030.

Publication C

Martin Reger and Heinz Hoffmann, Hydrophobin coated Boehmite Nanoparticles stabilizing oil in water emulsions, *J. Colloid Interface Sci.*, **2011**, doi: 10.1016/j.jcis.2011.10.050.

Publication D

Martin Reger, Tomoko Sekine and Heinz Hoffmann, Boosting the Stability of Protein Emulsions by the synergistic use of Proteins and Clays, *Colloid and Polymer Sci.*, **2011**, recommended for publication after revision.

Publication E

Martin Reger, Tomoko Sekine and Heinz Hoffmann, Pickering Emulsions stabilized by Amphiphile covered Clays, *Colloids and Surfaces A*, **2011**, accepted, Ms. Ref. No.: COLSUA-D-11-00925.

Publication F

Japanese patent, Oil in Water emulsion in cosmetic, **2011**

Patent about emulsions and Pickering emulsions that are stabilized by protein or protein clay synergism. My name is included as inventor #3.

Publication A

Martin Reger, Tomoko Sekine, Tohru Okamoto and Heinz Hoffmann, Unique emulsions based on biotechnically produced hydrophobins, *Soft Matter*, **2011**, 7, 8248-8257.

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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 $\mu l\ s^{-1}$. 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

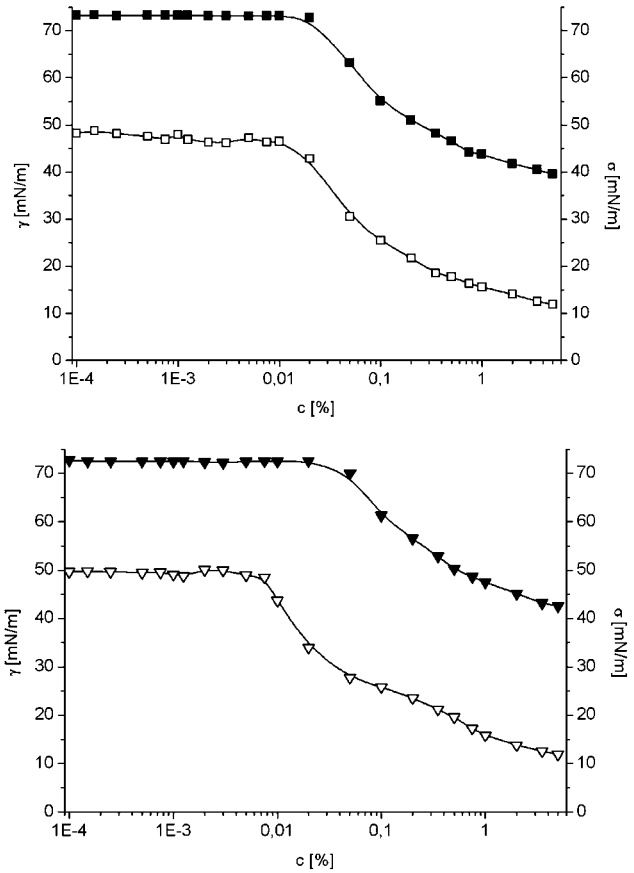

Fig. 1 Surface and interface tension against decane for increasing concentrations of HPA (squares) and HPB (triangles) determined by the drop volume technique. Filled symbols: surface tension γ; open symbols: interface tension σ.

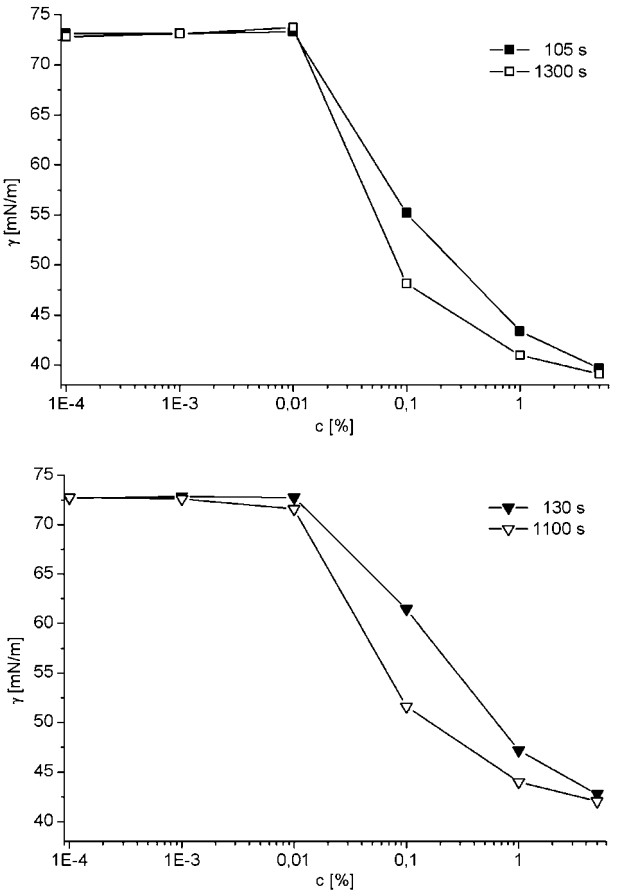

Fig. 2 Time-dependent surface tension profile for HPA (squares) and HPB (triangles). Plotted are the surface tensions γ for very short drop formation times (filled symbols: 1 s μl^{-1}) as well as for very long drop formation times (open symbols: 43 s μl^{-1}).

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]

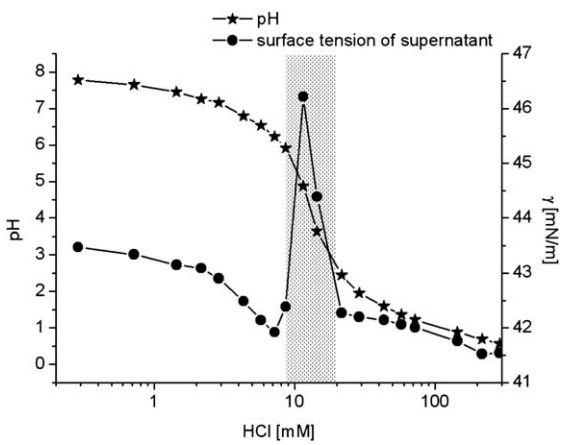

Fig. 3 Plot of pH (stars) and surface tension γ (circles) of 1% HPA (IEP: 5.65) solutions against HCl concentration (mM). The shaded area indicates the HCl concentrations where HPA shows strong flocculation.

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

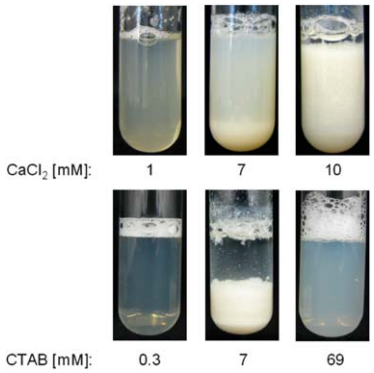

Fig. 4 Solutions of 1% HPB with increasing amounts of $CaCl_2$ and cationic surfactant CTAB. Excessive protein flocculation takes place at 10 mM $CaCl_2$ and 7 mM CTAB, respectively. Adding excess CTAB leads to HPB resolubilisation, whereas the flocculated state remains even at higher $CaCl_2$ concentrations.

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.

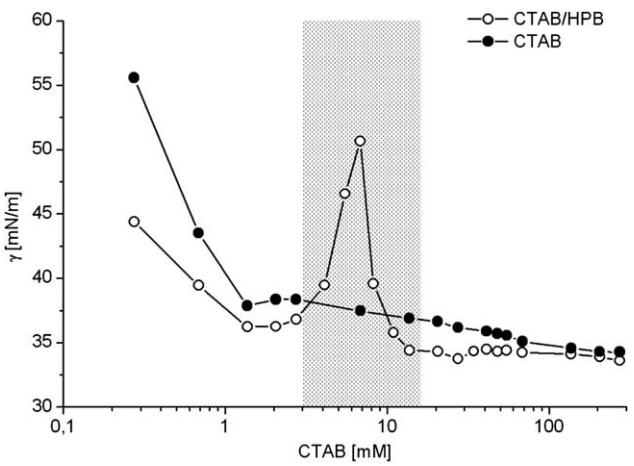

Fig. 5 Surface tension γ profile of the supernatants of mixtures from 1% HPB and increasing amount of CTAB (open circles) in comparison to the surface tension of a pure CTAB solution (closed circles). The shaded area indicates the CTAB concentration range where HPB is in the flocculated state.

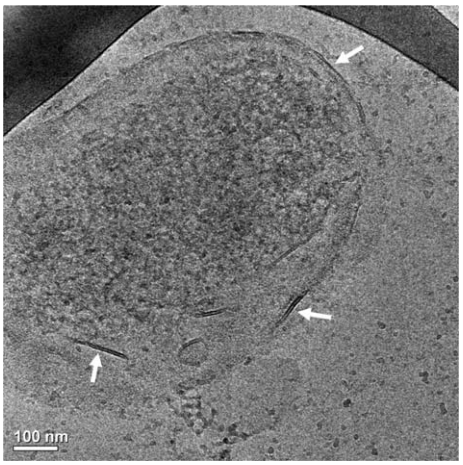

Fig. 6 Cryo-TEM micrograph of a 0.1% HPA solution. White arrows show membrane fragments formed by big protein aggregates.

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

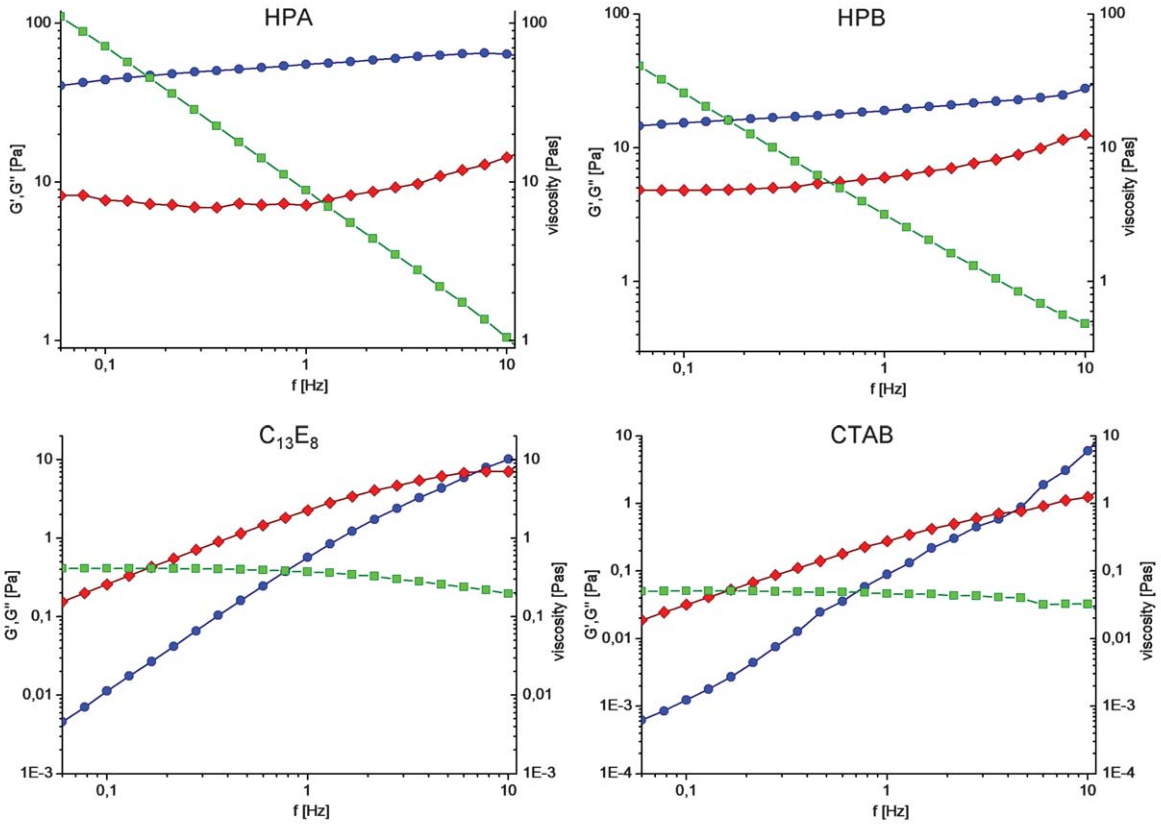

Fig. 7 Rheograms of the emulsion layers containing 1 wt% emulsifier and $\Phi = 0.2$ dodecane measured at $\tau = 0.5$ Pa one day after emulsification. Blue: storage modulus G' (Pa), red: loss modulus G'' (Pa) and green: viscosity η (Pa s).

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).

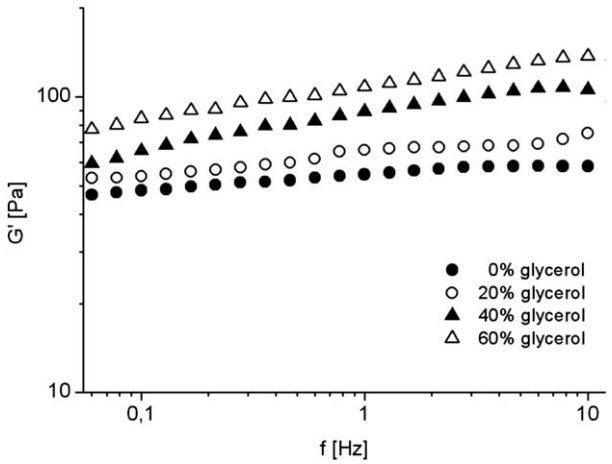

Fig. 8 Storage moduli G' (Pa) against frequency (Hz) measured at $\tau = 0.05$ Pa for emulsions prepared with various amounts of glycerol after 1 day. Sample composition: aqueous phase: 1% HPB and 0–60% glycerol; oil $\Phi = 0.2$ dodecane.

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

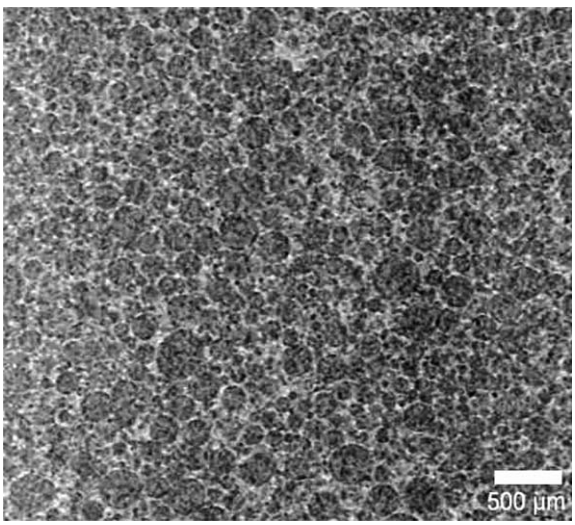

Fig. 9 Computer tomography of a homogeneous protein emulsion. The emulsion contained 0.5% HPB and 60% glycerol in the aqueous phase and $\Phi = 0.6$ dodecane, pH 6. The average droplet diameter is 50 μm.

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.

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

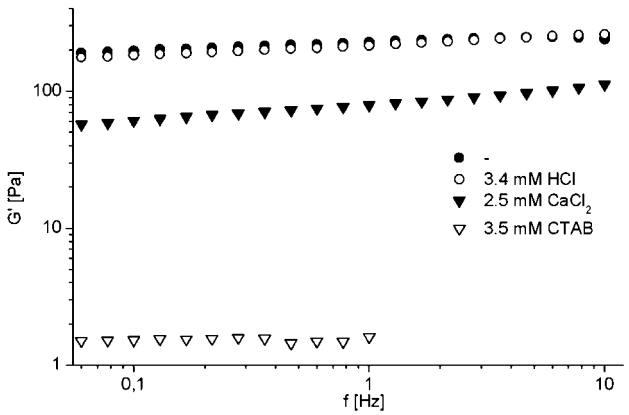

Fig. 10 Storage moduli G' ($\tau = 0.5$ Pa) of emulsions prepared from flocculated protein. Final concentrations: 0.5% HPB without and with flocculation agent (3.4 mM HCl, 2.5 mM $CaCl_2$ and 3.5 mM CTAB) and a mass ratio Φ of 0.65 dodecane.

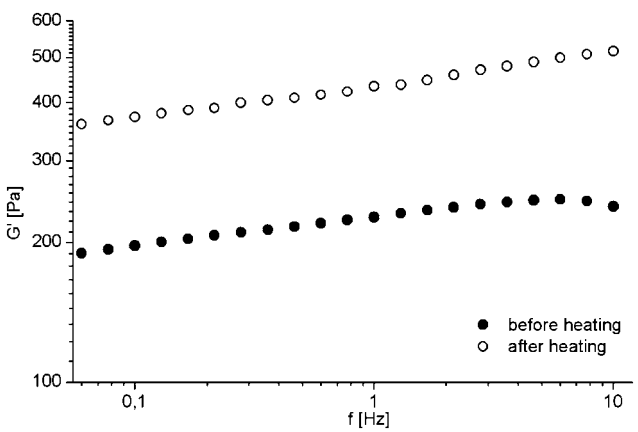

Fig. 11 Storage moduli G' ($\tau = 0.5$ Pa) of an emulsion with 0.5% HPB and $\Phi = 0.65$ dodecane before and after heating for 5 min at 92 °C.

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.

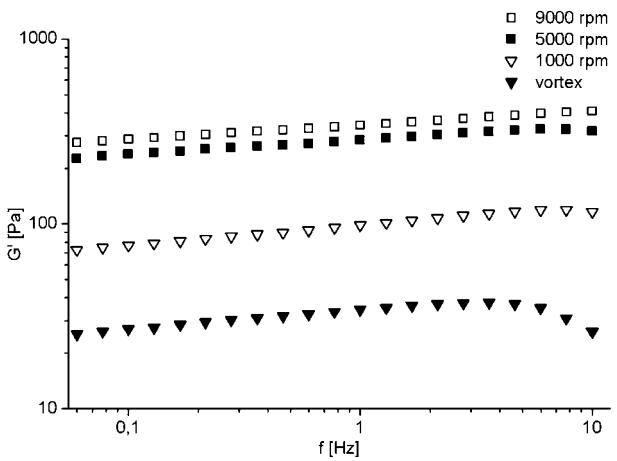

Fig. 12 Storage modulus G' ($\tau = 0.5$ Pa) of emulsions prepared with different mixing aids. Final concentrations: 1 wt% HPB and $\Phi = 0.65$ dodecane.

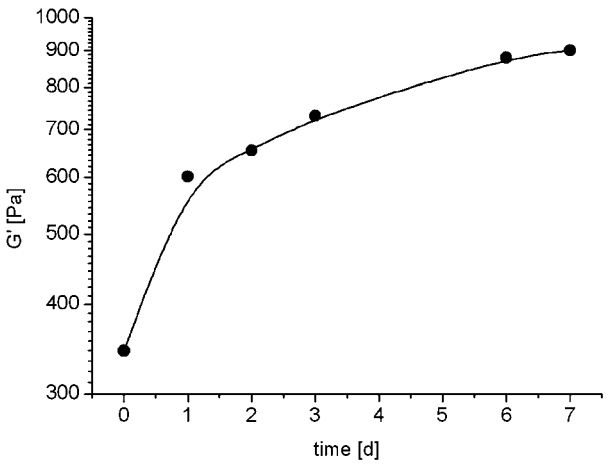

Fig. 13 G' (Pa) at $\tau = 0.5$ Pa and $f = 1$ Hz measured at different time points. Sample composition: 1% HPB and $\Phi = 0.65$ dodecane, prepared with the Homo Disper at 9000 rpm.

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.

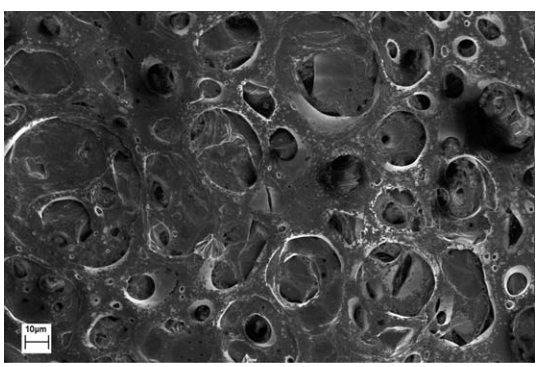

Fig. 14 REM micrograph of the drying residue of an emulsion containing 1% HPB and $\Phi = 0.65$ dodecane, prepared with the Homo Disper at 9000 rpm.

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

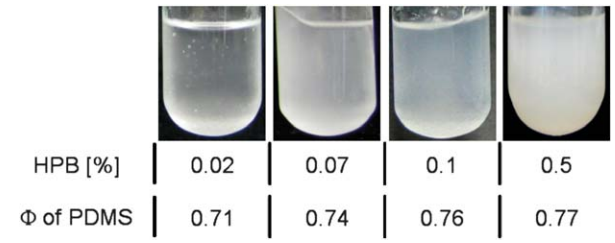

Fig. 15 Determination of the maximum oil content for homogeneous emulsions depending on the used protein concentration. Aqueous phase contained 60% glycerol.

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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PAPER

Pickering emulsions stabilized by novel clay–hydrophobin synergism

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We have studied the physico-chemical properties of high internal oil in water (o/w) emulsions, stabilized by synergistic interaction between hydrophobin and clay. As an emulsifying agent with biological background we used H Star Protein® B (HPB). Its emulsifying partner, Laponite XLG, is a synthetic layered silicate. One to one aqueous mixtures of HPB and Laponite XLG resulted in homogeneous emulsions with an oil mass fraction Φ of 0.65 PDMS. When used separately, both systems form unstable o/w emulsions. Moreover rheological measurements indicate the weak gel-like properties of their emulsions, whereas the simultaneous use of clay and hydrophobin results in long-term stable o/w emulsions with very pronounced gel-like properties. Characteristic rheological properties are their high storage modulus G' (>1000 Pa), a high yield stress value and viscosity (1 Pa s at a shear rate $\gamma = 100\ s^{-1}$). Despite a low polydispersity, a certain ripening of the emulsion matrix depending on the incubation time and shear rate was observed. It is concluded that the high storage moduli in the gel-like emulsions are due to the elasticity of the clay–protein films surrounding the oil droplets forming a self-supporting three-dimensional network. Our results highlight the relevance of the novel hydrophobin–clay synergism, resulting in excellently stabilized surfactant-free emulsions.

Introduction

Proteins are amphiphilic compounds that bind both on hydrophilic and on hydrophobic surfaces.[1] Like other surface-active polymers they also bind on clays which have hydrophilic surfaces.[2,3] Clays, which are negatively charged, can be made hydrophobic by adding cationic surfactants.[4] It is usually assumed that the binding of cationic surfactants is a consequence of their cationic nature. It is known however that both typical non-ionic and zwitterionic surfactants bind to clay surfaces.[5,6] By the adsorption of these surfactants the surface of clays can be turned from hydrophilic to hydrophobic and back to hydrophilic again. When the surface of the clays becomes hydrophobic, the clay–surfactant complexes usually precipitate from aqueous solutions.

Synthetic and natural clays are colloidal building blocks with a well-defined structure.[7] They are layered silicates with special ion-substitutions, like Si by Al, Al by Mg and Mg by Li.[8] Therefore the clays possess an excess negative charge. Clay particles have a thickness of 1 nm and their building blocks are separated by thin layers of cationic ions, usually alkali-metal ions. The clays can be exfoliated to single sheets by applying high shear rates. Solutions of exfoliated clays are transparent and have a low viscosity. With increasing concentration the solutions show an abrupt sol–gel transition.[9] In the older literature this transition was usually explained on the basis of a card-house structure for the gels.[10–12] It was assumed that the negatively charged surfaces of the clay sheets form a three-dimensional network with the positively charged sides of the clays. Other theories have been proposed for the sol–gel transitions.[13] It is also conceivable that the sol–gel transition is due to the interaction of small stacks of clays which are oriented parallel to each other and which become larger with time. Very often the theories do not take into account that the gels develop birefringent properties which become stronger with time. Clays are ideal compounds for the adsorption and removal of all kinds of waste products like dyes, multivalent cations or surfactants because they have such huge surfaces of up to 1000 $m^2\ g^{-1}$.[14,15]

Clay–surfactant complexes are perfect systems for the preparation of Pickering emulsions. Such emulsions from clay/non-ionic surfactant systems have proven to be quite stable.[16] In this article we prepare Pickering emulsions from clay–protein complexes and compare the properties of these emulsions to emulsions which are prepared from the proteins alone. The emulsions were formed with a recombinantly produced hydrophobin, called H Star Protein®.[17] It is produced as fusion protein harboring the hydrophobin protein of the fungi *Aspergillus nidulans*. Hydrophobins act as highly surface-active proteins[18,19] and are well known for their strong tendency to self-aggregate.[20,21] These properties combined with its now obtained high availability due to genetic engineering make the H Star Protein® interesting for industrial applications.

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Materials and methods

H Star Protein® B, from now abbreviated as HPB (19 kDa; IEP: 6.15), is a recombinant hydrophobin[17] and was a gift from BASF, Ludwigshafen. HPB consists 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 Protein® B please refer to ref. 17. The clay Laponite XLG[22] was purchased from Rockwood Clay Additives GmbH, Moosburg. Polydimethylsiloxane (PDMS) was purchased from Shinetsu Kagaku, Tokyo. It has general formulation: $(CH_3)_3SiO[(CH_3)2SiO]nSi(CH_3)_3$. The polymerization degree η is ranging from 5 to 19 (>98%) and the viscosity is approximately 6 mPa s. Merck, Darmstadt, supplied the nonpolar oils decane and dodecane, as well as the polar oil octyl-methoxycinnamate (OMC, brand name: Eusolex® 2292). Other chemicals not specified in the text were of analytical grade or equivalent.

The surface tension σ of the samples was measured with the volume-drop tensiometer TVT1 from Lauda Co., Königshofen, at a constant drop-formation speed of 1 μl s^{-1}. In order to determine the free amount of hydrophobin in the clay–hydrophobin mixtures the supernatant of the samples was used. Therefore the samples were centrifuged in a Medifuge from Heraeus Instruments GmbH, Hanau, for 10 minutes at 2000*g*.

For Cryo-Transmission Electron Microscopy (Cryo-TEM) a drop of the sample was placed on a TEM-grid (200 mesh, Science Services, Munich). Removing the majority of the liquid sample with blotting paper resulted in a thin stretched film over the grid holes. Afterwards the specimens were shock-vitrified by rapid immersion into liquid ethane and cooled to below −178 °C by liquid nitrogen in a Zeiss Cryobox freezing unit. The specimens, kept below −178 °C, were studied in a Zeiss EM922 Omega EFTEM transmission electron microscope, operated at 200 kV. All images were digitalized with the CCD camera system from Ultrascan 1000, Gatan.

All emulsions were prepared from aqueous solutions of hydrophobin and clay. In order to avoid microbial growth all samples contained 0.5 wt% phenoxyethanol. It turned out that one step oil addition to the aqueous phase led to emulsion breakdown, so it was only possible to produce high oil content emulsions with stepwise addition of oil. Samples emulsified with a Vortex shaker (IKA Genius 3, Staufen) were treated for 0.5 h at maximum power. High pressure emulsions were pre-emulsified using the Homo Disper (Tokushu Kika, Osaka) with about 1000 revolutions per minute (rpm). Afterwards the pre-emulsions were filled in the High Pressure Emulsifier (APV 1000, Albertslund) and passed three times through the device at the desired pressure (100–1000 bar).

For light microscopy the samples were trapped between a microscope slide and cover glass and investigated with a Zeiss light microscope (model: 47 60 05-9901). The micrographs were digitalized with the DFK 41F02 camera and analyzed with the IC capture 2.1 software (The Imaging Source, Bremen).

The rheology of the emulsion layers was measured with a cone-plate rheometer RheoStress 600 from Haake Thermo Scientific, Karlsruhe at 25 °C. The experimental data were analyzed with the Haake RheoWin Data Manager, Version 3.3.

For Cryo-Scanning Electron Microscopy (Cryo-SEM) the sample was trapped in aluminium specimens and rapidly frozen in liquid nitrogen in the Leica BalTec HFM-100 freeze device. Using the Leica EM VCT 100 Vacuum-Cryo-Transfer-System the sample was loaded under cold nitrogen atmosphere in the Leica EM MED 020 freeze fracture and sputter device. After cutting the specimens by a carbide metal knife, they were immediately covered with a platinum layer of desired thickness. Finally the Ultra Plus Zeiss SEM harboring a third-generation Gemini electron optical column was charged with the coated specimens. The integrated Thermo Scientific MagnaRay WDS spectrometer automatically handled alignment, analysis settings and data acquisition and eased the measurement procedure.

The emulsion was filled in a round-bottom flask and attached to the Freeze Dryer ALPHA 1-4 from Christ GmbH, Osterrode, until all of the water and oil had been removed. The freeze-dried emulsion was coated with a 1.3 nm iron layer in the Cressington Sputter Coater 208 HR and analyzed with a Zeiss 1530 scanning electron microscope (SEM).

Results and discussion

Interaction of clay and hydrophobin

Solutions of clays and of the hydrophobin are low viscous and transparent. Mixtures of the two compounds are turbid however (Fig. 1).

The turbidity could be due to depletion flocculation because both particles carry the same negative ionic charge but are of different size and shape. On the basis of surface tension measurements depletion flocculation can be ruled out because the free protein concentration is very much lower than the total protein concentration in the mixed samples. The turbidity must therefore be due to aggregates between the two particles even though the long range interaction between the particles is repulsive. The samples of the mixtures look very similar to mixtures between clays and polyvinyl alcohol (PVA). In such samples it had been shown that PVA adsorbed onto clay particles and the formed complexes aggregate in solutions to large clusters.[23] Obviously the situation between Laponite XLG and HPB is similar to the situation between clays and PVA. In both cases the polymers bind to the clay surface. The adsorption energy for the binding of the negatively charged hydrophobin to the negatively charged clay particles can overcome the repulsive interaction energy between the two particles.

It is interesting to note that the samples with mixing ratios of 8 : 2 and 3 : 7, 2 : 8 and 1 : 9 have separated into two layer systems while the other samples are turbid but have not separated on a macroscopic level.

The dependence of the amount of hydrophobin adsorbed to 0.5 wt% Laponite XLG on the used hydrophobin concentration was determined as follows. HPB solutions in the presence and absence of 0.5 wt% clay were prepared. The samples containing hydrophobin and clay were centrifuged. As clay particles covered with hydrophobin formed a precipitate, the remaining, non-adsorbed HPB was located in the supernatant. By determining the surface tension of the supernatant and comparing it to the values of HPB without clay, the amount of non-adsorbed HPB could easily be obtained. Consequently the other part of the initial HPB amount was adsorbed onto the clay particles. Surface tension measurements are shown in Fig. 2.

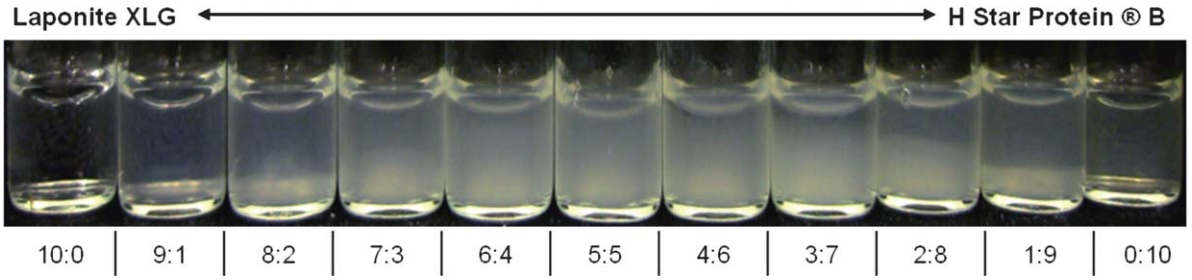

Fig. 1 Mixtures prepared from 1 wt% Laponite XLG and 1 wt% H Star Protein® B (HPB) at neutral pH.

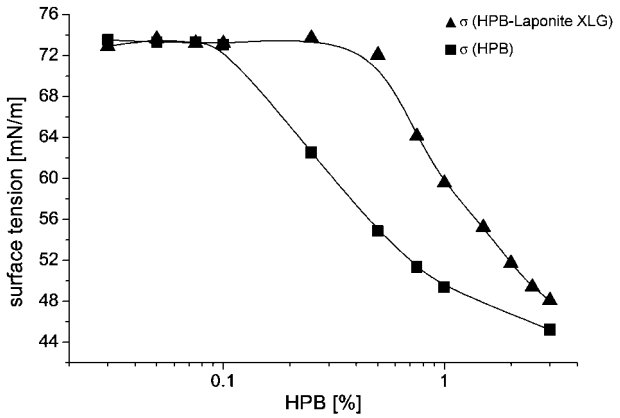

Fig. 2 Surface tension σ of the supernatants of samples with 0.5 wt% Laponite XLG and increasing concentrations of HPB in comparison to the surface tension of HPB alone. Drop-formation speed was 1 μl s^{-1}.

The results from Fig. 2 allow us to plot the adsorbed amount of hydrophobin against the used, total amount of hydrophobin (Fig. 3).

The results in Fig. 3 show that 0.5 wt% clay can bind at least three times as much hydrophobin. The large amount of adsorbed HPB makes it conceivable that not all of the adsorbed hydrophobin is accommodated in the two monolayers on each side of the clay but some hydrophobin is adsorbed in multilayers. The surface tension measurements indicate that the samples still contain some free hydrophobin when the total amount of hydrophobin and of clay is 0.5 wt%.

In Fig. 4 Cryo-TEM micrographs are shown of solutions of clays and of solutions of clays and hydrophobin. In the pure clay samples the platelets are homogeneously distributed over the whole area. With very few exceptions the clay platelets are in an exfoliated state and the platelets are of irregular shape. It is noteworthy to mention that the clays in the thin film are oriented either parallel or perpendicular to the film. It is likely that these limited orientations are a result of the electrostatic interaction between the clay particles. The diameter of the platelets varies from 10 to 50 nm. In the presence of equal amounts of hydrophobin the clay particles are no longer homogeneously distributed but are clustered into domains of the size of 0.5 μm.

Synergistic emulsifying action of clay and hydrophobin

In this chapter emulsions will be compared which have been prepared from silicon oil (PDMS) and water under the same conditions but with different emulsifiers. The emulsions were

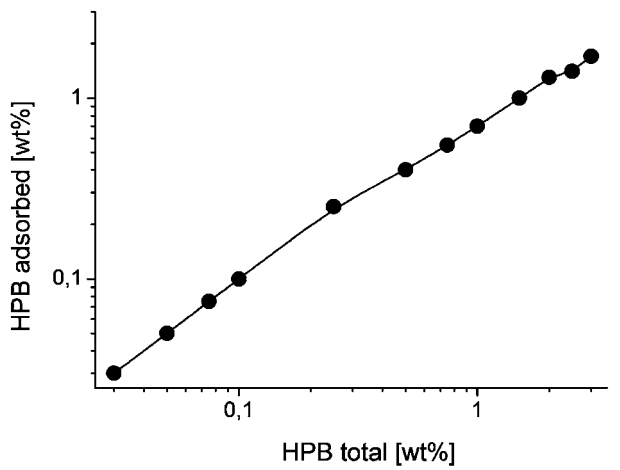

Fig. 3 Plot of the adsorbed amount of HPB (wt%) on 0.5 wt% Laponite XLG in dependence of the totally used HPB concentration (wt%).

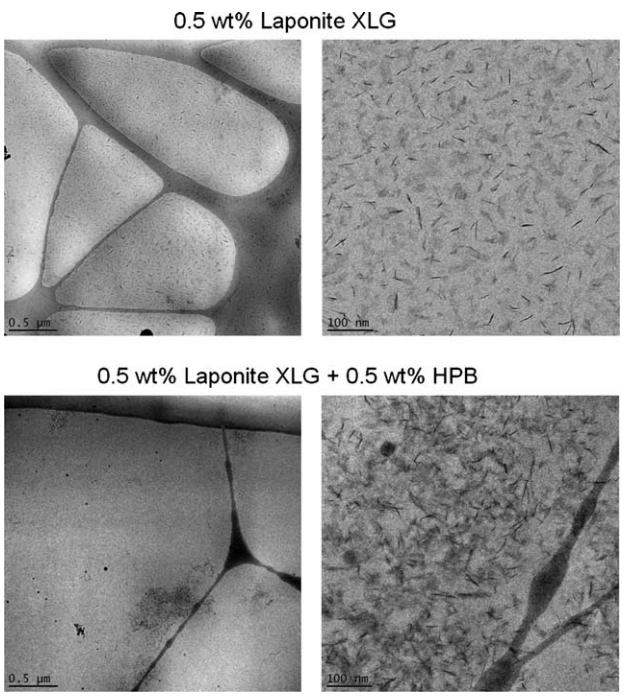

Fig. 4 Cryo-TEM micrographs of 0.5 wt% clay and of a mixture of 0.5 wt% clay and 0.5 wt% HPB at different magnifications.

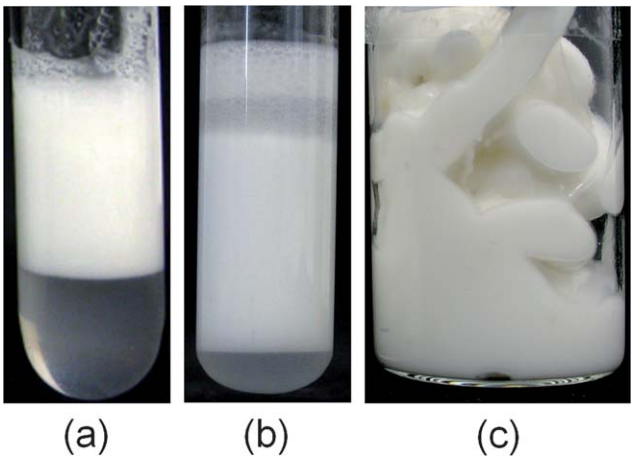

Fig. 5 Synergistic emulsifying action of hydrophobin and clay. Shown are high pressure (1000 bar), one day old Pickering emulsions prepared with 0.5 wt% HPB (a), 0.5 wt% Laponite XLG (b) and 0.5 wt% HPB/0.5 wt% Laponite XLG (c) as emulsifiers, oil mass fraction $\Phi = 0.65$ PDMS.

prepared with a high pressure emulsifier at 1000 bar and an oil mass fraction Φ of 0.65 PDMS. The used emulsifiers were: 0.5 wt% HPB, 0.5 wt% Laponite XLG and 0.5 wt% HPB in combination with 0.5 wt% Laponite XLG. The produced samples after 1 day are shown in Fig. 5.

The sample that was produced with hydrophobin (Fig. 5a) is a two layer system, with an upper gel-like emulsion and a lower aqueous layer. Emulsions with hydrophobin alone have been studied before with dodecane as oil.[24] Detailed measurements on the emulsions have shown that the systems are o/w emulsions.

In contrast to emulsions from surfactants which are low viscous layers, the emulsions from hydrophobins showed gel-like properties. This behavior is due to the fact that the protein-covered oil droplets behave like sticky particles and form densely packed systems which usually are called high internal phase emulsions or HIPE-systems. The dimensions of the droplets in such layers depend on the shear stress of the used emulsification method. With lower pressures as used for the samples in Fig. 5, homogeneous emulsions could be obtained. The two layer situation that is obtained in Fig. 5 is due to the fact that the hydrophobin concentration is not high enough to cover completely the surface of the oil droplets. With lower pressure for the preparation or with higher concentration of hydrophobin, homogeneous layers could also be obtained. The upper layer emulsion was also an o/w emulsion with gel-like properties. It could not be diluted with water. As can be judged from the volume fraction of the emulsion layer, it contained only a small amount of water in which the hydrophobin forms a network structure in which the oil is entrapped. The system that is obtained with the pure Laponite XLG (Fig. 5b) was an unstable multilayer emulsion which separated within one day into three layers: a lower aqueous layer, an aqueous emulsion and one upper oily layer. The emulsion that was produced from 0.5 wt% Laponite XLG and 0.5 wt% HPB was a homogeneous stable emulsion as it is also shown in Fig. 5, sample (c). As it is already obvious from the paste-like properties the dispersed oil droplets in the layer must be held in a three-dimensional network formed by the clay–hydrophobin particles even though both the clay particles and the hydrophobin carry excess anionic charges.

The rheological properties of the two emulsions with hydrophobin and with the clay–hydrophobin complexes were

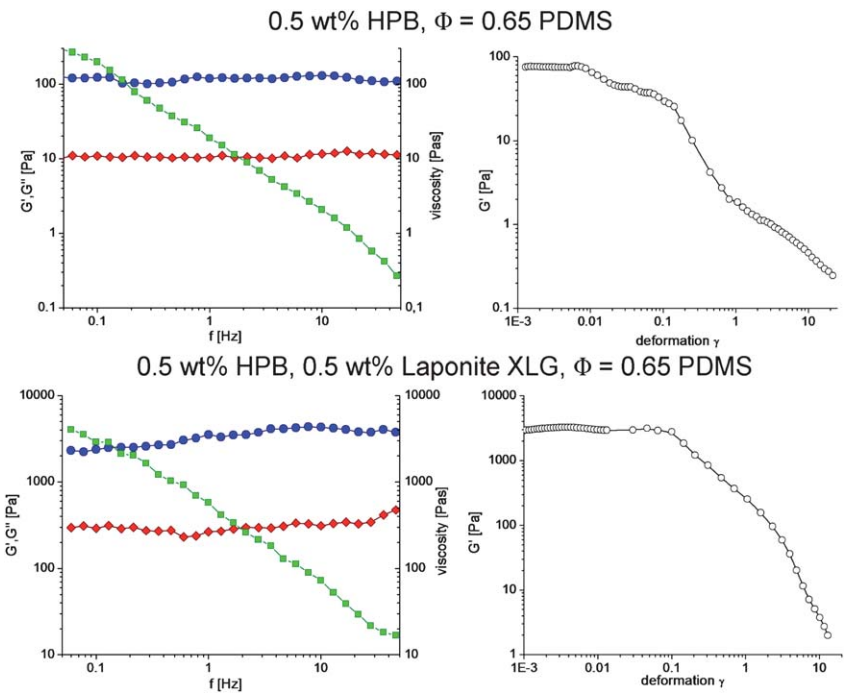

Fig. 6 Comparison of the rheological properties of emulsion layers prepared from 0.5 wt% HPB (upper row) and from a combination of 0.5 wt% HPB and 0.5 wt% Laponite XLG (lower row) acting as emulsifiers. Shown are, on the left side, rheograms ($\tau = 0.05$ Pa) with the color code: blue: storage modulus G' (Pa), red: loss modulus G'' (Pa) and green: viscosity η (Pa s). On the right side the storage modulus G' in dependence of the applied deformation γ is evaluated. The rheological properties were obtained one day after emulsification at 1000 bar.

characterized by oscillating rheological measurements. Some results are shown in Fig. 6.

As was known already from the previous investigations with dodecane–water emulsions, the emulsion with silicon oil and HPB protein behaves like a soft gel.[24] The storage modulus G' and the loss modulus G'' of the samples are independent of frequency and the storage modulus G' is an order of magnitude larger than the loss modulus G'' (Fig. 6, upper row). With 100 Pa the storage modulus is much higher than it should be if the modulus would be determined by the number density of the oil droplets. It had been concluded therefore that the storage modulus of the protein emulsions is due to the elasticity of the protein network that is formed by the interacting hydrophobin monolayers around the oil droplets. The results of the oscillating measurements on the sample with Laponite XLG and HPB look qualitatively very similar; the absolute values of storage and loss modulus are 30 times higher than those for the emulsions with hydrophobin alone (Fig. 6, bottom row). The incorporation of the clays into the hydrophobin has obviously increased the elasticity of the film. In view of the stiffness of the platelets this result is not so surprising. It is assumed that both sides of the clay particles are covered by the hydrophobin and the thus-formed hydrophobin sandwiches adsorb onto the oil droplets. It is therefore likely that the interaction between the hydrophobin and hydrophobin-covered clay sandwiches adsorbed at the oil droplets is very similar, because in each case the contact between the droplets is a hydrophobin–hydrophobin contact. It is interesting to note that both networks can be stretched by about 10% before they break down, which is obvious from the measurements in which the amplitude of the oscillating shear stress was increased at constant frequency (see Fig. 6, bottom row, right figure). Viscoelastic networks in general can differ strongly in the deformation to which they can be stretched before they break down. Entanglement networks from wormlike micelles can be stretched ten times before they break, while silica networks can only be stretched one % before they break.[25,26] Finally, as clays are known for sol–gel transitions, a rheological characterization of an aqueous solution of 0.5 wt% Laponite XLG was performed. With a storage modulus G' of 0.03 Pa at $\tau = 0.5$ Pa and $f = 1$ Hz, the Laponite XLG was far away from the gel-state at this concentration. Moreover the emulsion layer stabilized by clay (Fig. 5b) showed also no signs of gel-like properties. We therefore conclude that at these conditions only the synergistic use of hydrophobin and clay results in emulsions with highly gel-like properties.

Emulsion structure

The size of the droplets in the investigated emulsion can be resolved by light microscopy. The average emulsion droplet size was determined to be 2.3 ± 1.0 μm. The hydrophobin–clay based emulsion was also investigated with the Cryo-SEM technique (Fig. 7a). On a larger magnification, a single droplet is shown in Fig. 7b.

Small white spots can be seen inside the spherical cross-section of the droplet. The diameter of those spots is around 40 nm that is in the same range as the diameter of the individual clay particles (Fig. 4). It is therefore likely that the Cryo-SEM method can resolve the individual clay particles on the emulsion droplets.

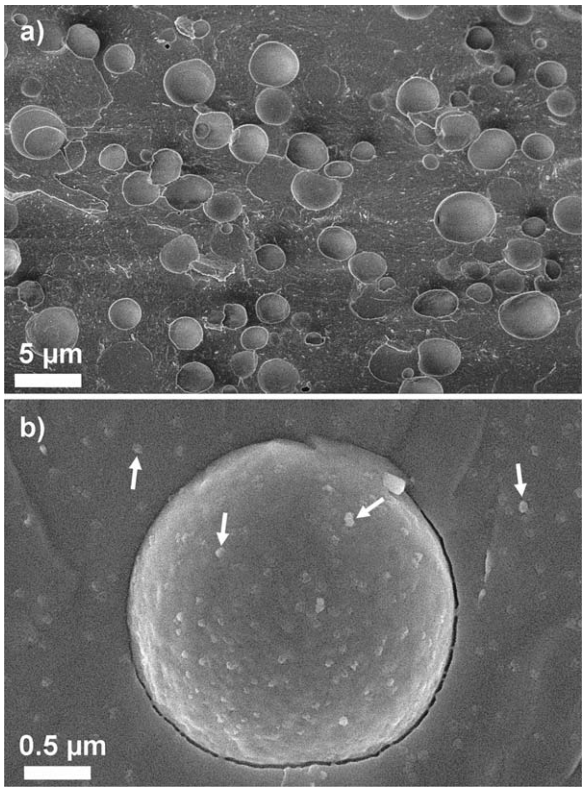

Fig. 7 Cryo-SEM micrographs of a hydrophobin–clay synergism based emulsion. An overview of the emulsion topology is provided in (a). White arrows in (b) indicate clay particles to be located at the interface and in the water layer. Sample composition: 0.5 wt% HPB, 0.5 wt% XLG, $\Phi =$ 0.65 PDMS, 1000 bar.

A few such spots are also visible in the aqueous bulk layer. It is therefore probable that not all of the clay particles have been used up for the formation of the droplets. In order to be aware of possible artifacts Cryo-SEM pictures of the same sample without clay did not show any white spots.

Shear influence on emulsion properties

The size of droplets in emulsions depends usually on the shear stress that is used in the emulsification process.[27] Under extremely high shear rates droplets with diameters in the range of tens of nm can be reached, but with standard emulsifying machines, like the Vortex shaker, droplets with diameters in the range of tens of μm are obtained.

This dependence of the diameter on the used shear rate comes about the shear stress acting on a droplet. It can deform and break the droplet if the shear stress is larger than the Laplace pressure of the droplet. The formed droplet can then only be stable if there is enough emulsifier available in the solution to cover and protect the freshly prepared oil/water interface against coalescence. In order to study the influence of shear stress on the emulsion we prepared emulsions with the same composition but with different emulsifying methods, respectively, a Vortex shaker and a high pressure emulsifier. The results of these studies are summarized in Table 1. It contains the storage modulus G' of the emulsions as well as the average droplet size, determined by statistic analysis of light microscopy images.

Table 1 Comparison of storage moduli G' (Pa) ($\tau = 0.5$ Pa, $f = 1$ Hz) and average droplet size (μm) for emulsions containing the same composition but having been prepared at different shear stresses. Sample composition: 0.5 wt% HPB, 0.5 wt% Laponite XLG, and oil mass fraction $\Phi = 0.65$ PDMS

	Vortex shaker	100 bar	300 bar	1000 bar
G'/Pa	199	1265	2518	3183
Droplet size/μm	14.2 ± 5.3	4.9 ± 2.1	2.8 ± 0.7	2.3 ± 1.0

The results show that increasing shear stress decreases the size of the emulsion droplets and increases the storage modulus of the emulsion. According to the core–shell model it is interesting to note that with the highest used pressure (1000 bar) the droplet size is reached where the clays just can cover the droplets. For the lower pressures some of the clays must be present as free particles in the aqueous layer between the emulsion droplets of the samples.

Emulsion stability and aging

Emulsions in general are not thermodynamically stable systems. For this reason they usually change with time and after long enough times they can separate into two layers, respectively oil and water. The aging can be due to coalescence of the droplets, Ostwald ripening process, up-creaming of the droplets or a combination of all three processes.[28] Investigations of the visual properties as well as the emulsion droplet size dependence on incubation time for these emulsions could not detect any changes over many weeks indicating the emulsions to be long-term stable.

Rheological measurements showed, however, that the storage modulus of the emulsions increased strongly within one day, but after this modestly with time. Some results of the aging process are shown in Fig. 8.

It is assumed that the mechanism of the aging process in the emulsion is similar as for the emulsions which were prepared by HPB alone as emulsifier.[24] It was concluded that the aging in these systems comes about by the evolution of the three-dimensional network of the cross-linked hydrophobin covered emulsion droplets while the size of the droplets does not change with time. The increase of the storage moduli for the present system is however much less than the changes for the hydrophobin emulsions were.

The aging is thus due to a change in the conformation of the hydrophobin molecules and of a partial entanglement of hydrophobin molecules with each other among the whole emulsion layer. As for any kinetically controlled process, the rate of change should increase with temperature. For this reason, the storage moduli of freshly prepared emulsions should have increased after short-time heating and then been constant with time. These changes in the storage moduli which are due to conformational changes are irreversible processes. The storage moduli therefore increased from 1265 Pa to 2438 Pa ($\tau = 0.5$ Pa; $f = 1$ Hz) after the heating procedure and then stayed constant.

Self-supporting network

For the self-supporting three-dimensional hydrophobin–clay network it was mentioned in the previous chapter at various places that the storage moduli of the emulsions were determined by the elasticity of the network and not from the droplets and their interaction. It was therefore concluded that the network should not collapse if the oil and the water would be removed from the emulsions. An emulsion was therefore freeze-dried and the dried samples were viewed with SEM. The result is shown in Fig. 9.

The SEM micrographs show a porous material with globular shaped holes with diameters around 3–4 μm. This is the same size as that of the emulsion droplets which could be seen in the emulsions (Table 1). This simple experiment confirms that the network in the emulsions is a self-supporting network and both

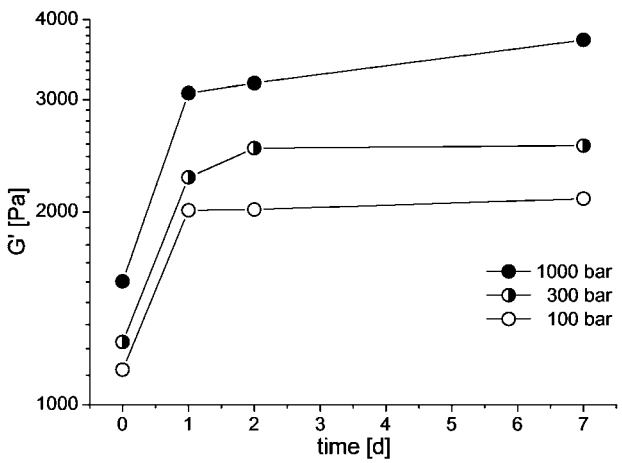

Fig. 8 Storage moduli G' (Pa) measured at $\tau = 0.5$ Pa and $f = 1$ Hz at different time points. Sample composition: 0.5 wt% HPB and 0.5 wt% Laponite XLG and oil mass fraction $\Phi = 0.65$ PDMS; emulsions prepared with the high pressure emulsifier.

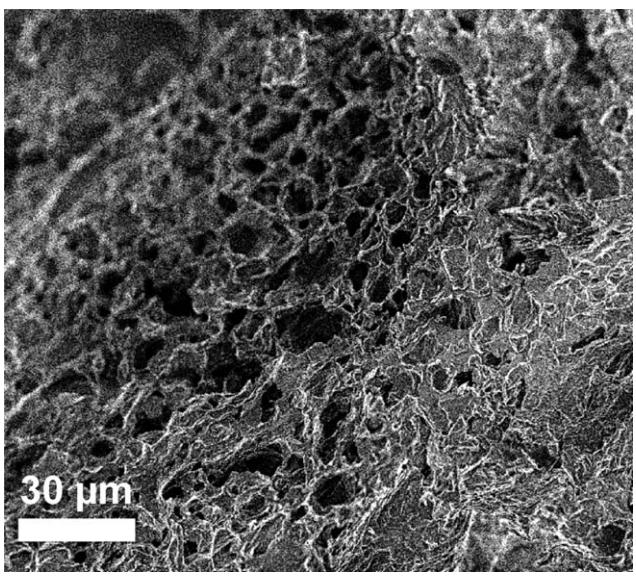

Fig. 9 SEM micrograph of the freeze-dried residue of an emulsion containing 0.5 wt% HPB, 0.5 wt% Laponite XLG and an oil mass fraction $\Phi = 0.65$ decane, prepared with the high pressure emulsifier at 300 bar.

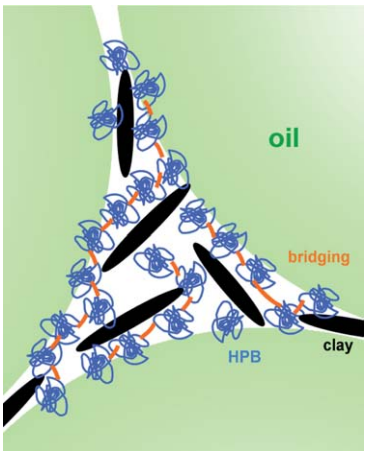

Fig. 10 Conceptual diagram of the self-supporting, three-dimensional network existing formed by hydrophobin covered clay particles. The HPB molecules (blue) absorbed on the clay (black) interact (orange) with each other. The clay particles serve as strengtheners of the hydrophobin network.

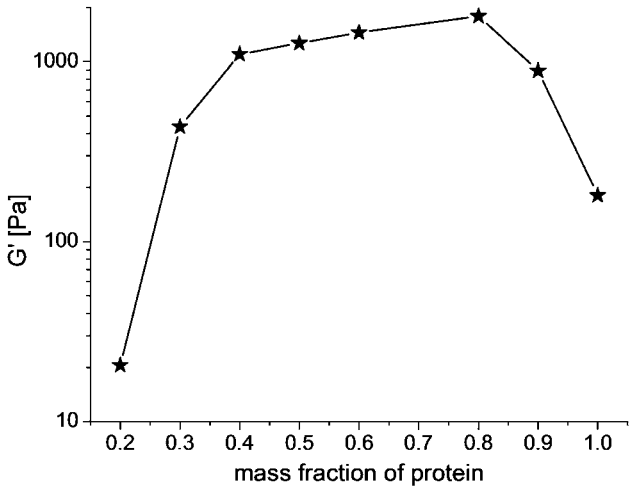

Fig. 12 Comparison of G' values ($\tau = 0.5$ Pa, $f = 1$ Hz) measured directly after emulsification (300 bar) for emulsions containing different protein/clay mass fractions. The emulsions contained 1 wt% of protein/clay as well as a PDMS mass fraction $\Phi = 0.65$.

oil and water layers can be removed without collapse and destruction of the network. This result also can be used as support that the storage moduli of the emulsions are given by the strength of the network.

A conceptual diagram of this self-supporting, three-dimensional network formed by the hydrophobin-covered clay particles is shown in Fig. 10.

Influence of the hydrophobin–clay ratio

Pickering emulsions were also prepared in which only the ratio between clay and protein was varied and all other conditions were kept the same. Prepared emulsions are shown in Fig. 11.

The emulsions seem to be very similar for the range where the hydrophobin mass fraction is varied between 0.4 and 0.8. This result is surprising because the coverage of the clays must change strongly with the hydrophobin/clay ratio. The results show however that this parameter does not influence very much the storage moduli G' as long as the total amount of protein and clay remains the same (Fig. 11). It is likely that this result is an indication that the size of the droplets in the emulsions of Fig. 11 is the same but the droplets are covered not only by hydrophobin/clay sandwiches but also by hydrophobin molecules.

Noteable decreases of the storage moduli G' were only observed at very small (0.2) and very high hydrophobin mass fractions (0.9 and 1.0). At small protein concentrations the entanglement of the three-dimensional hydrophobin–clay network is not as distinct as at higher hydrophobin mass fractions. A lower number of bridging points consequently would result in lower gel-like properties (Fig. 12). At higher hydrophobin mass fraction (0.9 and 1.0) the amount of clay acting as strengthener of the three-dimensional network has also decreased. Accordingly the network strength decreased (Fig. 12).

Oil polarity influence on Pickering emulsion properties

Pickering emulsions were prepared from diverse oils which differ in molecular weight and polarity (sample composition: 0.5% HPB, 0.5% Laponite XLG and $\Phi = 0.65$ oil). All other conditions like the amount of emulsifier and mixing conditions were kept constant. Three Pickering emulsions have been prepared from the apolar and low molecular weight oil dodecane, from the silicon oil PDMS and from the polar oil octyl-methoxycinnamate (OMC). Their visual appearance showed already that the rheological properties of the emulsions seem to be similar. All of them had a yield stress value which prevents the emulsions from forming a horizontal meniscus. Detailed rheological

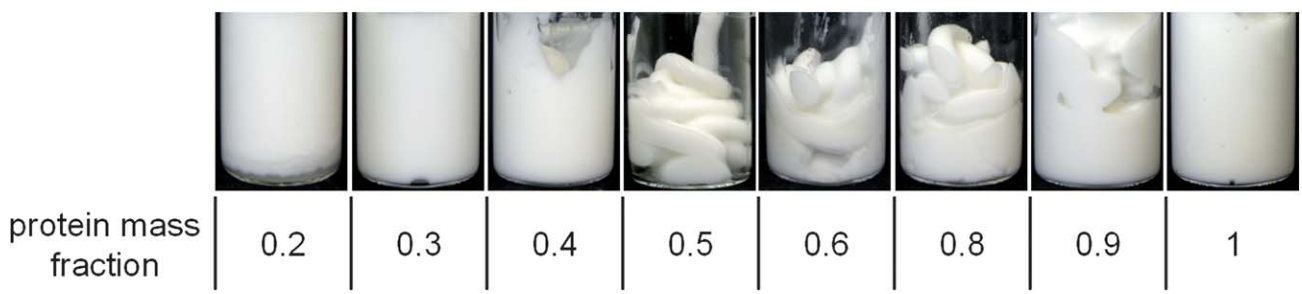

Fig. 11 High pressure emulsions (300 bar) containing different hydrophobin/clay mass fractions. The emulsions contained 1 wt% HPB/Laponite XLG as well as a PDMS mass fraction $\Phi = 0.65$.

measurements showed that even the storage moduli for the three oils did not differ much. The storage moduli (τ = 0.5 Pa; f = 1 Hz) of the emulsions were 1265 Pa for PDMS, 1693 Pa for dodecane and 1854 Pa for OMC. This is indeed a small change considering that the storage modulus of an emulsion is a parameter that can vary many orders of magnitude for emulsions with the same structure and the same dimension of the droplets (~2 μm). This little dependence on the oil is again a good confirmation of the previously made conclusion that the storage modulus of the emulsions is mainly controlled by the elastic properties of the films around the oil droplets. These results are also in agreement with investigations on the properties of bubbles and foam films stabilized by hydrophobin.[29]

Oil mass fraction influence on emulsion stability

The gel-like properties of the studied emulsions are a result of the attractive interaction between the oil droplets that are covered with the hydrophobin–clay particles. Dispersed oil droplets with such properties will therefore contract to a dense layer until the attractive forces are balanced by repulsive forces which come from the packing of the droplets. With this situation it is clear that dilute emulsions which are far away from dense packing will be stable but will separate into two layers. The described situation was confirmed with following experiments. Homogeneous looking emulsions containing 0.5 wt% HPB, 0.5 wt% Laponite XLG and an oil mass fraction Φ of 0.1 can be prepared, but they start to separate within a few minutes. The layer boundary moved within a few days, the emulsion layer got smaller due to the progressive dense packing of the emulsion droplets. Stable, homogeneous emulsions needed to have an oil mass fraction Φ of more than 0.3. The storage moduli G' of the emulsions containing different oil mass fractions Φ measured directly after preparation are shown in Fig. 13.

Influence of the charge reversed hydrophobin on emulsions

The negative charge of HPB can be reversed by adding corresponding amounts of HCl. On approaching the point of zero

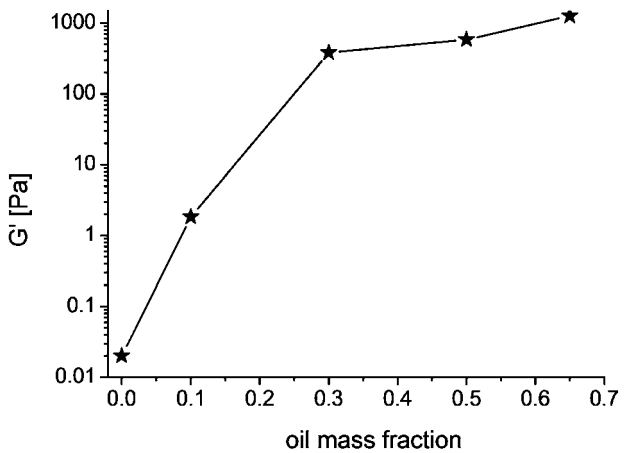

Fig. 13 Storage moduli G' (τ = 0.5 Pa, f = 1 Hz) measured directly after emulsification at 300 bar for emulsions containing different PDMS oil mass fractions Φ. The emulsions contained 0.5 wt% HPB and 0.5 wt% Laponite XLG as well as PDMS mass fractions Φ between 0.1 and 0.65.

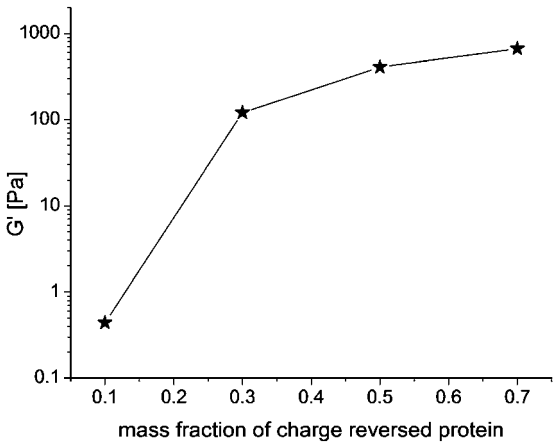

Fig. 14 Storage moduli G' (τ = 0.5 Pa, f = 1 Hz) measured directly after emulsification at 300 bar for emulsions prepared with charge reversed protein. The emulsions contained 0.1–0.7 wt% charge reversed HPB, 0.5 wt% Laponite XLG and an oil mass fraction Φ of 0.65 PDMS.

charge the hydrophobin flocculates but dissolves again when the hydrophobin becomes positively charged again. At a pH of 4 the hydrophobin solutions are clear again. The positively charged hydrophobins also bind to clays. In mixtures of clays and positively charged hydrophobins precipitates are formed.

Emulsions were prepared with such turbid solutions having different weight ratios of hydrophobin and clay, but a constant oil mass fraction Φ of 0.65 PDMS. The samples were prepared with the same amount of 0.5 wt% Laponite XLG and an oil mass fraction Φ of 0.65 PDMS, but with increasing amount of charge reversed hydrophobin. A two-phase sample was obtained with 0.1 wt% charge reversed hydrophobin, whereas single, homogeneous layer emulsions have been achieved between 0.3 and 0.7 wt% charge reversed hydrophobin. All emulsion layers had gel-like properties. Rheological measurements (Fig. 14) showed however that the strength of the storage moduli G' of these emulsions is considerably lower than the modulus of the emulsion in which the clays and protein molecules carry the same ionic charge (compare to Fig. 7, 300 bar).

The difference of the moduli G' of the emulsions with the same clay/hydrophobin ratio but with different charged hydrophobins was a factor of 5. This somewhat surprising behavior is probably due to the fact that the hydrophobin/clay interaction with charge reversed hydrophobin is stronger than in the other situation. As a consequence of the strong binding of the charge reversed hydrophobin to the clay the hydrophobin–hydrophobin interactions which also determine the strength of the whole network become weaker and therefore the storage moduli have decreased.

Comparison of different Pickering emulsions

Pickering emulsions have been known already for many years.[30–32] Different particles in combination with surfactants or other amphiphilic compounds have been used for emulsions from this investigation with properties of other systems. The most often used particles of Pickering emulsions have been fumed silica particles,[33,34] clays,[35–39] Latex particles[40] and Boehmite particles.[41] In most investigations the emphasis of the studies has been on the stability of the emulsions and on the

dimensions of the droplets in the emulsions. Little attention was usually paid to the rheological properties of the emulsions. It is therefore difficult to compare the properties of the previously obtained results with the properties of this investigation.

Pickering emulsions from clays and surfactants have been prepared by the group of Lagaly.[16,42] It was shown that non-ionic surfactants like glycerol monostearate $C_{16}E_{10}$, sugar surfactant and lecithin bind to clays and stable o/w emulsions with more than 50% of oil could be obtained. It was mentioned that the emulsions showed thixotropic behavior which means that the emulsions had a yield stress. The storage modulus of the emulsions was not measured and no information was given regarding the reason for the thixotropic behaviour. No linear viscoelastic region was observed. While no detailed comparison can be made the general remarks in the investigation indicate that the properties of the emulsions did not have such a strong gel-like behaviour as observed in this investigation. Detailed studies on Pickering emulsions from fumed silica particles and cationic surfactants were reported from the group of B. P. Binks.[43] In contrast to the clay particles which have a surface area around 1000 $m^2\ g^{-1}$, the used silica particles have only a surface of around 250 $m^2\ g^{-1}$. In the dispersed state the particles are negatively charged. Their isoelectric point is below pH 3. The particles could be dispersed in water to clear or bluish solutions. With increasing cationic surfactant precipitation of the silica–surfactant complexes occurred. The complexes did not redissolve with excess surfactant. Emulsions could be prepared from these complexes with a water/oil ratio of 1 : 1. It was mentioned that the most stable emulsions were obtained at the conditions where the amount of precipitate had a maximum. SEM measurements on the emulsions showed that the silica–surfactant complexes were not evenly distributed on the surface of the droplets and the droplets were considerably larger as in this investigation. A higher silica concentration had to be used than clays in this investigation to reach stable emulsions.

In view of the larger particles this is not surprising. No rheological measurements were made on the emulsions. The SEM micrographs of the diluted emulsions indicated however that the interaction between the droplets was attractive.

Pickering emulsions have also been prepared from needle-like particles as from surfactant coated Boehmite.[37] These particles are positively charged and have a thickness of around 20 nm. In spite of their large dimensions stable emulsions could be obtained with as little as 0.05% of Boehmite. The droplets had a diameter of 20 μm or more. No rheological properties of these emulsions were reported.

These comments on available investigations on Pickering emulsions make it likely that the studied emulsions did not have such gel-like properties as the emulsions of this investigation where the gel-like properties are most prominent and in contrast to normal emulsions which very often have a low viscous behaviour. As far as we know our investigation is the first one in which it was mentioned that the stability of the emulsion is due to a self-supporting three-dimensional network from the clay/protein particles.

Conclusions

Hydrophobins, in both the negatively charged and in the positively charged state, bind strongly to clay particles which are dispersed in aqueous solutions. The resulting clay–hydrophobin compounds can be used as emulsifiers for the formation of homogeneous o/w emulsions with oil content between 30 and 65% by weight. The emulsions are stabilized in a two stage process. Firstly the protein–clay particles are highly surface active due to the properties of hydrophobin and therefore prevent the freshly formed oil droplets from coalescence. In the next step the hydrophobin–clay network evolves due to hydrophobin–hydrophobin entanglement and interaction within one day and therefore provides long term stability of the produced emulsions. The emulsions have gel-like properties because the hydrophobin-sandwiched clay particles act as sticky particles and form three-dimensional networks in the emulsions. The elastic properties of the gels are due to the three-dimensional network of the clay–hydrophobin network. The oil and water can be removed from the emulsions by freeze-drying without collapse of the network structure.

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Hydrophobin coated boehmite nanoparticles stabilizing oil in water emulsions

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ABSTRACT

Hydrophobin coated boehmite nanoparticles have been used to establish tooth-paste like, homogenous emulsions. The surface-modified nanoparticles were simply obtained by mixing aqueous solutions of cationic boehmite particles with the anionic hydrophobin H Star Protein B® (HPB). Surface tension measurements clearly show that 1 wt.% boehmite binds up to 1 wt.% HPB. The strong interaction and aggregation of hydrophobin coated boehmite nanoparticles was proven by Cryo-TEM measurements, too. Interestingly, the combined use of 0.5 wt.% HPB and 0.5 wt.% boehmite as emulsifying agents resulted in very stable, homogenous, high internal phase emulsions (65 wt.% oil) that are stable over months. The established emulsions have also been characterized by rheological measurements. Storage moduli of more than 1000 Pa are characteristic for their high gel-like properties. Furthermore, light microscopy showed an average droplet size close to 1 μm with low polydispersity. Cryo-SEM confirmed that the hydrophobin coated nanoparticles are located at the interface of the oil droplets and therefore stabilize the emulsion systems.

1. Introduction

There is currently great scientific interest around hydrophobins and their possible applications [1–4]. Hydrophobins are tiny, cysteine-rich proteins that are built up from only about 100 amino acids. They are acting as highly surface active proteins [5,6] and are well known for their strong tendency for self-aggregation [7,8] even at interfaces [9]. They are now available in kilogram scale due to genetic engineering [10]. In nature, filamentous fungi produce hydrophobins for several reasons, like for the fungal attachment to hydrophobic surfaces [11]. Based on altered properties regarding their structure and function, hydrophobins have been classified in type I and II [12]. In this study we use H Star Protein B® (HPB) [10]. It is based on class I hydrophobin DewA from *Aspergillus nidulans*. DewA is connected to a fusion partner, respectively a truncated form of yaaD from *Bacillus subtilis*. Recently it was shown that o/w emulsions prepared with HPB as emulsifier have gel-like properties even at as low concentrations as 0.2 wt.%. The emulsions have long-term stability [13]. It was concluded that the hydrophobin coated oil droplets act as sticky droplets and are attracting each other. The hydrophobin molecules adsorbed at different oil droplets interact, penetrate and entangle with each other. As a consequence, a three dimensional protein network through the whole emulsion sample is formed. A scanning electron microscope micrograph of the drying residue of an emulsion containing 1% HPB and 65% dodecane confirmed this assumption [13]. The group of *Lips* recently observed similar effects for class II hydrophobin in aqueous solution. They report that a significant binding energy between two hydrophobin molecules exists. This attraction is due to short-range hydrophobic interaction [14,15]. In the case of proteins as emulsifying agents, like ß-lactoglobulin, it is usually assumed that the protein molecules form beside a sterical barrier also a repulsive one between the oil droplets [16–18]. Obviously emulsions based on hydrophobin are stabilized by attractive interaction between the sticky emulsion droplets.

Clay minerals are interesting compounds for different applications [19,20] and have recently been used in combination with HPB as emulsifier [21]. In this combination, even though both particles are negatively charged, surface tension measurements clearly indicated a strong binding between them. One to one aqueous mixtures of HPB and Laponite XLG in combination with all types of oils resulted in very stable, homogenous o/w emulsions with extraordinarily gel-like properties. A high storage modulus G' and viscosity are characteristic rheological properties of these emulsions. It was concluded that the elasticity of the sample is due to the formation of a self-supporting three dimensional network by the protein–clay particles. After removing the oil and water by freeze drying the protein–clay network remained.

Lately Tigges et al. investigated the properties of emulsions stabilized with surface-modified boehmite particles [22]. Boehmite is an alumina powder with a cationic surface. In that study, it was modified with p-dodecylbenzenesulfonic acid as ionic and p-toluensulfonic acid as non-ionic surfactant. Pickering emulsions prepared from these combinations were not stable for longer than

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a few days and showed typical emulsion instability signs like creaming.

In this paper, however, it will be shown that very stable, homogenous and tooth-paste like Pickering emulsions, that offer long term stability, can be prepared by the use of small amounts of boehmite particles in combination with hydrophobin. Rheological measurements of the obtained Pickering emulsions indicate their high gel-like character. The low amount of emulsifier as well as the outstanding stability makes these emulsions not only very interesting for science but also for industrial application, like in cosmetics.

The section with the results of this manuscript is organized as follows. The first part is about the characterization of boehmite regarding issues that are important for a deeper understatement of our further investigations. The second section part deals with the hydrophobin–boehmite interaction. The amount of adsorbed hydrophobin in dependency of the used concentration will be monitored by surface tension measurements. Moreover Cryo-TEM studies of boehmite without and with hydrophobin are shown, proving clearly their interaction. The third part is about Pickering emulsions prepared from hydrophobin coated boehmite particles. We determined the rheological properties of these emulsions. A certain ripening of the protein–boehmite matrix in dependency of storage time will be reported. The long-term stability of the emulsions will be confirmed by observing the emulsion homogeneity as well as the emulsion droplet size in dependency of storage time. Finally, the fourth part shows the effects of varying different parameters, like the protein concentration or oil polarity, to the appearance and rheological characteristics of the Pickering emulsions.

2. Materials and methods

2.1. Materials

H Star Protein® B, from now abbreviated as HPB (19 kDa; IEP: 6.15; Zeta Potential – 31 mV for 1 wt.% solution), is a recombinant hydrophobin [10] and was a gift from BASF, Ludwigshafen. The commercial boehmite powder, called Disperal P2, was purchased from Sasol GmbH, Hamburg. Boehmite is a γ–AlO(OH) with a cationic surface. Serva Electrophoresis GmbH, Heidelberg, provided the anionic surfactant Sodium-Dodecylsulfate (SDS). Polydimethylsiloxane (PDMS) was purchased from Shinetsu Kagaku, Tokyo. It has general formulation: $(CH_3)_3SiO[(CH_3)2SiO]nSi(CH_3)_3$. The polymerization degree η is ranging from 5 to 19 (>98%) and the viscosity is approximately 6 mPa s. Merck, Darmstadt, supplied the nonpolar oil Dodecane, as well as the polar oil Octyl-methoxycinnamate (OMC, brand name: Eusolex® 2292). Other chemicals not specified in the text were of analytical grade or equivalent.

2.2. Surface tension

The surface tension σ of the samples was measured with the volume-drop tensiometer TVT1 from Lauda Co., Königshofen, at a constant drop-formation speed of 3 s/μl. In order to determine the free amount of protein in the boehmite–hydrophobin mixtures, the supernatant of the samples was used. The samples were therefore centrifuged in a Medifuge from Heraeus Instruments GmbH, Hanau, for 10 min at 2000*g*.

2.3. Cryo-TEM

For Cryo-Transmission Electron Microscopy (Cryo-TEM) a drop of the sample was placed on a TEM-grid (200 mesh, Science Services, Munich). Removing the majority of the liquid sample with blotting paper resulted in a thin stretched film over the grid holes. Afterwards the specimens were shock-vitrified by rapid immersion into liquid ethane and cooled to below −178 °C by liquid nitrogen in a Zeiss Cryobox freezing unit. The specimens, kept below −178 °C, were studied in a Zeiss EM922 Omega EFTEM transmission electron microscope, operated at 200 kV. All images were digitalized with the CCD camera system from Ultrascan 1000, Gatan.

2.4. Emulsion preparation

All emulsions were prepared from aqueous solutions of hydrophobin and boehmite. As antimicrobial agent, all samples contained 0.5 wt.% phenoxyethanole. It was only possible to produce high oil content emulsions with stepwise addition of oil. Samples emulsified with Vortex shaker (IKA Genius 3, Staufen) were treated for 0.5 h at maximum power. High pressure emulsions were pre-emulsified using the Homo Disper (Tokushu Kika, Osaka) with about 1000 rpm (rpm). Afterwards the pre-emulsions were filled in the High Pressure Emulsifier (APV 1000, Albertslund) and passed three times through the device at the desired pressure (100–1000 bar).

2.5. Light microscopy

The samples were trapped between a microscope slide and cover glass and investigated with a Zeiss light microscope (model: 47 60 05-9901). The micrographs were digitalized with the DFK 41F02 camera and analysed with the IC capture 2.1 software (The Imaging Source, Bremen).

2.6. Rheology

The rheology of the emulsion layers was measured with a cone-plate rheometer RheoStress 600 from Haake Thermo Scientific, Karlsruhe at 25 °C. The experimental data were analysed with the Haake RheoWin Data Manager, Version 3.3.

2.7. Cryo-SEM

For Cryo-Scanning Electron Microscopy (Cryo-SEM) the sample was trapped in aluminum specimens and rapidly frozen in liquid nitrogen in the Leica BalTec HFM-100 freeze device. Using the Leica EM VCT 100 Vacuum-Cryo-Transfer-System the sample was loaded under cold nitrogen atmosphere in the Leica EM MED 020 freeze fracture and sputter device. After cutting the specimens by a carbide metal knife, they were immediately covered with a platinum layer of desired thickness. Finally the Ultra Plus Zeiss SEM harboring a third generation Gemini electron optical column was charged with the coated specimens. The integrated Thermo Scientific MagnaRay WDS spectrometer automatically handled alignment analysis.

3. Results and discussions

3.1. Characterization of boehmite

In the case of clays, it is basic scientific knowledge to have information about their cationic exchange capacity (cec). For example, the negatively charged clay Laponite XLG has a cationic cec of 65.7 meq/100 g [23]. By adding different amounts of cationic surfactant, one can easily adjust the charge type and extent of the mixture. Such important information, however, was still missing in the case of the positively charged boehmite nanoparticles. The determination of the anionic exchange capacity (aec) of 1 wt.% boehmite was done as follows. Samples from 1 wt.% boehmite with increasing concentrations of the anionic surfactant SDS have been prepared. By monitoring the surface tension behavior of the

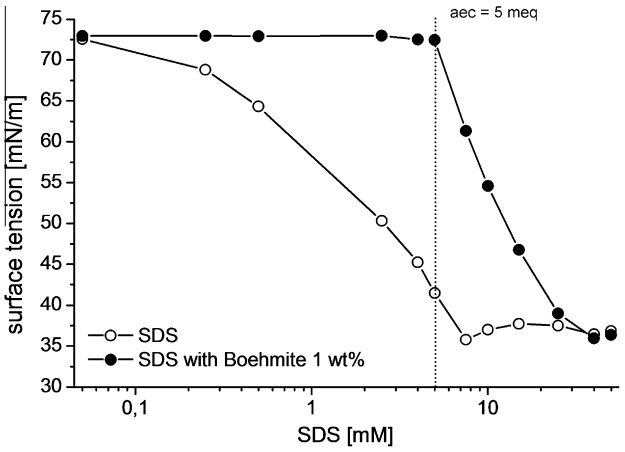

Fig. 1. Surface tension profile of the supernatants of samples with 1 wt.% boehmite and increasing amounts of the surfactant SDS in comparison to the surface tension of SDS alone. The anionic exchange capacity of 1% boehmite is 5 mM SDS.

supernatants of the boehmite–SDS mixtures, the aec could easily be determined (Fig. 1).

The aec of boehmite is determined to 50 meq/100 g. According to Fig. 1, up to 35 mM SDS can bind to 1 wt.% boehmite.

Clays are also well known for providing an abrupt sol–gel transition with increasing concentration of the solutions [24]. Consequently the viscosity η in dependency of the clay concentration increases at the sol–gel transition point rapidly. For our studies, it is important to check, whether something similar like this can be observed in pure boehmite solutions. We chose therefore a concentration range of 0.5–10 wt.% boehmite. To the naked eye the solutions appeared to be low viscous. In order to get exact values, the viscosity η was determined by oscillating measurements. Table 1 offers an overview of the viscosity η at a frequency f of 1 Hz at two different shear stresses τ (0.05 and 0.5 Pa) for 0.5–10 wt.% boehmite solutions.

The results summarized in Table 1 show clearly that boehmite solutions in this concentration range are shear thinning. Even with 10 wt.% boehmite the viscosity is only eight times higher than the one of water. Moreover frequency dependent measurements of the solutions did not show any sign of elastic behavior even at very low shear stresses (<0.05 Pa). Accordingly the boehmite solutions show no sol–gel transition in the used concentration range.

3.2. Interaction of boehmite and hydrophobin

1 wt.% solutions of boehmite and hydrophobin (HPB) are transparent and low viscous. Mixtures of the two compounds however show bicontinuous behavior with a swollen, turbid lower phase (Fig. 2).

The turbidity of the mixtures must be due to aggregates formed by the two particles. As both particles carry opposite charges, they obviously interact with each other. The hydrophobin adsorbs on the boehmite particles due to electrostatic attraction. The precipitates are not compact, but somehow swollen. We assume the hydrophobin covered boehmite units as sticky particles. Beside our previous investigations [13,21], other scientists have shown that hydrophobins self-aggregate and form monolayers [14,15]. We imagine that the hydrophobin does not loose these abilities due to adsorption on a solid surface. It could therefore be possible that the particles are forming a loose three-dimensional network. In order to prove this assumption, oscillating, rheological measurements of the swollen precipitate after centrifugation (2000 rpm, 5 min) of the 1:1 mixture (Fig. 2) have been performed. It turned out that the hydrophobin coated boehmite particles truly form a three-dimensional network. For detailed information consider the Supporting Information part. One of the most swollen samples, respectively the 1:1 mixture, will be taken as emulsifier for the formation of Pickering emulsions in Section 3 of the result part.

Table 1
Overview of the viscosity η at two different shear stresses τ (0.05 and 0.5 Pa) determined by oscillating measurements for boehmite solutions in the concentration range 0.5–10 wt.%. [Single column].

Boehmite (wt.%)	0.5	1	2.5	5	7.5	10
η (mPa s) at τ = 0.05 Pa	7.9	10.7	15.3	16.9	25.9	44.1
η (mPa s) τ = 0.5 Pa	5.4	4.8	5.7	5.2	6.7	7.9

Surface tension measurements of HPB solutions without and in the presence of 0.5 wt.% boehmite are shown in Fig. 3.

The results from the figure allow determining the adsorbed and free amount of hydrophobin at different concentrations. The results are summarized in Table 2.

The results in Table 2 show that 0.5 wt.% boehmite can bind up to 0.5 wt.% hydrophobin. The surface tension measurements indicate that the samples still contain some free protein when the total amount of protein and of boehmite is 0.5 wt.%.

Another important aspect comes up, if we remember the anionic exchange capacity (aec; 50 meq/100 g) of the boehmite. A concentration of 2.5 mM SDS is enough to compensate the charge of 0.5 wt.% boehmite. Referring to Fig. 3 0.1 wt.% HPB bind to 0.5 wt.% boehmite before the surface tension starts to decrease. 0.1 wt.% HPB corresponds to a molar concentration of 0.05 mM. Considering its amino acid composition, HPB contains five aspartic acid as well as twelve glutamine acid residues. If we assume all of them to be charged, the molar concentration would rise to 0.85 mM. Even this concentration is obvious not close to the aec. In order to understand this apparent discrepancy, one has to remind the differences between proteins and surfactants. The size of the folded HPB is about 5 nm [13]. Consequently, the required space per molecule for adsorption is much bigger compared to the surfactant SDS. The big size has two effects. On the one hand one protein molecule probably masks more than one positive charge of the boehmite. On the other hand sterical effects may play an important role preventing other protein molecules from adsorption. Moreover the possibility of conformational rearrangement upon adsorption of the protein in order to get a better electrostatic interaction with the boehmite is possible.

In Fig. 4 Cryo-TEM micrographs are shown from solutions of boehmite and of solutions of HPB covered boehmite nanoparticles.

The needle-like boehmite particles have sizes between 10 and 50 nm and are homogeneously distributed over the whole area in the pure boehmite sample. The boehmite particles in the thin film seem to show almost only parallel or perpendicular orientation to the film. These limited orientations are likely to be a result of the electrostatic repulsion between the boehmite particles. In the presence of equal amounts of hydrophobin, the boehmite particles are clustered into domains of 1 μm size. Moreover the hydrophobin coated boehmite nanoparticles seem to share many bridging points with each other. These multiple connections are an indication of the sticky character of the modified boehmite particles. Considering the Cryo-TEM micrographs, one can easily imagine the formation of a self-supporting three-dimensional network by the hydrophobin covered boehmite particles.

3.3. Synergistic emulsifying action of hydrophobin modified boehmite

In this chapter the influence of the used emulsifier type to the properties of the obtained emulsions will be investigated and compared to each other. All emulsions therefore have been prepared

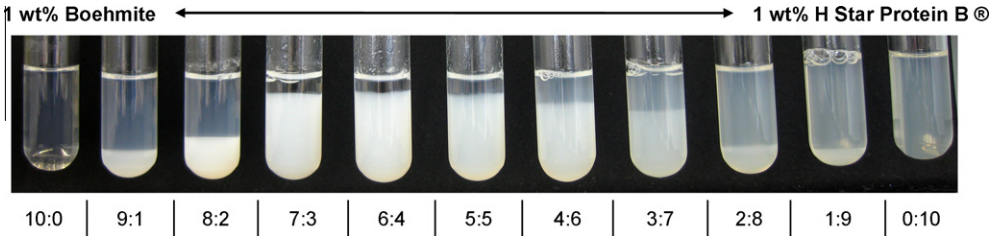

Fig. 2. Mixtures prepared from 1 wt.% boehmite and 1 wt.% H Star Protein® B (HPP) at pH 6.

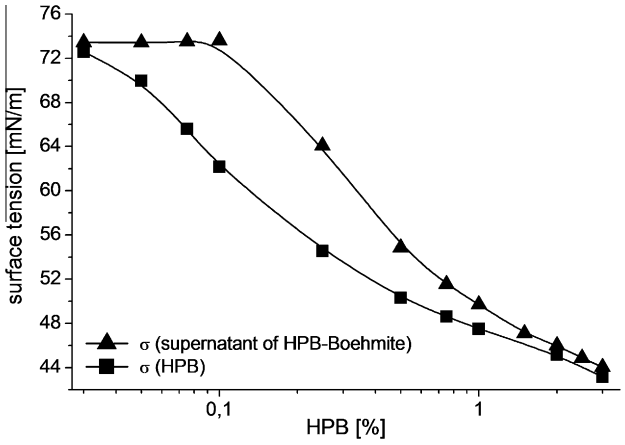

Fig. 3. Surface tension σ of the supernatants of samples with 0.5 wt.% boehmite and increasing concentrations of HPB in comparison with the surface tension of HPB alone. Drop-formation speed was 3 s/μl.

from water and silicon oil (PDMS) under the same conditions but with different emulsifiers. In Fig. 5 emulsions prepared with a high pressure emulsifier at a pressure of 1000 bar and an oil mass fraction Φ of 0.65 PDMS are shown. The used emulsifiers were 0.5 wt.% HPB (a), 0.5 wt.% boehmite (b) and 0.5 wt.% HPB in combination with 0.5 wt.% boehmite (c). The samples were photographed 1 day after preparation.

The sample with hydrophobin as emulsifying agent is a two layer system with an upper gel-like emulsion and a lower aqueous layer. Former investigations confirmed these emulsions to be of the o/w type [13]. Rheological measurements showed that the hydrophobin based emulsions have gel-like properties due to the formation of a self-supporting three-dimensional network. In fact, the emulsion shown in Fig. 5a is not a homogenous one. The two layer situation is probably due to the fact that the hydrophobin concentration is not high enough to cover completely the surface of the freshly sheared, smaller oil droplets. Using lower pressures (100 or 300 bar) resulted in homogeneous emulsions. The emulsion obtained from the pure boehmite (Fig. 5b) is an inhomogeneous, multilayer emulsion. It separates within one day in three layers, a lower aqueous layer, an aqueous emulsion and a very small, upper oily layer that can hardly be seen in Fig. 5b. It is noticeable, that a solution of 0.5 wt.% boehmite has a surface tension of 72.80 mN/m indicating its very hydrophilic character, but it can act at least as weak emulsifying agent. Fascinatingly, the emulsion based on 0.5 wt.% boehmite covered with 0.5 wt.% HPB is a homogenous, tooth-paste like emulsion (Fig. 5c). As it is already obvious from the picture, the oil droplets in the emulsion must be held in a three-dimensional network built up by the hydrophobin covered boehmite particles. They act as sticky particles that are interacting with each other giving the emulsion gel-like properties.

In order to compare the rheological properties of the emulsion layers from Fig. 5, their corresponding storage moduli G' at a shear stress $\tau = 0.05$ Pa are plotted against the frequency f (Fig. 6).All storage moduli are independent from the used frequency indicating every emulsion to have gel-like properties.

Obviously there are big differences in the absolute values of G'. Interestingly, the storage modulus G' increased from 100 Pa (use of HPB alone) to more than 2000 Pa due to synergistic use of HPB and boehmite. If the storage moduli would be determined by the number density of the oil droplets, both storage moduli of the emulsions prepared with HPB are simply much too high. It had been concluded therefore that the storage modulus of the protein emulsions are due to the elasticity of the protein network that is formed by the interacting protein monolayers around the oil droplets [13,21] As the boehmite particles are covered by the protein, it is likely that the interaction in HPB based emulsions is very similar, because in each case the contact between the emulsion droplets is a protein–protein contact. The rigid, needle-like boehmite particles are obviously acting as stiffener of the self-supporting, three-dimensional hydrophobin network. Viscoelastic networks in general show a big diversity in the needed deformation to induce their breakdown [25,26]. Deeper rheological investigations showed that addition of boehmite increases the tolerance against deformation of emulsions based on HPB from 10% to almost 20%.

The emulsion prepared from hydrophobin coated boehmite particles (Fig. 5c) was also investigated with the Cryo-SEM technique (Fig. 7).

Fig. 7a provides an overview of the emulsion droplet structure. Due to the almost high internal oil phase content, the droplets are closed packed and touch each other. This spatial limitation will also support the entanglement of the hydrophobin–boehmite nanoparticles coating the emulsion droplets resulting in the formation of the rigid network. Furthermore the emulsion droplets are of a round shape and they seem to be hardly polydisperse. Their size is in good agreement as well as with theoretical calculations using the core–shell model as with result from light microscopy investigations. The average emulsion droplet size using light microscopy was determined as 1.05 ± 0.24 μm.

In Fig. 7b some of the emulsion droplets are covered with bright spots that are forming structures (white arrows). From their size they could be regarded as the hydrophobin coated boehmite particles. A few such spots are also visible in the aqueous bulk layer.

Table 2
Amounts of adsorbed and free hydrophobin (HPB) in wt.% for samples at a fixed boehmite concentration of 0.5 wt.%.

HPB total (wt.%)	0.03	0.05	0.08	0.1	0.25	0.5	0.75	1	1.5	2	2.5	3
HPB free (wt.%)	0	0	0	0	0.08	0.2	0.4	0.55	0.9	1.5	2	2.5
HPB adsorbed (wt.%)	0.03	0.05	0.08	0.1	0.17	0.3	0.35	0.45	0.6	0.5	0.5	0.5

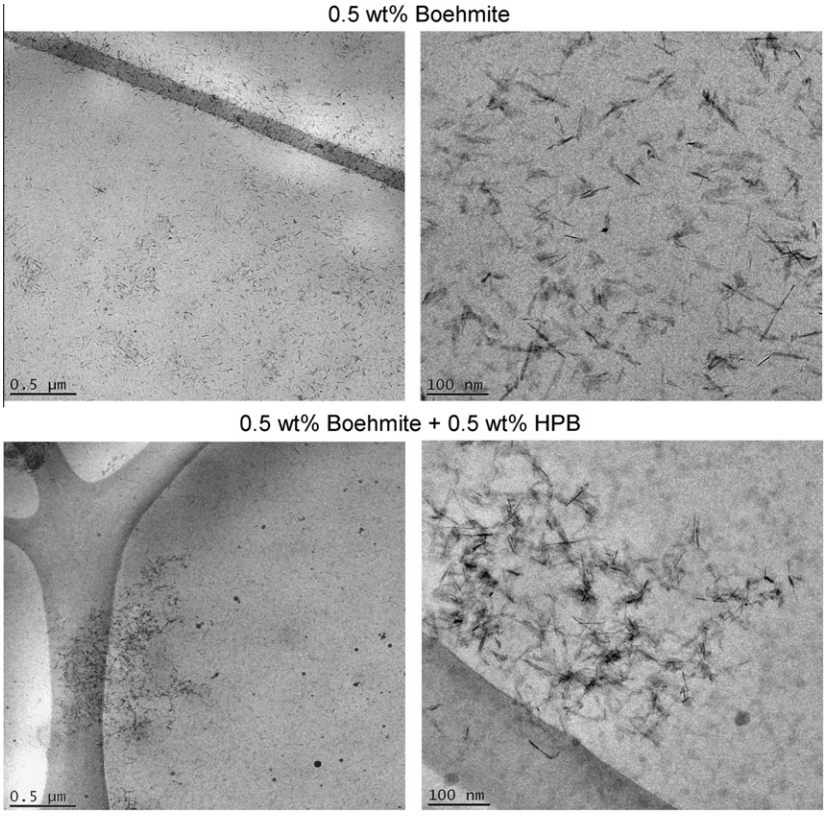

Fig. 4. Cryo-TEM micrographs of 0.5 wt.% boehmite and of a mixture of 0.5 wt.% boehmite and 0.5 wt.% HPB at different magnifications.

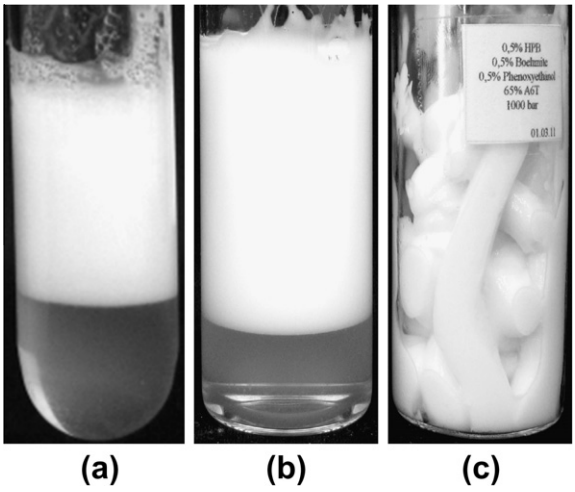

Fig. 5. Demonstration of synergistic emulsifying action. Shown are high pressure (1000 bar), 1 day old emulsions prepared from 0.5 wt.% HPB (a), 0.5 wt.% boehmite (b) and 0.5 wt.% HPB/0.5 wt.% boehmite (c) as emulsifiers, oil mass fraction $\Phi = 0.65$ PDMS.

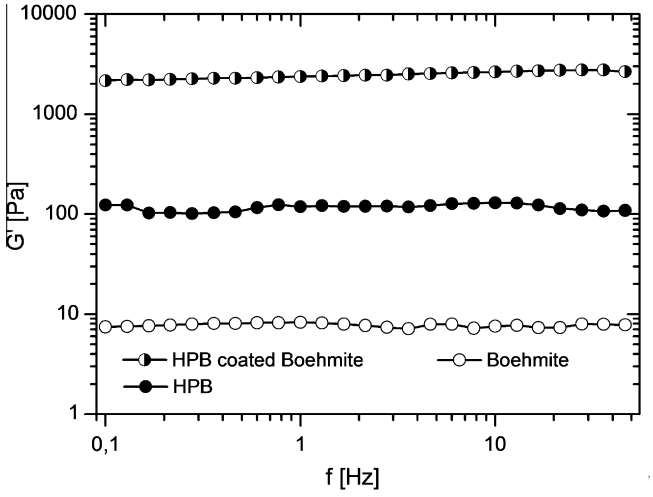

Fig. 6. Trend of storage moduli G' (Pa) of emulsion prepared from 0.5 wt.% boehmite (open circles), 0.5 wt.% HPB (closed circles) and 0.5 wt.% HPB and 0.5 wt.% boehmite (half-closed circles) acting as emulsifier. The data were obtained 1 day after emulsification at a shear stress τ of 0.05 Pa.

Interestingly, the sample without boehmite did not show any sign of these spots. Nevertheless, their number is quite low and their distribution among the emulsion droplets is not homogenous. It is therefore not possible to say definitely that these spots are the modified boehmite particles. Unfortunately, it was not possible to obtain Cryo-SEM micrographs at higher magnifications as the sample tend to strong charging effects.

It is also important to investigate the influence of the used emulsification machine, respectively vortexer and high pressure emulsifier. The emulsion droplet size depends typically on the shear stress used in the emulsification process [27]. Therefore, the emulsion droplet size and the storage moduli G' in dependency of the used shear stress for a Pickering emulsion prepared with the same amounts of emulsifier, water and oil was investigated (Table 3). According to Table 3 with increasing shear stress the emulsion droplet size as well as the droplets polydispersity decreased. The biggest change was obtained by switching from Vortex shaker to the high pressure emulsifier at 100 bar. A second effect can be observed by monitoring the storage moduli G' in dependency of increasing shear stress. The behavior of G' is contrary to it of the emulsion droplet

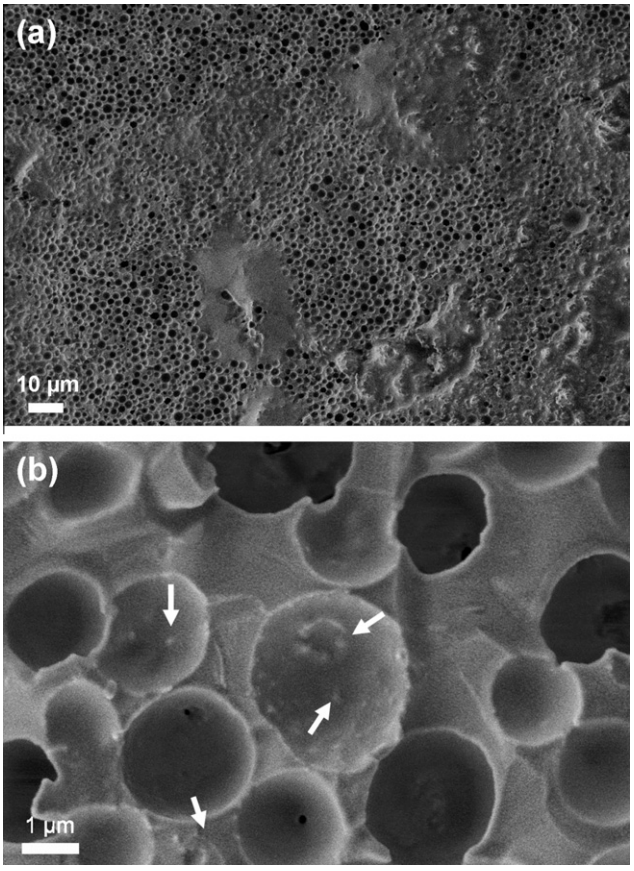

Fig. 7. Cryo-SEM investigation of the Pickering emulsion containing 0.5 wt.% HPB, 0.5 wt.% boehmite and an oil mass fraction Φ of 0.65 PDMS prepared at 1000 bar. White arrows in (b) show structures that could be formed by the hydrophobin coated nanoparticles.

size. With using higher shear stresses, G' increases, too. It is obvious, that a smaller emulsion droplet size corresponds to a higher number of oil droplets. This affects the formation and stiffness of the self supporting, three-dimensional hydrophobin–boehmite network. The enlarged number of oil droplets can fill up the network more densely. Moreover it promotes the stiffness of the network by creating more tie points for protein–protein entanglement between individual emulsion droplets.

In emulsion science and rather in its application in industrial products, like cosmetic creams, there is a great interest to obtain a long-term stable, homogenous emulsion layer. Therefore, it is indispensable to investigate the hydrophobin coated boehmite based emulsion for typical emulsion instability processes like creaming, Ostwald Ripening or droplet coalescence [28–30]. Typical ways to follow emulsion stability include monitoring the emulsion droplet size and the emulsions' visual appearance in dependency of time. It is noteworthy that the homogenous, single layer Pickering emulsions prepared with the high pressure emulsifier (Table 4) did not show any visual sign of instability, like oil creaming or phase separation, even after 170 days storage at room temperature. Moreover the emulsion droplet size did not change at all (Table 4), indicating the Pickering emulsions to have long-term durability.

Our former investigations [13,21] proved already that emulsions based on hydrophobin show ageing effects monitored by the storage moduli G' in dependence of time. We observed similar effects in HPB coated boehmite based emulsions (Fig. 8). The Pickering emulsions show a strong ripening within the first day after preparation indicated by the doubling of the G' values. The aging in these is due to the evolution of a three-dimensional network of the cross-linked hydrophobin–boehmite covered droplets while the size of the droplets does not change with time. Changes in the conformation of the protein molecules and of a partial entanglement of protein molecules with each other among the whole emulsion layer are likely to be responsible for the ageing phenomena. The sticky hydrophobin coated boehmite adsorbed at the o/w interface can bind to each other by hydrogen-bonding, van-der Waals attraction or the formation of chemical bonds by cysteine residues. The strong tendency for self-aggregation of hydrophobins was proven in our former study, showing the surface tension of HPB is strongly time dependent indicating some molecular rearrangements to take place. Indeed, the presence of four disulfide bridges make HPB truly a rigid molecule [10], but obviously without loosing its flexibility to undergo adsorption induced molecular reorganizations and interactions. As lysozyme also has four disulfide bridges and is proven to undergo similar refolding processes after adsorption, it is a good comparison to HPB [31]. Interestingly, however, is the fact that the storage moduli quadrupled after 170 days (Fig. 8). As the samples have been closed tightly, no water should have been evaporated. It seems that the final formation of the network is a long-time process.

As the hydrophobins contain four conserved disulfide bridges, it was interesting to note the influence of the addition of a typical disulfide breaker like dithiothreitol (DTT) on the Pickering emulsion. As no significant changes in the emulsion appearance as well as in its rheological properties could be observed, we assume the disulfide bonds not to be involved in the formation of the self-supporting protein network.

3.4. Effects of parameter variation to the emulsions

In this section we want to study the influence of varying different parameters to the Pickering emulsions based on hydrophobin coated boehmite particles. An important point is the investigation of the oil mass fraction dependence on the emulsion homogeneity and elastic properties. We assume the gel-like properties of the emulsions result from the attractive interaction between the hydrophobin–boehmite covered oil droplets. The dense layer of the protein covered particles on the oil droplets will try to increase their contacts, until the repulsive forces originating from the packing will overcome the attractive forces. Therefore diluted, freshly prepared, homogenously looking emulsion layers will contract, until both forces are balanced. The result will be a two layer situation, respectively in our case an upper emulsion layer and a lower aqueous phase. Experiments with varying the PDMS oil mass

Table 3
Average emulsion droplet size (µm) and storage moduli G' (Pa) in dependency of the emulsification method, respectively Vortex shaker and high pressure emulsifier. The emulsion contained 0.5 wt.% HPB, 0.5 wt.% boehmite and an oil mass fraction Φ of 0.65 PDMS and was investigated directly after preparation.

	Vortex shaker	High pressure emulsifier		
		100 bar	300 bar	1000 bar
G' (Pa) at τ = 0.5 Pa and f = 1 Hz	12.8	761	1166	1352
Average droplet size (µm)	17.67 ± 14.40	1.39 ± 0.47	1.19 ± 0.24	1.05 ± 0.24

Table 4
Average emulsion droplet size (μm) in dependency of storage time at 25 °C for Pickering emulsions prepared with the high pressure emulsifier. The emulsions contained 0.5 wt.% HPB, 0.5 wt.% boehmite and an oil mass fraction Φ of 0.65 PDMS.

Storage time (d) at 25 °C	100 bar	300 bar	1000 bar
0	1.39 ± 0.47	1.19 ± 0.24	1.05 ± 0.24
7	1.40 ± 0.43	1.19 ± 0.24	1.05 ± 0.29
170	1.43 ± 0.34	1.21 ± 0.21	1.08 ± 0.28

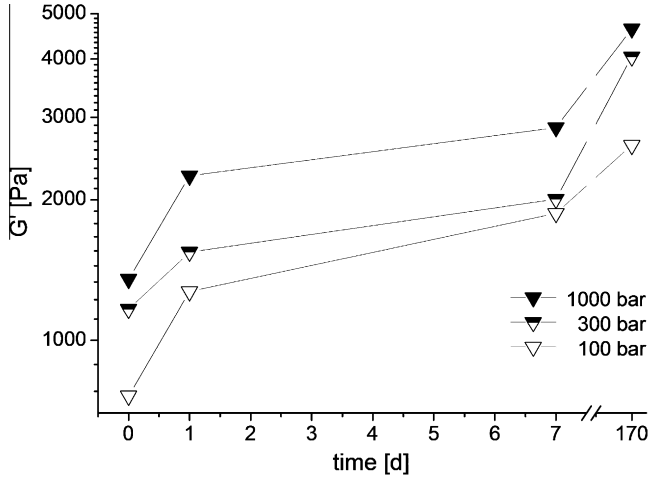

Fig. 8. Trend of storage moduli G' (Pa) (τ = 0.5 Pa; f = 1 Hz) in dependency of storage time for high pressure emulsions. The emulsions contained 0.5 wt.% HPB, 0.5 wt.% boehmite and an oil mass fraction Φ of 0.65 PDMS.

fraction Φ were performed in order to determine the minimum PDMS mass fraction for obtaining a single layer emulsion. All emulsion contained 0.5 wt.% HPB, 0.5 wt.% boehmite and have been prepared with the high pressure emulsifier at 300 bar. With using oil mass fraction Φ lower than 0.3, the homogenous emulsion layer contract within a short time, resulting in a two layer system. With increasing the oil mass fraction, the progress of the emulsion layer's contraction was weaker. Long-term stable, homogenous Pickering emulsions need to have an oil mass fraction Φ higher than 0.3. The trend of the storage moduli G' in dependency of the used oil mass fraction Φ is shown in Fig. 9. In order to be free from aging effects, the rheological measurements were immediately performed after the emulsification process.

The storage moduli G' is increasing with higher oil mass fractions Φ, until almost a G'-plateau at $\Phi \geqslant 0.5$ is reached. At these oil mass fractions the freshly formed, loose three-dimensional network of the hydrophobin covered boehmite is almost filled. A further increase in the storage moduli due to aging effects will therefore be detected with longer incubation times as shown in Fig. 8.

For possible industrial or cosmetic applications, it is also of great interest to check what type of oils can be emulsified by the system. In order to investigate homogenous emulsion layers, Pickering emulsions based on 0.5 wt.% HPB, 0.5 wt.% boehmite and an oil mass fraction Φ of 0.65 have been prepared with the high pressure emulsifier at 300 bar. The chosen oils were Dodecane, an apolar and low molecular weight oil, the silicon oil PDMS and the polar oil Octyl-methoxycinnamate (OMC). Using our system enabled to emulsify all three types of oil. By eye all emulsions looked quite similar: They were homogenous single layer emulsions with toothpaste like appearance and a yield stress value that prevents the emulsions from forming a horizontal meniscus. In Table 5 the average droplet size and the storage moduli G' (τ = 0.5 Pa; f = 1 Hz) for each oil type is summarized.

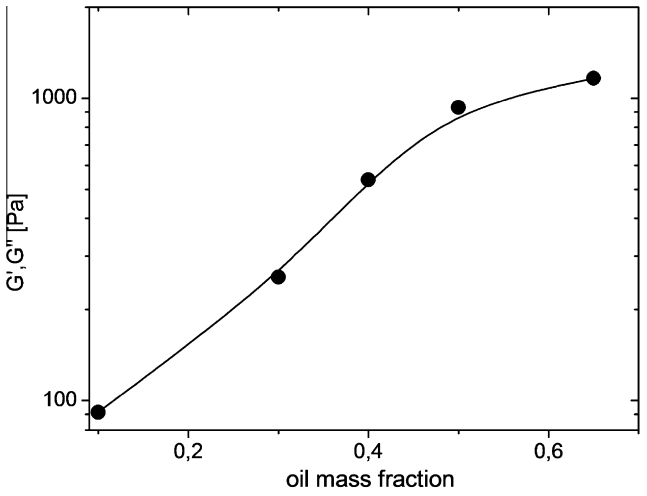

Fig. 9. Trend of storage moduli G' (Pa) (τ = 0.5 Pa; f = 1 Hz) in dependency of the used oil mass fraction Φ for high pressure emulsion measured directly after preparation. The emulsions contained 0.5 wt.% HPB, 0.5 wt.% boehmite and an oil mass fraction of 0.65 PDMS.

Table 5
Average droplet size (μm) and storage moduli G' (Pa) (τ = 0.5 Pa; f = 1 Hz) for Pickering emulsions prepared with different types of oil, respectively dodecane, PDMS and Octyl-methoxycinnamate (OMC). All emulsions were prepared from 0.5 wt.% HPB, 0.5 wt.% boehmite and an oil mass fraction Φ of 0.65 at 300 bar. The investigations were directly after emulsification performed.

Oil type	Dodecane	PDMS	OMC
Average droplet size (μm)	1.51 ± 0.51	1.19 ± 0.24	1.27 ± 0.31
G' (Pa) (τ = 0.5 Pa; f = 1 Hz)	1718	1166	1707

The average droplet sizes as well as the storage moduli for the three oils do not differ significantly (Table 5). The storage modulus of an emulsion is a parameter that can vary many orders of magnitude for emulsions with the same structure and the same dimension of the droplets (~2 μm). As the oil type obviously does not influence the storage moduli dramatically, our assumption that the storage modulus of the emulsions is mainly controlled by the elastic properties of the films around the oil droplets is again confirmed.

Finally, it is also very important to determine the minimum concentration of the hydrophobin coated boehmite particles that are needed to maintain the ability to emulsify high mass fractions of oil. Emulsification experiments were performed with keeping the ratio of hydrophobin to boehmite 1:1, but lowering their total concentration. The obtained samples are shown in Fig. 10. The emulsions containing 0.05 and 0.1 wt.% of each, HPB and boehmite, are unstable, multilayer systems. Some oil has immediately creamed up, the emulsion layer itself is inhomogeneous. The emulsion prepared with 0.25 wt.%, however, is homogenous and shows signs of the already observed tooth-paste character for emulsion containing 0.5 wt.% of HPB and boehmite (Fig. 5). Even the yield stress is significantly higher, as this emulsion does not form a horizontal meniscus.

It has to be reported that the appearance of the emulsions containing 0.05 and 0.1 wt.% HPB and boehmite showed a big shear rate dependency. It was possible to emulsify 65 wt.% PDMS using the Homo Disper at low shear rates of 1000 rpm. Applying these emulsions to the high pressure emulsifier, however, lead to partial breakdown of its emulsifying abilities (Fig. 10). This phenomenon can be explained as follows: The higher the shear stress the more the oil droplet size decreases and the more the freshly produced interface increases resulting in a much higher oil droplet number.

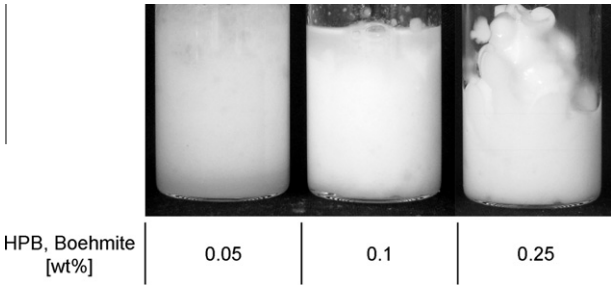

Fig. 10. Pickering emulsions with keeping the ratio of hydrophobin to boehmite 1:1, but varying their total concentration from 0.05 to 0.25 wt.%. The PDMS oil mass fraction was 0.65. The emulsions were prepared with the high pressure emulsifier at 300 bar and directly photographed.

Consequently, coalescence is more likely to happen faster at higher shear stresses, if the fresh interface is not fast enough protected by the emulsifier. Therefore, the ratio between the adsorption kinetic of the hydrophobin coated boehmite to the interface and the coalescence kinetic is the critical parameter for emulsion stability.

Table 6 provides an overview of the storage moduli in dependence of the used concentration of HPB and boehmite.

Increasing the concentration of HPB and boehmite does also result in higher storage moduli and consequently in higher elastic properties of the Pickering emulsions. It is possible to obtain homogenous, single layer Pickering emulsions with high elastic properties with already 0.25 wt.% of HPB and boehmite.

3.5. Comparison to the system clay and hydrophobin

Previously we used HPB in combination with the clay Laponite XLG in order to stabilize Pickering emulsions. Besides the fact that both particles carry an excess negative charge, the hydrophobin adsorbs to the clay surface. Nevertheless, very stable Pickering emulsions could be obtained [21].

In this study we replaced clay to boehmite. Two main differences exist between both particles. On the one hand the boehmite has a needle-like appearance in comparison with the disc-like shape of the Laponite clay. On the other hand the boehmite carries an excess positive charge. Therefore it was interesting to study Pickering emulsions prepared from both combinations. The obtained Pickering emulsions in both cases have a homogenous appearance, are very stable and show similar rheological behavior. On the microscopic level the average emulsion droplet size for the combination of hydrophobin and clay is twice (2.3 ± 1.0 μm) as the one determined for hydrophobin and boehmite (1.1 ± 0.2 μm). Even the polydispersity is quite lower due to the use of boehmite. These two phenomena might be correlated with the different shape of the clay and boehmite particles. The needle-like boehmite could be more effectively in surrounding droplets than the disc-like clay particles.

As the hydrophobins effectively adsorb on positive as well as negative loaded surfaces, the hydrophobin adsorption is independent of the surface charge type. Its ability to form gel-like emulsions is not affected, too. The clays or boehmite particles act obviously as additional stiffener of the self-supporting hydrophobin network promoting an increased yield stress to the emulsion.

Table 6
Storage moduli G' (Pa) (τ = 0.5 Pa; f = 1 Hz) for Pickering Emulsions keeping the ratio of hydrophobin to boehmite 1:1, but varying their total concentration from 0.05 to 0.5 wt.%. The PDMS oil mass fraction was 0.65. The emulsions were prepared with the high pressure emulsifier at 300 bar.

HPB, boehmite (wt.%)	0.05	0.1	0.25	0.5
G' (Pa)	3.7	136	913	1166

4. Conclusion

A novel way stabilizing oil in water emulsions using the synergistic action of hydrophobin and boehmite nanoparticles has been developed and characterized. This combination offers three major features that clearly separate it from most other common emulsions or Pickering emulsions: At first, the active emulsifier concentration is very low, with not more than 1 wt.%. Furthermore the system is independent from the used oil type, and long-term stability of the Pickering emulsions is maintained by its intrinsic gel-like property.

Ordinary emulsion stability approaches include the introduction of a repulsive energy between the emulsion droplets in order to prevent coalescence events [16–18]. Our study, however, presents an extraordinary and new way guaranteeing long-term emulsion stability. The hydrophobin is known for self-aggregation and film formation [7,8] indicating the existence of an attractive adsorption energy overcoming the repulsive electrostatic force in the short range distance. The adsorption is referred to be irreversible [14]. The hydrophobins do not loose their tendency of self-assembly after adsorption to an interface. As a consequence we assume the hydrophobin coated boehmite nanoparticles to be sticky particles. The emulsion droplets surrounded by them aggregate with each other without showing typical emulsion instability mechanisms [13]. The self-supporting hydrophobin–boehmite network entraps the oil droplets and provides gel-like properties.

The hydrophobin effectively adsorbs to negatively charged surfaces like clays [21] as well as now shown to positively charged ones, too. The resulting Pickering emulsions are in both cases gel-like. Therefore a lot of new opportunities in the surface modification field as well as the use of other combinations in Pickering emulsion science are unclosing for scientists as well as industrial users.

This work may contribute significantly to emulsion science by offering a novel emulsion stabilizing mechanism. Obviously, emulsion stability and simultaneous emulsion droplet aggregation are no longer considered to be antipodes. In order to address the question if the hydrophobin can be replaced by other proteins or polymers, an additional paper for the combination of amphiphile and clay is in preparation.

Acknowledgment

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jcis.2011.10.050.

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Supplementary Figures

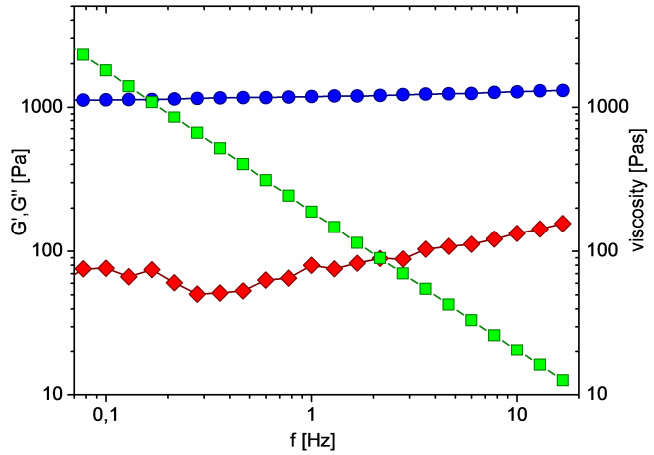

Fig. S1: Rheogram of the pellet prepared from the mixture of 0.5 wt% HPB and 0.5 wt% boehmite. Blue: storage modulus G' [Pa], red: loss modulus G'' [Pa] and green: viscosity η [Pas]. [Single column].

Publication D

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Boosting the Stability of Protein Emulsions by the synergistic use of Proteins and Clays

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Abstract The preparation and the properties of high pressure emulsions based on five different proteins are reported. As proteins we used the well-studied bovine serum albumin (BSA), a biotechnical produced hydrophobin called H Star Protein B ® (HPB), a protein isolate from soybeans, a wheat protein isolate (Plantasol W) and a commercially available yeast extract. All emulsions were characterized by visual appearance, light microscopy, conductivity and rheological measurements. Beside the emulsion based on soy protein isolate all other samples showed phase separation under the used conditions (0.5 wt% protein; 50 wt% oil). Plantasol W and yeast extract formed the most unstable emulsions showing typical instability processes like coalescence. Gel-like properties have been observed for emulsions based on BSA, soy protein isolate and HPB. The same proteins were also used to stabilize emulsions after their adsorption on clay particles. Interestingly, all emulsions had gel-like properties with a yield stress value and were stable to the used conditions. It is concluded that the gel character results from the stickiness of the protein covered oil droplets and is independent from the used protein type. The proteins which are adsorbed on the oil droplets can still interact and bind to proteins on other oil droplets.

Keywords: BSA, emulsion, hydrophobin, protein-clay synergism, rheology

Introduction

Strategies to stabilize emulsions are based among other things on the use of effective emulsifying agents. Good emulsifiers are known to provide a sterical and repulsive barrier [1-4] between the oil droplets in order to prevent the emulsion droplets from emulsion instability processes like coalescence [5,6] or Ostwald Ripening [7]. Usually the use of surfactants [8,9] or hydrophobic modified polymers [10-13] should help to overcome or at least to decelerate these phenomena. As a consequence of the ionic charges of the surfactant coated oil droplets their interaction is usually repulsive. For moderate oil mass concentrations (up to 40 %) such emulsions systems have a low viscosity and can easily be diluted with water [14].

Recently, we have reported emulsions which had been stabilized by a bio-technologically produced hydrophobin, called H Star Protein B ® (HPB) [15,16]. In spite of the fact that HPB

carried an excess negative charge, surprisingly, the interaction between the hydrophobin covered oil droplets was attractive and therefore contrary to the stabilization approaches described above. A self-supporting, three-dimensional network was built up by the HPB covered oil droplets. The rigidity of the network was due to partial interpenetration, entanglement and bridging of hydrophobins located on different emulsion droplets. Therefore, the visual appearance as well as the rheological characterization of emulsions based on HPB showed that they behaved like soft gels. Furthermore, the joint use of hydrophobin and clay particles resulted in homogenous emulsions with tooth-paste like character [17]. Rheological measurements proved that theses emulsions had even stronger gel-like behaviour than without the use of clay. It was therefore concluded that hydrophobin and clay offer a synergistic emulsifying performance. The rigid clay particles provide extra stiffness to the network. The hydrophobin adsorption on the clays was confirmed by surface tension measurements and by Cryo-TEM observations. The disc-like clay particles can be coated with hydrophobin at both sides. The resulting clay-hydrophobin sandwiches are now amphiphilic and bind strongly on oil droplets. It was possible to obtain long-term stable, homogenous oil in water (o/w) emulsions with as little as 0.5 wt% of each, clay and hydrophobin. The emulsions offered storage moduli *G'* values above 1000 Pa. In addition the emulsions had a high yield stress value. In summary, these properties of the emulsions based on hydrophobin and clay can only be explained by the existence of attractive forces between the clay-hydrophobin coated oil droplets. In literature hydrophobins are well known as versatile proteins of fungal origin [18-20]. One of their unique properties is their tendency to self-aggregation [21-23], even at interfaces [24-27]. Recently the group of Lips also confirmed the existence of an adhesion energy acting between hydrophobin molecules [28]. It turned out that the attraction between hydrophobins is less due to charge-charge interaction than rather to short-range hydrophobic interaction.

In our previous investigations hydrophobin was exclusively used as protein, because it is known to provide the lowest surface tension among proteins [29,30]. This study concerns the questions, if hydrophobin can be replaced by four other commercially available proteins. We therefore used the well characterized bovine serum albumin (BSA), a wheat protein isolate called Plantasol W, a soy protein isolate as well as cheap yeast extract. We compare their emulsifying abilities (straight away from the laboratory bench) to them of hydrophobin for emulsions containing equal amounts of oil and water. Particular attention is paid to the emulsion stability against phase separation, the emulsion droplet size and the rheological properties in dependency of time. Moreover we evaluate if these emulsions based on clay and

other proteins beside hydrophobin have similar properties as the ones prepared with hydrophobin coated clay particles [17].

Materials and Methods

H Star Protein ® B, abbreviated as HPB, is a recombinant hydrophobin[15] and was provided by BASF SE, Ludwigshafen. HPB has a molecular weight of 19 kDa and an isoelectric point (IEP) of 6.15. HPB consists of the class I hydrophobin DewA from the fungi *Aspergillus nidulans* and a truncated form of the *Bacillus subtilis* protein yaaD. Lyophilized bovine serum albumin (BSA) was purchased from Serva Electrophoresis, Heidelberg. The molecular weight of BSA is around 67 kDa and has at 4.7 its IEP. Plantasol W is a hydrolyzed wheat protein produced by Gelita GmbH, Eberbach. The protein content is given as 77-85 wt% with an average molecular weight of 1-5 kDa with an IEP of 4 to 4.5. NutritionRx, Buchholz, supplied the soy protein isolate with an average protein content of 87 wt%. The yeast extract was bought from Carl Roth GmbH & Co KG, Karlsruhe. The protein content is reported to be around 70 wt%. One fifth of the dry substance of *Saccharomyces cerevisiae* is referred to be originated from the cell wall. A major component of it is the mannoprotein (50%), which has long been known as effective bioemulsifier[31]. As Plantasol W, soy protein isolate and yeast extract are quiet cheap materials, we just used them as received. The clay Laponite XLG[32] was bought from Rockwood Clay Additives GmbH, Moosburg. Polydimethylsiloxane (PDMS) with a general formulation of $(CH_3)_3SiO[(CH_3)_2SiO]nSi(CH_3)_3$ was purchased from Shinetsu Kagaku, Tokyo. Its polymerization degree η is ranging from 5 to 19 (>98 %) and the viscosity is approximately 6 mPas. Other chemicals not specified in the text were of analytical grade or equivalent.

With the volume-drop tensiometer TVT1 from Lauda Co., Königshofen, the surface tension σ of the samples was measured at a constant drop-formation speed of 1 µl/s. In order to determine the free amount of protein in the clay-protein mixtures the supernatant of the samples was used. Therefore the samples were centrifuged in a Medifuge from Heraeus Instruments GmbH, Hanau, for 10 minutes at 2000 g.

All emulsions were prepared from aqueous solutions of proteins alone or in combination with clay. All emulsions contained 0.5 wt% phenoxyethanole in order to suppress microbial growth. As one step oil addition to the aqueous phase led to emulsion breakdown, high oil content emulsions could only be obtained by stepwise addition of oil. Before using the High Pressure Emulsifier (APV 1000, Albertslund) the emulsions were pre-emulsified with the Homo Disper (Tokushu Kika, Osaka) at an emulsification speed of about 1000 revolutions per

minute (rpm). The pre-emulsions were transferred to the High Pressure Emulsifier and passed three times through the device at the desired pressure of 300 bar.

For Light Microscopy investigations following protocol was used. As most of the emulsions have a gel-like behaviour due to the sticky character of the emulsion droplets, it was not possible to dilute the samples directly with water. Therefore another way had to be chosen. After applying the emulsion layer to the microscope slide and closing it with the cover slide, it was just gently shifted a bit, in order to get a thin emulsion layer. Then focusing through the sample never resolved more than two droplet layers at the same time. Consequently, using one sharpness level gave a good overview about the average droplet size. Moreover the polydispersity was low and indicates the light microscopy to be appropriate in order to determine the average droplet size. The light microscope was from Zeiss, respectively the model: 47 60 05 – 9901. Using the DFK 41F02 camera allowed to digitalize the micrographs. The analysis was performed with the IC capture 2.1 software (The Imaging Source, Bremen).

The rheology of the emulsion layers was measured at 25 °C with a cone-plate rheometer RheoStress 600 from Haake Thermo Scientific, Karlsruhe. The Haake RheoWin Data Manager, Version 3.3, was used to analyze the experimental data.

Results and Discussion

Surface tension measurements

Proteins are amphoteric molecules and therefore lower the surface tension of aqueous solutions [33;34]. This property is provided by the building blocks that proteins are made from. In order to characterize the surface activity of the five proteins used in this study, we present here their surface tension profile in the concentration range from 0.01 to 5 wt% (Fig. 1). The drop formation speed was adjusted to 1 s/µl.

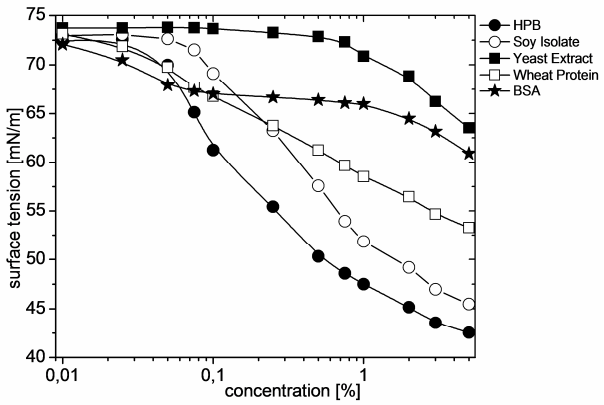

Fig. 1 Surface tension profile of HPB (closed circles), Soy Isolate (open circles), Yeast extract (closed squares), Wheat Protein-Plantasol W (open squares) and BSA (stars). The surface tension was measured at a drop formation speed of 1 s/µl.

All used proteins lower the surface tension of aqueous solutions. The surface tension decreasing ability increases from yeast extract over BSA, Plantasol W (wheat protein) and soy protein isolate to HPB. HPB is therefore the most surface active protein in our study. In literature hydrophobins are referred to be the most surface active proteins to be known at the moment [35,36]. The detailed experimental surface tension values for concentration of 0.5 wt% and 5 wt% are summarized in tab. 1.

Tab. 1 Overview of the surface tension σ of five different proteins at concentrations of 0.5 and 5 wt%. The drop formation speed was adjusted at 1 s/µl.

	yeast extract	**BSA**	**Plantasol W**	**soy protein isolate**	**HPB**
0.5 wt%	72.86 ± 0.04 mN/m	66.39 ± 0.03 mN/m	61.25 ± 0.08 mN/m	57.56 ± 0.05 mN/m	50.31 ± 0.06 mN/m
5 wt%	63.52 ± 0.02 mN/m	60.91 ± 0.60 mN/m	53.28 ± 0.22 mN/m	45.49 ± 0.18 mN/m	42.57 ± 0.10 mN/m

It should be noted that preliminary studies showed that aqueous solutions of soy protein isolate are two layer systems. The swollen, turbid lower phase makes approximately 30% of the total sample volume. We tried to overcome this two phase separation by changing the pH to lower (+HCl) and higher (+NaOH) values. It turned out that at a pH of 12 the sample became homogenous. Surface tension measurements however pointed out, that σ for 1 wt% solutions of soy protein isolate increased from 51.84 ± 0.07 (pH 6) to 55.69 ± 0.36 (pH 12). As we assume the emulsion properties to be directly linked to the surface activity, we used for the emulsion preparation the soy protein isolate in the two layer state.

Emulsions prepared from different proteins

In this section we want to compare the visual properties, stability, emulsion droplet size and rheological behaviour of emulsions based on the proteins used in this study. Therefore we kept the conditions similar for all emulsions, but just changed the protein type. For the

emulsification process the high pressure emulsifier was used at 300 bar. An emulsifier concentration of 0.5 wt% was chosen, the oil content was adjusted to 50 wt% polydimethylsiloxane (PDMS). In fig. 2 the obtained emulsions are illustrated directly after preparation (upper row) as well as three days after incubation at room temperature (lower row). Conductivity measurements indicated all emulsions to be of the oil in water type. Immediately after preparation the emulsions based on Plantasol W and yeast extract start to phase separate. After 3 days the emulsion layer disappeared, an upper oily and a lower aqueous phase remained. Although the oil mass fraction should be 0.5, these two emulsions do not show equal fractions of oil and water (lower row). This is probably a consequence of the fact that both proteins have weak emulsifying abilities. While filling the high pressure emulsifier with the pre-emulsion, it broke almost completely up. Consequently due to the lower density of PDMS, it moved upwards in the solution. A direct mixture of oil and water in the emulsification machine was not possible anymore. The two bottled emulsions therefore showed no equal volume ratios of oil and water.

Fig. 2 Emulsions containing 50 wt% PDMS stabilized by 0.5 wt% of different proteins. The upper row shows the emulsion directly after preparation, while the lower row demonstrates the situation after 3d incubation at room temperature. The dotted line in the Plantasol W based emulsion is as guide for the eye in order to demonstrate the phase boundary. The filling level is different for technical reasons.

Somewhat different is the situation for the emulsions based on soy protein isolate, BSA and HPB. Directly after preparation all of them are homogenous emulsions. After three days incubation at room temperature, however, the BSA and HPB based emulsions have phase separated indicated by the appearance of lower water like phases. Moreover the BSA based emulsion provided an additional instability process, known as oil creaming. Contrary to all

other emulsions the one based on soy protein isolate was still homogenous and did not present any signs of instability.

As noted before, emulsion destabilisation mechanisms include processes that are known as coalescence or Ostwald-Ripening. These changes in the emulsion structure are accompanied by an increase in the average emulsion droplet size (ads). In order to check the studied emulsions for droplet size altering, their average droplet size was determined directly after preparation and three days later by light microscopy (tab. 2).

Tab. 2 Overview of the average droplet size (ads) for emulsions based on different proteins. The size was evaluated directly after preparation and three days later by light microscopy. The emulsion contained 0.5 wt% protein and an oil mass fraction Φ of 0.5 PDMS.

	yeast extract	**BSA**	**Plantasol W**	**soy protein isolate**	**HPB**
0 days	not determinable	2.6 ± 1.2	2.0 ± 0.7	2.6 ± 0.5	2.0 ± 0.9
3 days	not determinable	3.7 ± 3.8	not determinable	2.8 ± 1.1	1.9 ± 1.0

The average emulsion droplet size could not be determined in the case of the emulsion based on 0.5 wt% yeast extract as the emulsion immediately broke. Even the ads with 2.0 ± 0.7 for the Plantasol W emulsion might be much too low for the real one as spontaneous coalescence was observed. Nevertheless the ads (0 days) in the case of BSA, HPB and soy protein isolate seemed to be very similar and in the range of theoretical calculations using the core shell model (see supporting information). Moreover the polydispersity of the droplets is quite small. After 3 days the ads and mainly the polydispersity increased in the case of BSA, while they stayed almost constant for HPB and soy protein isolate. We conclude that the emulsions prepared from HPB and soy protein isolate are stable, maybe even for longer incubation times. This was already proven in the case of HPB [16]. Interestingly the only emulsion that stayed homogenous under the used conditions was obtained from soy protein isolate.

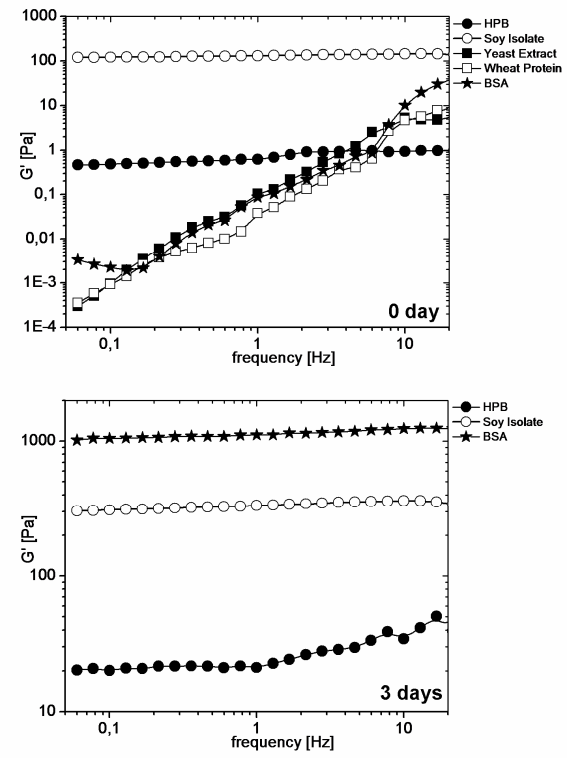

Fig. 3 Trend of the storage moduli *G'* [Pa] at τ = 0.5 Pa for emulsions based on 0.5 wt% of five different proteins. The storage moduli of the emulsion layers were evaluated directly after emulsification (above) and three days later (down). The emulsion contained 0.5 wt% protein and an oil mass fraction Φ of 0.5 PDMS.

In the case of emulsions prepared from HPB, we concluded the HPB covered emulsion droplets act as sticky particles. Their interaction resulted in the formation of a three-dimensional network [16]. Accordingly the storage moduli *G'* of these emulsions was much higher than it would be, if it was determined by the volume fraction of the droplets and their size distribution (see supporting information). The question arises, if similar effects can be observed in the emulsions from fig. 2. Therefore the rheograms at a shear stress τ = 0.5 Pa were measured. Fig. 3 shows the trend of the storage moduli *G'* for the five emulsion systems used in this study directly after emulsification and three days later.

From the frequency independent behaviour of *G'* in fig. 3 for the emulsion based on HPB and soy protein isolate one can directly conclude that these emulsions have elastic properties directly after preparation. Moreover the gel-like character increased remarkable with further incubation time. Whereas the emulsions based on BSA, Plantasol W and yeast extract show clearly viscous behaviour as the storage moduli *G'* is frequency-dependent. Interestingly the biggest change in the storage moduli *G'* seemed to be observed for the emulsion based on BSA, respectively from viscous to strong elastic appearance. It turned out, however, that the oil was squeezed out of the emulsion while measuring the rheogram after 3 days (fig. 4). The emulsion was agglomerated and sticky, but no longer homogenous.

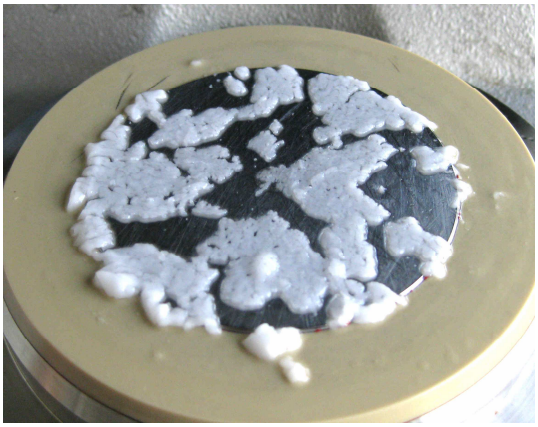

Fig. 4 Visual appearance of the emulsion based on 0.5 wt% BSA and an oil mass fraction Φ of 0.5 PDMS after rheological investigations.

From the obtained results in this chapter, we claim the unmodified soy protein isolate to have the best emulsification abilities under the used conditions of all proteins examined in this study.

Emulsions prepared from clays covered with different proteins

As mentioned already in the introduction part, recently we reported a novel hydrophobin clay synergism resulting in tooth-paste like emulsions [17]. Homogenous emulsions could be obtained with an oil mass fraction of more than 0.3. In order to check if such emulsions can also be prepared from other proteins than HPB, we replaced it with Plantasol W, soy protein isolate, BSA and yeast extract. All remaining conditions were kept the same; every emulsion contained 0.5 wt% protein and an oil mass fraction Φ of 0.5 PDMS. The used pressure was 300 bar. The visual appearance of these o/w emulsions directly after preparation and three days later is shown in fig. 5. The first noteworthy thing is that the emulsifying ability of Plantasol W and yeast extract boosted in comparison to the situation in fig. 2. Moreover for each protein-clay combination the corresponding emulsion was homogenous directly after preparation (fig. 5; upper row). A certain ripening of the emulsion layer after three days incubation at room temperature, however, was observed for all emulsions beside the one prepared with the hydrophobin HPB (fig. 5,; lower row). Furthermore this emulsion did clearly not form a horizontal meniscus indicating its gel-like properties can already be recognized by eye.

Fig. 5 Emulsions containing 50 wt% PDMS and stabilized by 0.5 wt% of different proteins and 0.5 wt% Laponite XLG. The upper row shows the emulsion directly after preparation, while the lower row demonstrates the situation after 3d incubation at room temperature.

As done in the case for the emulsions prepared from the proteins alone, the average droplet size (ads) was also measured in dependency of incubation time for the emulsions (tab. 3). The ads stayed constant if BSA, soy protein isolate or HPB was used in combination with clay. Interestingly the two proteins that form unstable emulsions, did also provide an increase in the ads as well in the droplet polydispersity in dependency of time. Coalescence as typical emulsion destabilization mechanism was therefore likely to have happened.

Tab. 3 Overview of the average droplet size (ads) for emulsions based on different protein-clay combinations. The size was evaluated directly after preparation and three days later by light microscopy. The emulsion contained 0.5 wt% protein, 0.5 wt% Laponite XLG and an oil mass fraction Φ of 0.5 PDMS.

	yeast extract	**BSA**	**Plantasol W**	**soy protein isolate**	**HPB**
0 days	5.1 ± 0.8	1.4 ± 0.4	5.3 ± 1.1	2.5 ± 0.7	1.9 ± 0.4
3 days	8.9 ± 3.1	1.3 ± 0.3	8.5 ± 2.2	2.7 ± 0.8	1.8 ± 0.4

For emulsions prepared from HPB, the additional use of clay resulted in an increase of the gel-like properties of the corresponding emulsions. The storage modulus *G'* rose to values ten times higher than without clay [17]. As the concentration of the clay was with 0.5 wt% far from its sol-gel transition, we concluded that the hydrophobin covered clay particles, act as sticky sandwiches. The clay introduced therefore an additional reinforcement of the self-supporting, three-dimensional hydrophobin network that showed an increased stiffness with

longer incubation times. In fig. 6 the trend of the storage moduli *G'* for the five emulsion systems (fig. 5) used in this study directly after emulsification and three days later is given.

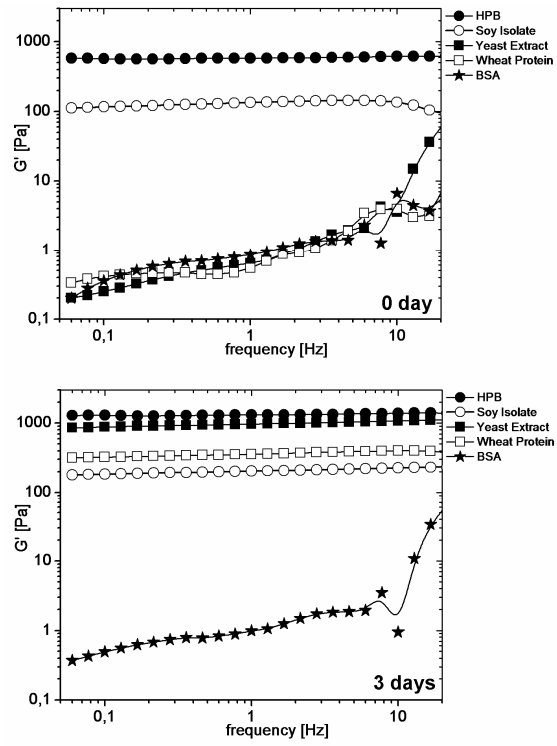

Fig. 6 Trend of the storage moduli G' [Pa] (τ= 0.5Pa) for the emulsions layers prepared from different protein-clay mixtures in dependency of time (0 days: upper row; 3 days: lower row). All emulsions were prepared from 0.5 wt% protein, 0.5 wt% Laponite XLG and an oil mass fraction Φ of 0.5 PDMS at a pressure of 300 bar.

Referring to the upper row of fig. 6 the storage moduli of the emulsions based on HPB or soy protein in combination with Laponite were frequency independent directly after preparation. In contrast the other emulsions did not show any signs of gel-like behaviour as their storage moduli were quite frequency-dependent. Interestingly, even the emulsion based on BSA that appeared to be very low gel-like after three days storage, showed aging effects (fig. 6; lower row). The strongest effects were surely observed in the case of the wheat protein (Plantasol W) and yeast extract. These emulsions changed from a rather viscous state directly after preparation to a high elastic behaviour after three days incubation at room temperature. The increase of the storage modulus G' for all protein-clay combination without BSA is due to the formation of a time-dependent, self-supporting protein-clay network surrounding the oil droplets. The surface active protein covered clay particles adsorb rapidly to the freshly prepared oil water interface during homogenization. Thereafter the protein molecules undergo time-dependent, conformational rearrangements in order to interact more effectively with the new oil-water environment and interpenetrate with each other [37-38]. The protein covered clay particles act as sticky sandwiches and form a time-dependent, self supporting network

that strengthens with incubation time. For the combination of clay and hydrophobin it was recently shown that this network remains after removing the oil and water by using freeze drying [17].

From the different elastic behaviour, it is obvious that the emulsion droplet-droplet interaction was different and depending on the used protein in combination with clay. The most effective interaction was observed in the case of HPB. Nevertheless, beside BSA, all other proteins showed a time-dependent evolving of their gel-like properties. It has to be noted that in the case of the wheat protein, the emulsion started to smell somehow suspect. Even though 0.5 wt% phenoxyethanole was used to prevent bacterial or fungal growth, its amount was probably not sufficient enough. However we did not want to increase the amount of it over the amount of the used protein, because from the chemical point of view phenoxyethanole can be considered as an amphiphile, too. In order to prove this, a emulsion replacing protein by 0.5 wt% phenoxyethanole was prepared. The other parameters were kept the same. The obtained emulsion separated quickly in a two layer system and was low-viscous as judged by its visual appearance. Rheological measurements confirmed the viscous behaviour as the storage modulus was frequency dependent. At a shear stress τ = 0.5 Pa and a frequency f = 1 Hz, *G'* was about 0.09 Pa. Moreover no sign of aging effects were observed.

Finally in order to affirm that all of the used proteins bind to the clay particles surface tension measurements were conducted. Therefore the surface tension of the supernatant of the protein-clay samples was measured. The obtained value corresponded to the one of the non-adsorbed, free protein concentration (fig. 1). By subtraction this concentration from the totally used protein concentration (0.5 wt%), the adsorbed protein concentration could be determined. Tab. 4 shows the measured surface tension of the supernatant of clay-protein mixtures as well as the concentration of adsorbed and non-adsorbed protein.

Tab. 4 Overview of the surface tension σ of the supernatants of mixtures of 0.5 wt% protein and 0.5 wt% Laponite XLG as well as the concentration of adsorbed and non-adsorbed protein.

	yeast extract	**BSA**	**Plantasol W**	**soy isolate**	**HPB**
σ [mN/m]	73.66 ± 0.05	73.21 ± 0.02	71.31 ± 0.02	71.48 ± 0.05	72.07 ± 0.03
adsorbed protein [wt%]	0.4-0.5	0.5	0.47	0.42	< 0.45
free protein [wt%]	0-0.1	0	0.03	0.08	< 0.05

The results in tab. 4 clearly show that in each case more than 80% of the used protein concentration is adsorbed at the clay particles. Interestingly in the case of BSA all protein is

adsorbed, nevertheless the corresponding emulsion has very weak gel-like properties. Obviously the interaction or attractive force between the adsorbed BSA molecules was quite low.

From the results obtained in this chapter we can conclude the following: Each of the used proteins adsorbs to clay particles. All of the obtained emulsions are different in their rheological behaviour. Moreover the one based on BSA has a low gel-like character. Therefore it can finally be ruled out, that the strong gel-like behaviour observed in the other cases was due to the gelation of the clay particles. Furthermore the synergistic use of clay in combination with proteins (beside BSA) led to a significant increase of their emulsifying abilities. It is also important to note that phenoxyethanole has truly amphiphilic properties and can act as emulsifying agent. Emulsions with gel-like properties however by using only it in combination with clay could not be obtained. The gel-like character of the emulsion in fig. 6 is due to the time-dependent formation of a three-dimensional protein network that is strengthened by the incorporation of stiff clay particles. The only emulsion that did not phase separate under the used conditions was the one prepared with hydrophobin indicating the obvious benefits of its use. However as it will be shown later, it is worth mentioning that by changing the conditions, stable homogenous emulsions can be obtained from the other proteins, too.

All emulsions investigated in this study show more or less gel-like properties that are a due to the amphiphilic character of proteins that coat the clay. Therefore the emulsion droplets that are covered by these protein-clay sandwiches show attraction in the short term distance. This assumption is also proven by the aging and contraction of the emulsion layer in dependency of time (fig. 5). An interesting question arises: Is it possible to obtain homogenous emulsions with higher gel-like properties by simply increasing the oil mass fraction Φ? Therefore emulsions containing 65wt% PDMS have been prepared from every protein-clay combination. The final concentration was: 0.5 wt% protein, 0.5 wt% Laponite XLG and 65 wt% PDMS. The obtained emulsions are illustrated in fig. 7. All emulsions had a homogenous look. Fascinatingly the visual appearance of the emulsions prepared from BSA and especially the one from HPB showed impressively their gel-like properties. With HPB a tooth-paste like emulsion had been established. Conductivity measurements proved that all emulsions still were of the o/w type.

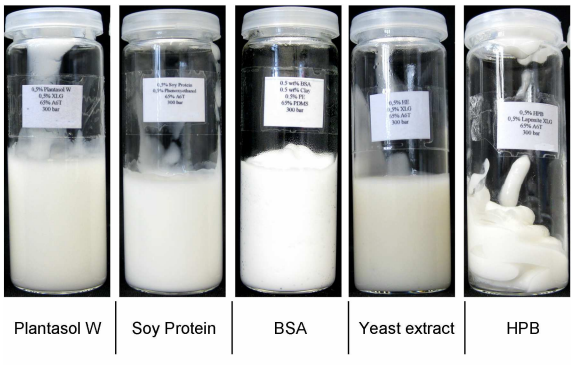

Fig. 7 Overview of emulsions (300 bar) prepared from 0.5 wt% protein and 0.5 wt% Laponite XLG directly after preparation. The used oil mass fraction Φ was 0.65 PDMS.

As the visual appearance did not change with further incubation time, only the emulsions directly after preparation are shown in fig. 7. It is also noteworthy that the emulsions, beside the one prepared from yeast extract, did not show significant changes in their emulsion droplet size in dependency of the incubation time (tab. 5). Light microscopy studies of the emulsion based on the synergism of yeast extract and clay after being incubated for three days showed explicit that microbial growth had already taken place. The increase in the ads as well as the emulsion's bad smell was probably the consequence of the emulsifier digestion by microbial life-forms.

Tab. 5 Average emulsion droplet size (ads) for emulsions based on protein-clay synergism in dependency of the incubation time. All emulsions contained 0.5 wt% protein, 0.5 wt% clay and an oil mass fraction Φ of 0.65 PDMS.

	yeast extract	**BSA**	**Plantasol W**	**soy protein isolate**	**HPB**
0 days	3.8 ± 0.5	1.3 ± 0.3	7.2 ± 2.7	1.6 ± 0.7	2.8 ± 0.7
3 days	6.2 ± 1.4	1.4 ± 0.3	7.1 ± 1.0	1.7 ± 0.6	2.4 ± 0.8

In order to determine and compare the elastic behaviour of all emulsions, rheological measurements had been conducted. The storage moduli G' against the frequency f at a shear stress τ of 0.5 Pa at two different time points, respectively directly after preparation and three days later, are presented in fig. 8.

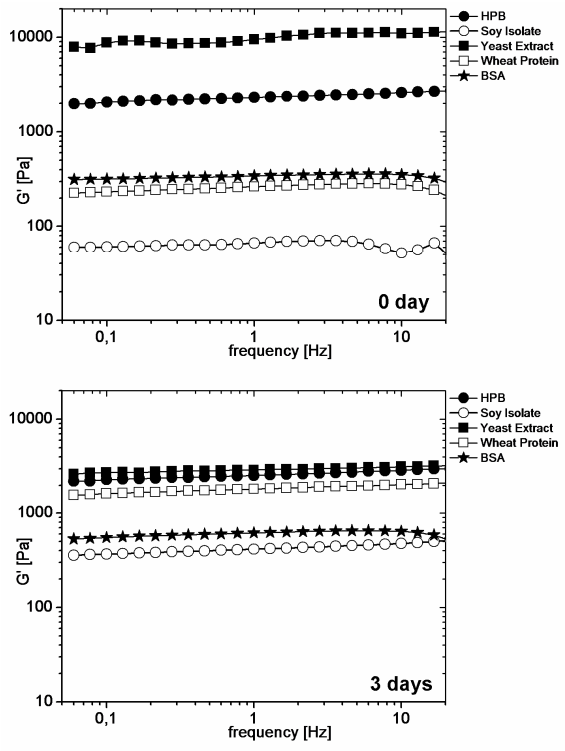

Fig. 8 Trend of the storage moduli *G'* (τ= 0.5Pa) evaluated in dependency of the storage time, respectively directly after preparation and after three days incubation at room temperature. All emulsions contained 0.5 wt% protein, 0.5 wt% Laponite XLG and an oil mass fraction Φ of 0.65 PDMS and were prepared at 300 bar.

As one can conclude from fig. 8, it is possible to obtain gel-like emulsions containing high amounts of oil by using only 0.5 wt% protein and 0.5 wt% clay. The absolute value of *G'* is depending on the used protein. Referring to fig. 8, upper row, the most gel-like emulsion is prepared from clay and yeast extract. The order for the gel-like character is: yeast extract, HPB, BSA, Plantasol W and soy protein. Nevertheless, as already mentioned, the emulsion prepared from yeast extract was prone to fast microbial growth. This is also proven by the weakening of its storage moduli after three days (fig. 8, lower row). All other emulsion, however, show aging effects due to the progressed protein-clay network formation. It is noteworthy to mention that the emulsions without the one prepared from yeast extract (fig. 7) offer long-term stability as one could assume from their gel-like behavior. For example the one prepared from hydrophobin and Laponite is now already stable for more than nine months.

Conclusions

Proteins, like hydrophobin or soy protein isolate, offer a new way of emulsion stabilization. In contrast to surfactants the oil droplets coated by them attract each other in the short term distance and form a self-supporting protein network with the emulsion droplets entrapped.

Due to the synergistic use of protein and clay at a total concentration of only 1 wt%, the emulsifying abilities of proteins, like from yeast extract or wheat protein, can be boosted. The obtained emulsions provide a long-term stability due to their intrinsic gel-like character.

All five proteins used in this study were surface active and therefore lower the surface tension of water. The surface activity of the used proteins depends on the amphiphilic character of the individual protein. Due to their amphiphilicity, it is likely that proteins bind to clays particles. The proteins used in this study differed in their emulsion abilities. The most stable emulsions under the used conditions were prepared from soy protein. Nevertheless, by changing the emulsifier concentration or the oil mass fraction it would probably be possible to obtain homogenous emulsions from all of them. Consequently, all proteins can be used for the preparation of emulsions. Contrary to ionic surfactants which form surfactant covered oil droplets that repel each other, these protein covered droplets attract each other. The resulting emulsions have therefore gel-like properties and cannot be diluted with water. Emulsions from protein covered clay particles are even more stable then emulsions prepared from the proteins alone. The shear modulus and the yield stress value of the protein covered clay emulsions are higher than the parameter from the protein emulsions. The storage moduli of the emulsions seem to be determined from the three dimensional network that surrounds and connects the individual emulsion droplets.

We conclude that by the choice of the protein in combination with or without clay and by adjusting the oil mass fraction Φ, one has an effective tool to adjust the properties of the desired emulsion. Surely the most stable, homogenous emulsions can be obtained by the use of hydrophobin, but nevertheless it is possible to replace it by other proteins.

Supporting Information – Theoretical considerations

a) Core-shell model

In the emulsification process the bulk organic liquid, the oil, is divided into little droplets with the radius r. As soon as the hydrophobic surface is formed, the amphiphilic molecules like surfactants or proteins will adsorb at the hydrophobic surface and prevent the droplets from coalescence and forming larger droplets again. The size of the droplets depends on the shearing rates that are produced by the homogenizer. Even with equal amounts of oil and water the formed emulsions should be of the o/w type because the used amphiphilic compounds are hydrophilic molecules and the curvature of the emulsion droplets should be

the same as in normal micelles, namely convex when looking from the oil to the water. It is conceivable that for high enough shear rates the complete amount of amphiphilic compounds will adsorb at the interface of the droplets and the amount of the amphiphilic compound will determine the final size of the droplets. For this situation the following equations will result.

(1) $\frac{m(oil)}{m(amphiphile)} = \mathrm{R}$

(2) $\frac{V(droplet)}{V(core)} = \frac{\frac{4}{3}\cdot\pi\cdot r^3}{4\cdot r^2\cdot d} = \frac{r}{3\cdot d}$

Where d is the thickness of the adsorbed layer, V is the volume and R is the mass ratio of oil to amphiphile. From the two equations the radius of the emulsion droplets r can be calculated.

(3) $\frac{r}{3\cdot d} = \mathrm{R}$

We obtain: $r = 3\cdot d\cdot \mathrm{R}$

For a given amphiphilic molecule the size of the emulsion droplets should increase linearly with the mass ratio of oil to amphiphile.

In our study about protein emulsions we used a ratio R of 100. The size of HPB is 5 nm. Inserting these values in equation (3) results in a theoretical radius of 1.5 µm, corresponding to a droplet size of 3 µm.

b) Storage module interpretation of the gel-like emulsions

Concentrated o/w emulsions can be looked as a system with densely packed droplets of oil in water. From this point of view the emulsions are similar systems as ringing gels from swollen globular micelles. The shear modulus of these systems can be understood on the basis of a theoretical model in which one globule represents one particle in the thermodynamic sense and the storage modulus of the system is proportional to the number density ν of the particles and indirect proportional to the structure factor S:

(4) $G = \frac{\nu\, kT}{S}$

The structure factor S is > 1 for attractive particle interaction and < 1 but > 0 for repulsive interaction [39].

The number density ν is simply given by the diameter of the globules:

$$\nu = \frac{1}{d^3}$$

(5)

The diameter of swollen micelles is in the range of 10 nm while the diameter of emulsion droplets is in the range of 1 µm. 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 [40].

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Publication E

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Pickering Emulsions stabilized by Amphiphile covered Clays

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A B S T R A C T

It is shown that all water soluble amphiphilic compounds like surfactants, polymers, block copolymers and proteins bind strongly to clay particles in water. On saturation, the clays bind up to the multifold weight of the additives of their own weight. Prior to saturation some amphiphilic compounds form precipitates with the clays while others do not. The soluble clay-amphiphile complexes do not lower the surface tension of water even though these particles must obviously be hydrophobic. It is concluded from these results that the clays alone are already hydrophobic particles. Solutions or precipitates of these amphiphile covered clay particles are ideal systems for the formation of stable Pickering emulsions. Depending on the used type and amount of amphiphile, the corresponding Pickering emulsions can have either viscous or gel-like properties with large storage moduli. For some combinations both the water and the oil can be removed from the emulsions without collapse of the three dimensional network of the films.

Keywords: Clay, Hydrophobin, Pickering Emulsion, Synergism, TTMABr

1. Introduction

Clays are interesting compounds for a wide range of applications [1-3]. The minerals can be exfoliated to single sheets in aqueous solutions. In this state the thin sheets have a thickness of one nm. Because of their large surface of about 1000 m^2/g, the clay particles can adsorb the manifold weight as their own weight. Clays are therefore used as adsorbers of many waste products like surfactants [4], dyes [5] or heavy metals [6] in the purification process of water. Clays are available either as natural or synthetic products. Natural clays are known under different names like Montmorillonite and Saponite. The diameter of the plate-like clay-particles ranges from 20 nm for synthetic clays to many µm for natural clays. Because of their large anisometry and possible surface modification, clays are often used for various purposes, for example as rheology modifiers [7]. The rheology of industrial formulations can be changed from shear thinning solutions to stiff gels with a few wt% of clays. In recent times clays have been used to strengthen the stiffness of polymers [8] and to improve the impermeability of gases through thin polymer films [9]. In combination with surfactants, clays can be made hydrophobic and these hydrophobic clay-particles can be dispersed in hydrocarbons [10]. In mixture with non-ionic surfactants clays have also been used for the preparation of Pickering emulsions [11]. This application brings up an interesting basic question. The surface of clays is generally considered to be a hydrophilic surface. Dispersions of clays have the same surface tension as water [12]. It is therefore assumed that clays are not surface active, but this conclusion may not be completely correct. It is known that polymers like polyethylene glycol which are considered to be hydrophilic bind on clays [13-14]. This result makes only sense, if the surface of the clays has somehow hydrophobic features.

Recently it was shown that one to one mixtures of clay-hydrophobin particles stabilize high internal oil in water (o/w) emulsions by synergistic interaction [15]. Only 1 wt% of hydrophobin and clay were needed to prepare homogenous emulsions. However, it was remarkable that both, the clay and hydrophobin protein are negatively charged. Rheological measurements showed that the Pickering emulsion from this combination has a high storage modulus G' (> 1000 Pa) and viscosity (1 Pas at $\gamma = 100\ s^{-1}$).

This manuscript focuses on three main aspects. The first part will be about the interaction between amphiphilic compounds and clay. Therefore especially the surface tension behaviour of the amphiphile-clay particles will be discussed in general terms. The second part deals with the question, what kind of one to one mixtures of amphiphile and clay will result in similar emulsions as obtained with the hydrophobin, called H Star Protein B ® [16]. The influence of the clay coverage with amphiphile on the visual appearance, stability and rheological behaviour to the emulsion is investigated. As a model system we used the cationic surfactant C_{14}-trimethylammoniumbromid (TTMABr) with Laponite XLG as clay compound. Finally, in the third part, we discriminated our emulsion systems from Pickering emulsions prepared with silica particles [17].

2. Materials and Methods

2.1 Materials

The cationic surfactant C_{16}-Trimethylammoniumbromid (CTAB) was bought from Merck, Darmstadt, whereas C_{14}-Trimethylammoniumbromid (TTMABr) was purchased from Clariant, Gendorf. Serva Electrophoresis GmbH, Heidelberg, provided the anionic surfactant Sodium-Dodecylsulfate (SDS). As non-ionic surfactant we chose alkyl polyoxyethylene glycol monomer type $C_{12}EO_{7.8}$ from Clariant, Gendorf, and Isotridecyloctaethylenglycolether ($C_{13}O_8$) from Sasol, Hamburg. As zwitterionic surfactant we used Tetradecyldimethylaminoxide (TDMAO) from Clariant, Gendorf, and Distearyldiammoniumchloride (DSDAC) from

Sanyo Chemical Industries, Japan. The non-ionic triblock copolymers Pluronic F38 and F127 were purchased from BASF, Ludwigshafen. The copolymer Polyoxyethylene$_{17}$-polyoxyporpylene$_4$-dimethylether (AQ 1704) was from Yuka Sangyo Co, Japan. The recombinant protein hydrophobin, called H Star Protein ® B (HPB)[16], was a gift from BASF, Ludwigshafen. The polyvinylalcohole (PVA, trade name Polyviol® LL 2860) was acquired from Wacker, Burghausen. Polyvinylpyrrolidon (PVP) was bought from Henkel, Düsseldorf, and Poly-diallyldimethylammoniumchloride (DADMAC) was obtained from Aldrich, Steinheim. The clay Laponite XLG [18] was purchased from Rockwood Clay Additives GmbH, Moosburg. It has a cationic exchange capacity (cec) of 65.7 meq/100g Polydimethylsiloxane (PDMS) was acquired from Shinetsu Kagaku, Tokyo. It has general formulation: $(CH_3)_3SiO[(CH_3)2SiO]nSi(CH_3)_3$. The polymerization degree η is ranging from 5 to 19 (>98 %) and the viscosity is approximately 6 mPas. Other chemicals not specified in the text were of analytical grade or equivalent.

2.2 Surface Tension

The surface tension σ of the samples was measured with the volume-drop tensiometer TVT1 from Lauda Co., Königshofen at a constant drop-formation speed of 1 s/µl. In order to determine the free amount of amphiphile in the clay-amphiphile mixtures, the supernatant of the samples was used. Therefore the samples were centrifuged in a Medifuge from Heraeus Instruments GmbH, Hanau, for 10 min at 2000 g.

2.3 Emulsion Preparation.

All emulsions were prepared from aqueous solutions of amphiphile and clay. Additionally all protein emulsions contained 0.5 wt% phenoxyethanole in order to prevent microbial growth. Using the High Pressure Emulsifier (APV 1000, Albertslund) required pre-emulsification of the sample using the Homo Disper (Tokushu Kika, Japan) at low rpm values of around 100. Afterwards the sample was emulsified three times at a pressure of 300 bar.

2.4 Rheology

The rheology of the emulsions was measured with a cone-plate rheometer RheoStress 600 from Haake Thermo Scientific, Karlsruhe, at 25 °C. The experimental data was analyzed with the Haake RheoWin Data Manager, Version 3.3.

3. Results and discussions

3.1. Clays and Amphiphiles in water

3.1.1 Clays and surfactants

In this segment we explain the general behaviour of surfactant-clay mixtures in relation to their visual appearance and their effects on surface tension. The schematic surface tension profile of a surfactant-clay mixture is compared to the one of a pure surfactant solution in fig. 1. With increasing surfactant concentration, the surface tension of water starts to decrease continuously until the critical micellar concentration (cmc) of the surfactant is reached. Whereas the clay-surfactant mixtures maintain the same surface tension as water, until the surfactant monolayer adsorption concentration (smac) is reached. All added surfactant is adsorbed on the clay particles. After reaching the smac, still surfactant binds to the clay-surfactant particles, but not completely as is obvious from the now decreasing surface tension. At the surfactant-clay cmc (scc), the free surfactant in the solution finally reaches the surface tension of a pure surfactant solution at and above its cmc. The amount of adsorbed surfactant can easily be determined by subtraction of the cmc from the ssc. Fig. 1 shows also some details which are of special interest for the formulation of Pickering emulsions. A concentration region of the surfactant exists in which the clay-surfactant particles already are formed, but the solutions are clear and no precipitate is formed in the solution. This concentration region or single phase area is marked as 1 Φ. The adsorption of the surfactant molecules must have made the surface of the clays more hydrophobic, but the remaining cationic charge of the clays is still strong enough to prevent precipitation. These could be conditions in which already stable Pickering emulsions are formed. With increasing adsorption of the surfactant molecules the clay-particle become so hydrophobic that they form a precipitate. This region is indicated in Fig. 1 as 2 Φ. The concentration of surfactant that is needed to proceed from 1 Φ to 2 Φ, is called phase transition point (ptp). Clays have a surface charge density of about one charge per nm^2. Cationic surfactants, like TTMABr, bind to the surface and compensate the charge density of the clay particles. In the case of 1 wt% Saponite 7 mM TTMABr are necessary [18]. Clay particles are stiff and do not show any undulation forces like surfactant bilayers. It is conceivable that bound surfactants are not randomly distributed to all available clay surfaces and ionic charges, but the surfactants bind in a cooperative manner. This means that some clay platelets might be completely covered while others are still completely free of surfactants.

We also examined the interaction between a typical anionic surfactant like Sodium-Dodecylsulfate (SDS) and the clay Saponite. Surface tension measurements of the supernatants of SDS-Clay mixtures resulted in the same values as pure SDS solutions. The attractive hydrophobic interaction between the C_{12}-chain of SDS and the clay is obviously too small to overcome the electrostatic repulsion between both. The ratio of both energies is determining, if adsorption to the clay particle is happening or not.

It is also well known that non-ionic surfactants bind to clays [19]. This adsorption can only be explained by hydrophobic interaction between both particles. For these experiments we used a typical non-ionic surfactant like $C_{12}EO_{7.8}$. With increasing the surfactant concentration in clay solutions we observe adsorption resulting in clear solutions, followed by precipitation and re-dissolution. Therefore three phase transitions points can be detected (tab. 1). Even the use of zwitterionic surfactants like the alkylaminoxides results in similar phase behaviour like in fig. 1. In our study we used Tetradecyldimethylaminoxide (TDMAO). This surfactant can not compensate the charge of the clay, but yet it binds to the

clay, the binding leads to precipitation and with excess of TDMAO leads to re-dissolutions of the precipitates. The binding of TDMAO and $C_{12}EO_{7.8}$ can not occur due to electrostatic interaction. It is therefore likely that these surfactants bind due to hydrophobic interaction. This means that the surface of the clays has to be at least weakly hydrophobic. In table 1 the ptp, smac, cmc and scc for different surfactants are summarized.
These results show unambiguously that with the exception of anionic surfactants all other types of surfactants bind to clays at low concentrations without forming any precipitates in the samples. With increasing surfactant adsorption the repulsive interaction due to the ionic charge of the clays can be compensated by the attractive interaction due to the bound surfactant molecules. It can be argued that the surfactants do not bind randomly on the surfaces of the clays but form hemi-micelles. This may be possible, but these hemi-micelles are then formed at concentrations where the surfactants do not form micelles in the bulk phase. The strong binding is therefore also an indication that the surface of the clays is already hydrophobic. Clays should therefore have surface active properties and adsorb at the surface of an aqueous phase. The reason why they do not lower the surface tension has to do with their large size and their small number density what results in a low surface pressure (see theoretical part of the paper).

3.1.2 Clays and polymers

What has been shown for the binding of surfactants to clay particles is also true for amphiphilic polymers. All water-soluble amphiphilic polymers bind to clays. At high concentrations they saturate the clay surfaces completely. Polymers can be classified into (block) copolymers, proteins and other polymers, like polyvinyl alcohol. The surface tension profile for clay-polymer solutions is similar to the one shown for surfactants (fig. 1). Of course polymers are large molecules with high molecular weights compared to surfactants. As a consequence the required molar concentration for polymer monolayer adsorption (pmac) is lower compared to smac. It is therefore more convenient to give the polymers' concentrations in % than in molar concentrations. Moreover some polymers have no cmc, while other like Pluronics [20] or ß-casein [21] have.
The surface activity and lipophilicity of blockcopolymers like polaxomers, also known as Pluronics, depend very much on the temperature [22-23]. The compounds form micelles and at higher concentrations liquid crystalline phases. Solutions of the compounds show a sol/gel transition with increasing temperature, which is caused by the dehydration of the PO-groups. It has been shown that a representative from this class of compounds, namely Pluronics F 127, adsorbs strongly on Saponite [24]. On saturation about the same weight of the blockcopolymer is bound to the clay. Precipitates of clay/blockcopolymers were also observed in the solutions.
Furthermore we investigated the interaction between the copolymer polyoxyethylene$_{17}$polyoxypropylene$_4$dimethylether (AQ 1704) and Saponite. Surface tension measurements resulted in similar results as performed before indicating that a strong adsorption of AQ 1704 to 1 wt% Saponite takes place. Up to 0.1 wt% AQ 1704 can adsorb to 1 wt% Saponite before the surface tension starts to decrease from the water value.
An interesting group of polymers from biological origin are proteins. These biopolymers are usually surface active and therefore lower the surface tension of water [25]. It has recently been shown that even negatively charged proteins like H Star Protein B ® (HPB) bind on negatively charged clays. Soft precipitates were observed in these samples even though both components are water soluble [15].
Other polymers like polyvinyl alcohol (PVA) [26], polyvinylpyrrolidon (PVP) [27] and even the rather hydrophilic polymer polyethylenglycol [13] bind to clay particles. The adsorption can easily be detected by surface tension measurements. When the polymers carry positive charges like in DADMAC, the complexes precipitate and re-dissolve with excess polymer [28]. The charge on the clays can therefore be reversed by such polymers.
Tab. 2 provides an overview about the phase transition point (ptp), the polymer monolayer adsorption concentration (pmac), the critical micellar concentration (cmc) and the polymer-clay critical micellar concentration (pcc) for different polymers.

3.2 Pickering emulsions

3.2.1 Using Amphiphile covered clays

Pickering emulsions prepared from recombinantly produced hydrophobin (HPB) and clay (Laponite XLG) with amazing properties have recently been reported [15]. The obtained high internal (o/w) phase emulsions (65 wt% oil) are very stable and have gel-like properties. The storage modulus G' is ten times larger than the one of the emulsion, which has been prepared from the hydrophobin alone. The clay-hydrophobin ratio can be varied from 6:4 to 2:8 without undergoing big changes of the gel properties. The size of the oil droplets in the emulsion depends very much on the shear rate that is used in the emulsification process. The diameter can easily be varied from 20 to 1 µm. The polarity of the oil did not seem to influence the stability and the elasticity of the samples [15]. In the previous investigation, it was shown that practically all of the clay and the protein can end up at the surface of the droplets, if the shear rate is high enough. When proteins bind to clays, the other side of the attached protein remains surface active. If these particles bind to an interface, the other side of the attached side is still surface active. It is therefore not unexpected that such systems have ideal properties to form stable Pickering emulsions. Surprisingly theses systems have not been recognized earlier in emulsion science. Due to their sticky character, the protein-clay particle covered oil droplets can bind to each other and form in this way stable, homogenous emulsions with gel-like properties. The storage moduli are determined by the elastic properties of the thin film. The films are extremely stable and elastic, even the oil and water can be removed without collapse of the three dimensional film structures [15].
In this investigation we want to find out, if this novel protein-clay synergism is unique or if it is possible to replace the

hydrophobin to either surfactant or polymer compounds. Therefore we prepared high pressure emulsions from 1:1 mixtures of amphiphile (0.5 wt%) and clay (0.5 wt%). The obtained samples prepared at a pressure of 300 bar are shown in Fig. 2. All samples contained a final composition of 0.5 wt% amphiphile, 0.5 wt% Laponite XLG and 50 wt% of the oil polydimethylsiloxane (PDMS).

The used amphiphiles for the Pickering emulsions in fig. 2 ranged from C_{16}-Trimethylammoniumbromid (CTAB), Isotridecyloctaethylenglycolether ($C_{13}O_8$), Tetradecyldimethylaminoxide (TDMAO), Pluronic F38, to H Star Protein ® B (HPB), polyvinylalcohole (PVA; Polyviol® LL 2860), polyvinylpyrrolidon (PVP) and polydiallyldimethylammoniumchloride (DADMAC). Interestingly all samples beside the sample prepared with DADMAC are homogenous emulsions. Moreover the samples with CTAB, $C_{13}O_8$, TDMAO, HPB, PVA and PVP do not form a smooth, horizontal meniscus, indicating their gel-like properties. Especially the toothpaste like performance of the Pickering emulsion prepared from PVA can be clearly seen. In order to make a deeper comparison of these Pickering emulsions, tab. 3 contains the storage modulus G' (τ= 0.5 Pa and f = 1 Hz) and the needed shear stress τ [Pa] to break the elastic (if present) behaviour of the emulsion.

Tab. 3 confirms what was already seen from Fig. 2. Fascinatingly, the samples with CTAB, $C_{13}O_8$, TDMAO, PVA and PVP have all gel-like properties and therefore show a similar synergism like in the case of HPB and clay. For CTAB only 14 mM in combination with 0.5 wt% clay are enough to obtain gel-like properties with already 50 wt% oil. One could argue that the clay concentration in the water phase is already 1 wt% and therefore somehow near to the clay gel-transition. However, the synergistic use of Pluronic F38 or DADMAC with clay did not result in gel-like Pickering emulsions, the measured G' values are very low. So the argument that the clay could be gel-like in the water phase and therefore stabilize the emulsion is not true.

In emulsion science the samples' appearance in dependence of storage time is of great interest. Therefore the samples were incubated at room temperature for 2 months (Fig. 3). Beside the samples containing TDMAO and Pluronic F38 all other emulsions did not change after 2 month storage at room temperature. The emulsion with TDMAO released oil, while the emulsion prepared with Pluronic F38 separated into two phases.

We conclude that it is indeed possible to obtain similar gel-like Pickering emulsions with other amphiphiles beside HPB. Moreover our studies show that by the choice of the amphiphile one can easily adjust the G' value of the Pickering emulsion. These results offer a lot of possibilities, chances and independence for applicants. However, the general question about the origin of that amphiphile-clay synergism comes up for scientists. In the case of the protein HPB, we argued that the oil droplets are covered with protein-clay sandwiches acting as sticky droplets. These particles interact, interpenetrate and entangle with each other resulting in the formation of a rigid, three-dimensional network. This assumption was proved by several experiments. First of all the Pickering emulsion aged with time. The storage moduli doubled after one day indicating that a progressed stiffening of the protein network took place. Moreover it was possible to remove all of the oil and water by freeze drying. The white drying residue was observed with Scanning electron microscopy (SEM). A network structure with holes was observed. The size of the holes was in agreement with the emulsion droplet size. Finally the Pickering emulsions contract after being diluted with the same amount of water again. Obviously an attractive force between the emulsion droplets must exist. If this sticky droplet concept is also true for other amphiphiles will be checked now.

3.2.2 Variation of amphiphile concentration

The type of amphiphilic compounds used in this study differs a lot in size, molar mass and behaviour. Consequently the coverage of the clays was quite different. In order to study the influence of the clay coverage by amphiphile, we prepared samples with increasing amount of amphiphile, while the clay and oil content were kept the same. As the Pickering emulsion prepared from the simple, cationic surfactant CTAB and clay had gel-like properties, it is interesting to look closer to this phenomenon. In order to prove, if even other cationic surfactants are able to produce stable gel-like emulsion in synergism with clay, we used TTMABr in this section. First of all it is essential to know the coverage of the clay at a defined amphiphile concentration. Therefore we performed surface tension measurements of TTMABr-Laponite XLG solutions and compared them to a pure TTMABr solution (Fig. 4). The phase transition point (ptp) from 1 Φ to 2 Φ of clay-TTMABr solutions is reached with already 0.6 mM TTMABr. 3 mM TTMABr are needed to cover the surface of 0.5 wt% Laponite XLG with a monolayer (smac). The cmc of TTMABr is reached at 3.7 mM, whereas the surfactant-clay cmc (scc) is obtained at 15 mM TTMABr. Consequently 0.5 wt% Laponite XLG can bind 9 mM surfactant. In order to investigate the influence of the clay coverage ratio on the emulsion properties, high pressure emulsions with increasing amounts of TTMABr have been obtained.

Pickering emulsions containing 0.5 mM (1 Φ), 1.5 mM (2 Φ), 5 mM (above smac), 10 mM and 45 mM TTMABr (above scc) are shown in Fig. 5. All samples contain 0.5 wt% Laponite XLG and 50 wt% PDMS as an oil. With increasing concentration of TTMABr the volume of the emulsion layer enlarged. The extension of the emulsion layer can be explained with the decreasing concentration of uncovered clay in the water phase. The osmotic pressure therefore would be getting smaller, the emulsion layer can swell. Presumption for this is a cooperative binding of the surfactant to the clay. Moreover it is also possible that an enhanced entanglement of the oil droplets due to the more covered clay particles with surfactant is responsible that the tightening of emulsion network is getting lower. Nevertheless, already as less as 5 mM TTMABr were enough to obtain a homogenous emulsion. The samples with 5 and 10 mM TTMABr show no horizontal meniscus indicating their gel-like properties. Increasing the TTMABr concentration over the scc (15 mM) leads to decreased elastic properties as can be seen from flat meniscus

of the sample containing 45 mM TTMABr. In order to look closer to the rheological properties of the Pickering emulsions prepared from differently surfactant-covered clay particles we measured and compared the storage moduli G' (τ = 0.05 Pa and f = 1 Hz) against the used TTMABr concentration (Fig. 6).

Adding little amounts of TTMABr leads to increased storage moduli. After reaching the smac of the clay particles, G' is even getting slightly higher than before, but then starts to decrease dramatically. Adding more and more surfactant (above scc) leads finally to 100 times smaller G', indicating the breakdown of the emulsion gel-like properties. As it is known from our previous investigation Laponite XLG does neither stabilize an emulsion itself nor does show any gel-transition at as low concentrations of 0.5 wt% [15]. Therefore the shown result in Fig. 6 clearly indicates that the clay coverage has immense influence on the rheological properties of the sample. While adding small amount of surfactant can boost the emulsifying properties of the solution, high amounts decrease them rapidly. Before reaching the smac, the clay particles became more hydrophobic due to enhanced TTMABr binding. Consequently the clay-surfactant particles are more likely to bind to an oil-water interface. Their adsorption energy becomes larger than the repulsive energy. Clay particles covered with a surfactant monolayer are completely hydrophobic and therefore form emulsions with the highest gel-like properties. In Fig. 7 a rheogram at τ = 0.5 Pa of the emulsion containing 5 mM TTMABr, 0.5 wt% Laponite XLG and 50 wt% PDMS is shown, indicating its high gel-like properties.

Adding more surfactant leads to minor gel-like properties as surfactant bilayers build up, resulting in increased hydrophilic properties of the clay-surfactant particles again. After reaching the scc, surfactant bilayers are adsorbed on each side of the clay molecules, making them positively charged. These particles are likely to bind hardly to an oil-water interface. Therefore the emulsion prepared from 45 mM TTMABr is only viscous, no sticky droplets are forming a network. The oil droplets are covered by TTMABr molecules, making the emulsion droplets positively charged. The emulsion is now stabilized by electrostatic repulsion and not longer by emulsion droplet attraction as it was before. Clay-surfactant particles do not act longer as sticky droplets.

Finally we can conclude that by adjusting the concentration of surfactant, one has an advantageous tool to regulate easily the rheological properties of the desired emulsion.

3. Comparison of Pickering emulsions from clays with other Pickering emulsions

It is interesting to compare the preparation and properties of Pickering emulsions from this investigation with Pickering emulsions which have been prepared from silica Nanoparticles [29]. The group of Binks has used commercially available silica particles for the preparation of Pickering emulsions. The silica particles had an average diameter of 10 nm and at a pH = 9.5 a similar cec like clays. Aqueous solutions of the particles were transparent and of low viscosity. With increasing concentration of CTAB a precipitate formed in the samples which reached a maximum around the cec and then decreased again with excess CTAB. The sediment is however not redispersed up to 100 mM CTAB in 2 % silica solutions. This behaviour is the same as for the observed Laponite/TTMABr interaction in our investigation. The samples with 2 % silica particles showed even a CTAB concentration region in which the system was still clear and no precipitate was observed even though the CTAB was adsorbed on the silica particles. Surface tension measurements showed that the silica particles did not lower the surface tension of water like the clay particles. The samples with CTAB below the cec showed however a reduced surface tension of 68 mN/m. It is conceivable that this reduced surface tension was due to the silica particles which had become slightly hydrophobic by the adsorbed surfactant. It is unlikely that it was due to free CTA-ions. Emulsions were prepared in samples with 2 % silica particles and increasing CTAB concentration from 0.1 mM to 100 mM. All samples consisting out of 50 % dodecane and 50 % aqueous phase. They resulted in a two layer system, a lower aqueous layer and an upper o/w layer. The volume fraction of the emulsion phase increased with the surfactant concentration up to the cec and decreased again for higher surfactant concentration. The stability of the emulsions against coalescence was highest at the CTAB concentration around the cec that is when the silica/surfactant particles had no charge. No detailed rheological data were reported. It was mentioned however in the manuscript that the viscosity of the emulsions passed over a maximum with the CTAB concentration.

The most remarkable difference between the Pickering emulsions from clay and from silica particles is that the one with clays have strong gel-like properties at small coverage of the particles with surfactant while the Pickering emulsions with silica particles behave like viscous solutions. In the first situation the interaction between the droplets is attractive while it is repulsive in the second case. While it is not completely clear at present where this difference in behaviour comes from, it is conceivable that it lies in the difference in sizes of the two interacting particles. For the clays, the surfaces are flat and have a size of about 40 nm x 40 nm = 1600 nm^2 while for the silica particles the interacting surface of the sphere is in the range of r^2 = 25 nm^2. Each clay particle is covered with much more amphiphile molecules and therefore a stickier particle than the ones prepared with silica. This surely influences the gel-like properties of emulsions based on amphiphile covered clay or silica particles.

4. Theoretical part - Why soluble hydrophobic clay particles seem to be not surface active

Hydrophobic molecules like surfactants, proteins or amphiphilic polymers are surface active and lower the surface tension of water. These molecules form monolayers at the aqueous surface and their surface pressure π of the monolayers acts against the surface tension of water $\sigma°$. The surface tension σ of the solution is thus $\sigma° - \pi$. The surface pressure π is given by the equation

$$\pi = \Gamma \cdot RT \qquad (1)$$

where Γ is the surface concentration that is the number of moles per area A. For small molecules like surfactant and polymers the surface concentration Γ is high enough when the molecules form a monolayer so that the surface tension is reduced. Even when the adsorbed molecules are still in the gaseous state the surface pressure is already appreciable and can be measured by the Langmuir Blodgett technique with molecules that are not soluble in water.

When hydrophobic particles like clays are adsorbed their surface concentration Γ is so low that the resulting surface pressure is so small that it does not affect the surface tension. This situation must exist with the clay solution with 0.5 wt % of clay where up to 3 mM of TTMABr could be adsorbed without any detectable lowering of the surface tension (Fig. 4). Obviously, all the added surfactant had been adsorbed by the clay because the cec of the clay is 0.6 meq/g. The clay-samples with more than 0.6 mM TTMABr form a precipitate. Obviously, they have become so hydrophobic by the adsorption that they precipitate in spite of the fact that the platelets still have an electric double layer and the long range interaction between them is repulsive. With less then 0.6 mM TTMABr the clay solutions are transparent even though the surfactant is adsorbed as is shown by the value of the surface tension (Fig. 4). We can therefore conclude that the hydrophobicity in this case is not big enough to overcome the electrostatic repulsion between the clays. Clays with 20 % adsorbed surfactant of their cec are already so hydrophobic to precipitate. It is conceivable that the clays themselves are already hydrophobic and their hydrophobicity is one of the reasons why surfactants bind on clays.

This situation may exist only when the clays are dispersed in water and most of the counter-ions are dissociated from the clay surface. The situation can be different with dry clays when the counter-ions are bound to the surface. It is thus conceivable that dissolved clays with adsorbed surfactants strongly bind to oil droplets in emulsions while naked clays can not well adsorb on oil droplets because the counter-ions in this case are in between the oil and the clays (Fig. 8).

5. Conclusion

It was shown that almost all studied amphiphilic compounds bind on negatively charged clay platelets. Negatively charged surfactants like SDS do not bind. Far from the saturation capacity, the clay-amphiphile particles are soluble. Around saturation the particles precipitate. Stable Pickering emulsions can be prepared from samples with 50 % water and 50 % oil with as little as 0.5 % clay and low amounts of amphiphile. The Pickering emulsions are of the o/w-type and have gel-like properties. The shear modulus of these phases can be varied between a few Pascal and several thousand Pascal for small changes in the composition on the clay surface.

It is normally assumed that clays have hydrophilic surfaces because clay particles do not lower the surface tension of water. Amphiphilic molecules like non-ionic surfactants or surface active water soluble polymers bind to clay surfaces. The resulting amphiphile covered clay particles again do not lower the surface tension of water even though these particles should now have hydrophobic properties. To explain their conflicting evidence, it is concluded that the clay particles and the surface modified clay particles are really hydrophobic but do not lower the surface tension of water because their surface pressure is not big enough to reduce the surface tension of water. The surface pressure is given by the number density of the adsorbed particles and in the case of the clays their number density is several orders of magnitude lower than the number density of surfactants. The surface tension is therefore practically not affected. It is for this hydrophobicity of the modified clay particles that they form very stable Pickering emulsions with oil and the emulsions have gel-like properties. The clay particles are cross-linking the oil droplets.

6. Acknowledgement

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8. Figures

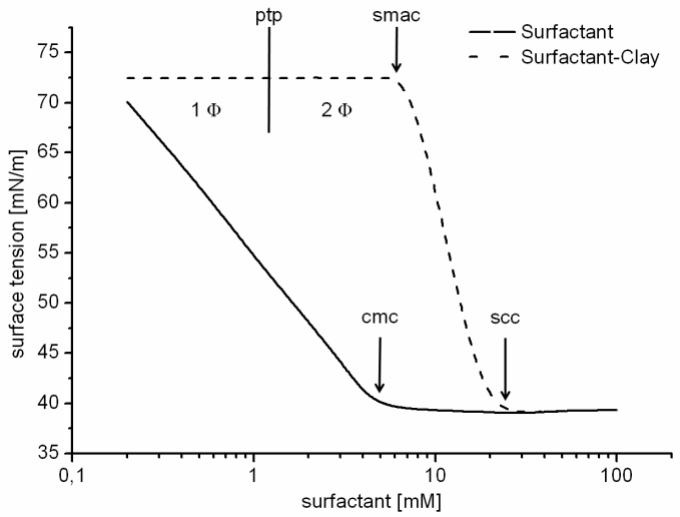

Fig. 1: Schematic surface tension profile of surfactants compared to surfactant-clay mixtures. Important surfactant concentrations are indicated as: Phase transition point (ptp), surfactant monolayer adsorption concentration (smac), critical micellar concentration (cmc) and surfactant-clay cmc (scc). [Single column].

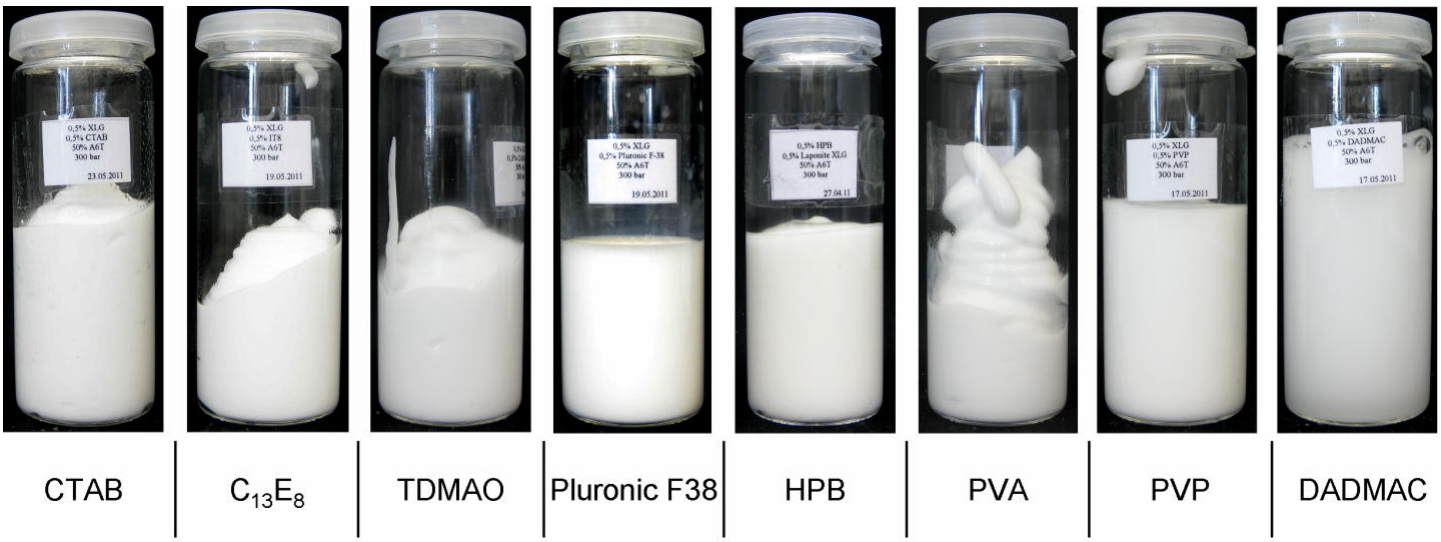

Fig. 2: Pickering emulsions from different 1:1 mixtures of amphiphile and clay prepared at 300 bar. All samples contained 0.5 wt% amphiphile, 0.5 wt% Laponite XLG and 50 wt% polydimethylsiloxane (PDMS) and were photographed after 1 d. [Full width].

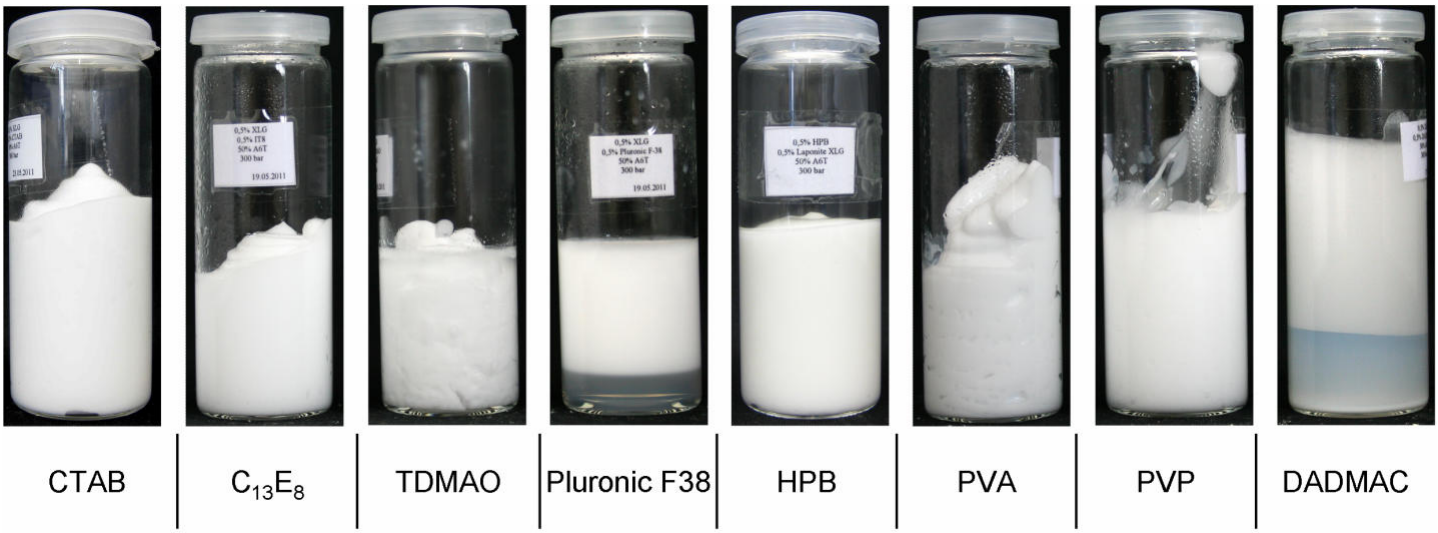

Fig. 3: Pickering emulsions from different 1:1 mixtures of amphiphile and clay prepared at 300 bar. All samples contained 0.5 wt% amphiphile, 0.5 wt% Laponite XLG and 50 wt% polydimethylsiloxane and were photographed after 2 month incubation at room temperature. [Full width].

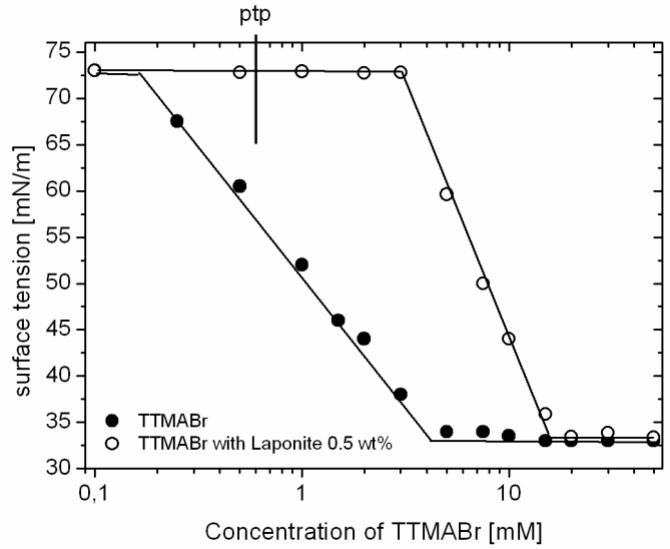

Fig. 4: Surface tension profile of the supernatants of samples with 0.5 wt% Laponite XLG and increasing amounts of TTMABr in comparison to the surface tension of TTMABr alone. [Single column].

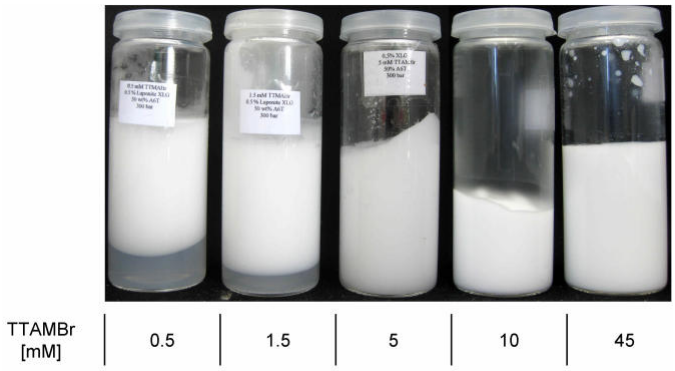

Fig. 5: Pickering emulsions stabilized by clay-surfactant particles at different surfactant concentration of TTMABr [mM], prepared at 300 bar. All samples contained 0.5 wt% Laponite XLG and 50 wt% polydimethylsiloxane and were photographed after 2 day incubation at room temperature. [Single column].

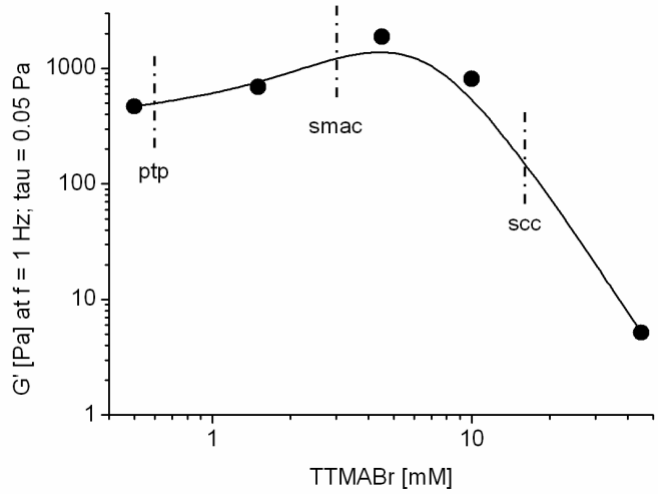

Fig. 6: Storage modulus G' [Pa] in dependency of TTMABr concentration [mM] in the emulsions, measured at τ = 0.05 Pa and f = 1 Hz directly after preparation. All Pickering emulsions contained 0.5 wt% Laponite XLG and 50 wt% PDMS. [Single column].

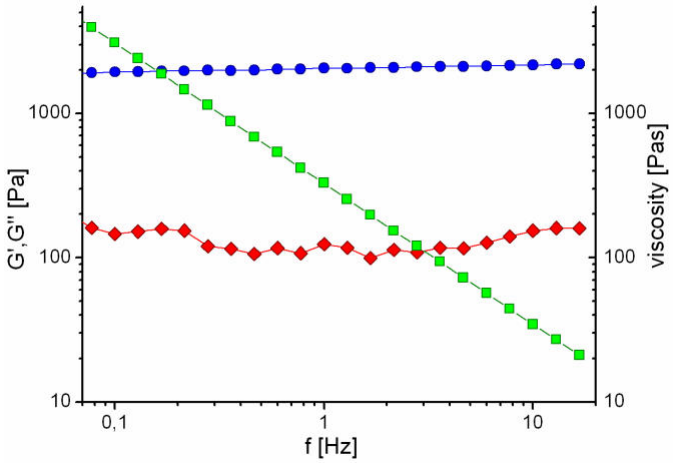

Fig. 7: Rheogram (τ = 0.5 Pa) of emulsion prepared from 5 mM TTMABr and 0.5 wt% Laponite XLG and 50 wt% PDMS, evaluated directly after emulsification at 300 bar. Blue: storage modulus G' [Pa], red: loss modulus G'' [Pa] and green: viscosity η [Pas]. [Single column].

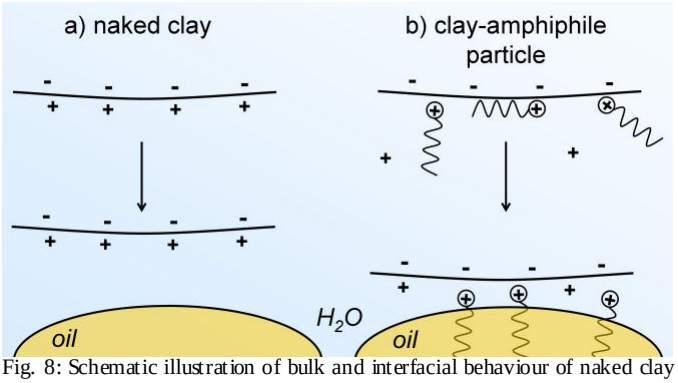

Fig. 8: Schematic illustration of bulk and interfacial behaviour of naked clay particles (a) in contrast to amphiphile covered clay particles (b). [Single column].

Tab. 1: Summary of phase transition point (ptp), surfactant monolayer adsorption concentration (smac), critical micellar concentration (cmc) and surfactant-clay cmc (scc) for different surfactant-clay mixtures. We chose TTMABr as cationic, SDS as anionic, $C_{12}EO_{7.8}$ as non-ionic and TDMAO as zwitterionic model surfactant. [Single column].

surfactant & 1 wt% Saponite	ptp [mM]	smac [mM]	cmc [mM]	scc [mM]
TTMABr	1.2	7	3.7	20
SDS	-	-	8	-
$C_{12}EO_{7.8}$	3; 12; 50	0.8	0.035	12
TDAMO	4; 25	0.5	0.15	50

Tab. 2: Summary of phase transition point (ptp), polymer monolayer adsorption concentration (pmac), critical micellar concentration (cmc) and polymer-clay cmc (pcc) for different polymer-clay mixtures. We chose Pluronic F127 as blockcopolymer, AQ 1704 as copolymer, H Star Protein B ® (HPB) as protein and polyvinyl alcohol (PVA; Polyviol® LL 2860) as synthetic polymer. [Single column].

polymer & 1% clay	ptp [%]	pmac [%]	cmc [%]	pcc [%]
Pl. F127 [28]	-	0.1	0.03	1
AQ 1704	-	0.1	-	0.5
HPB [15]	0.1	0.3	-	3
PVA [26]	-	0.02	0.01	3

Tab. 3: Overview of the storage moduli G' [Pa] (τ = 0.5 Pa and f = 1 Hz) and the needed shear stress τ to break the elastic behaviour of Pickering emulsions prepared from different amphiphiles measured directly after preparation. Final composition: 0.5 wt% amphiphile, 0.5 wt% Laponite XLG and 50 wt% polydimethylsiloxane. [Full width].

amphiphile	CTAB	$C_{13}O_8$	TDAMO	Pluronic F38	HPB	PVA	PVP	DADMAC
G' [Pa]	494	284	1188	0.062	589	1158	373	0.129
$\tau_{breakdown}$	15	2	10	-	5	30	30	-

Publication F

Japanese patent, Oil in Water emulsion in cosmetic, **2011**

Patent about emulsions and Pickering emulsions that are stabilized by protein or protein clay synergism. My name is included as inventor #3.

宣　誓　書

平成２３年　　月　　日

下記の出願について、甲１～甲４が真の発明者であることを、ここに宣誓します。

We declare that Kou 1 (甲１) to Kou 4 (甲４) are the original inventors of the below-described patent application.

記

１．出願番号　　特願２０１１－１６９３８２
Patent application No. 2011-169382

２．発明の名称　　水中油型乳化化粧料
Title　　Oil-in-water emulsion cosmetic

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Heinz Hoffmann

7 Presentations at international meetings

- ***Bayreuther Polymer Symposium BPS 2011***, 11-13. September, 2011, University of Bayreuth, Germany.

Poster presentation: Novel clay-hydrophobin stabilized surfactant-free emulsions.

- ***58th SEPAWA Congress***, 12-14. October 2011, Forum for Innovations, Fulda, Germany.

Oral presentation: Novel clay-hydrophobin stabilized emulsions.

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9 Erklärung

Hiermit erkläre ich, diese Arbeit selbstständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt zu haben.

Ferner erkläre ich, dass ich nicht anderweitig mit oder ohne Erfolg versucht habe, diese Dissertation einzureichen. Ich habe keine gleichartige Doktorprüfung an einer anderen Hochschule endgültig nicht bestanden.

Immenreuth, den 5. Dezember 2011