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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.

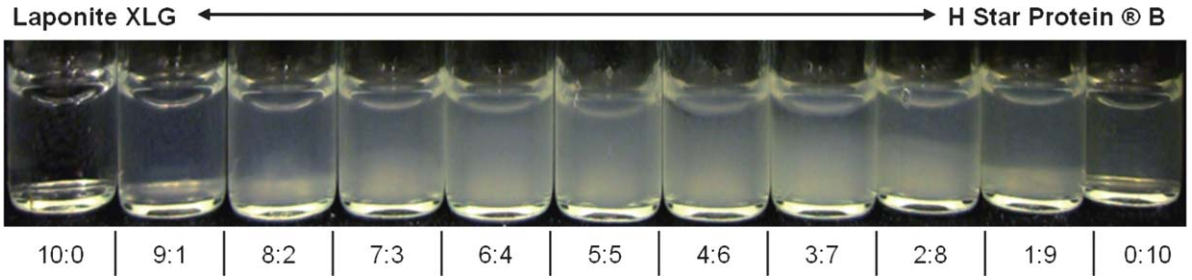

Fig. 1 Mixtures prepared from 1 wt% Laponite XLG and 1 wt% H Star Protein® B (HPB) at neutral pH.

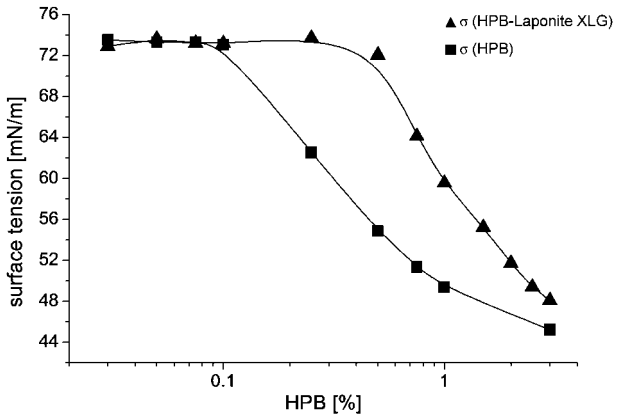

Fig. 2 Surface tension σ of the supernatants of samples with 0.5 wt% Laponite XLG and increasing concentrations of HPB in comparison to the surface tension of HPB alone. Drop-formation speed was 1 μl s^{-1}.

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%.

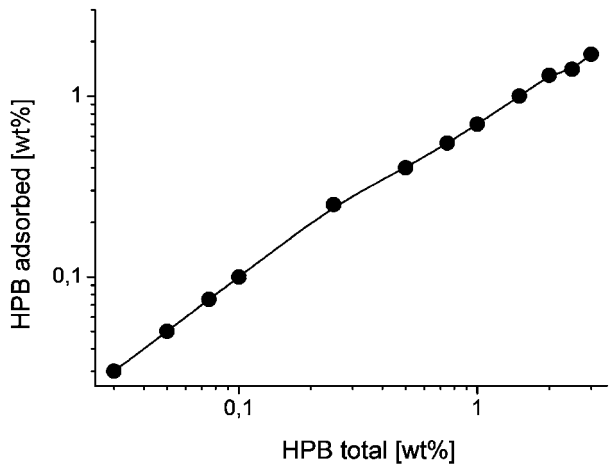

Fig. 3 Plot of the adsorbed amount of HPB (wt%) on 0.5 wt% Laponite XLG in dependence of the totally used HPB concentration (wt%).

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

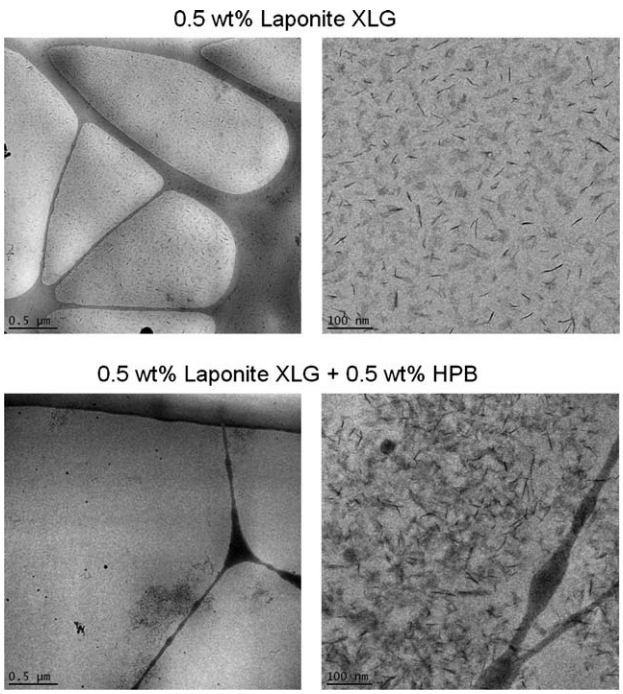

Fig. 4 Cryo-TEM micrographs of 0.5 wt% clay and of a mixture of 0.5 wt% clay and 0.5 wt% HPB at different magnifications.

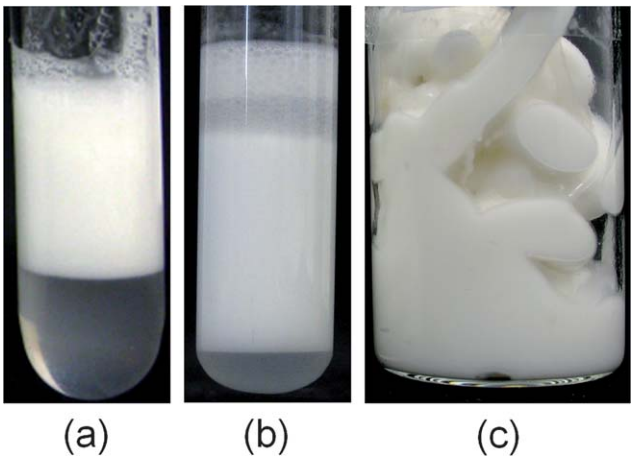

Fig. 5 Synergistic emulsifying action of hydrophobin and clay. Shown are high pressure (1000 bar), one day old Pickering emulsions prepared with 0.5 wt% HPB (a), 0.5 wt% Laponite XLG (b) and 0.5 wt% HPB/0.5 wt% Laponite XLG (c) as emulsifiers, oil mass fraction $\Phi = 0.65$ PDMS.

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

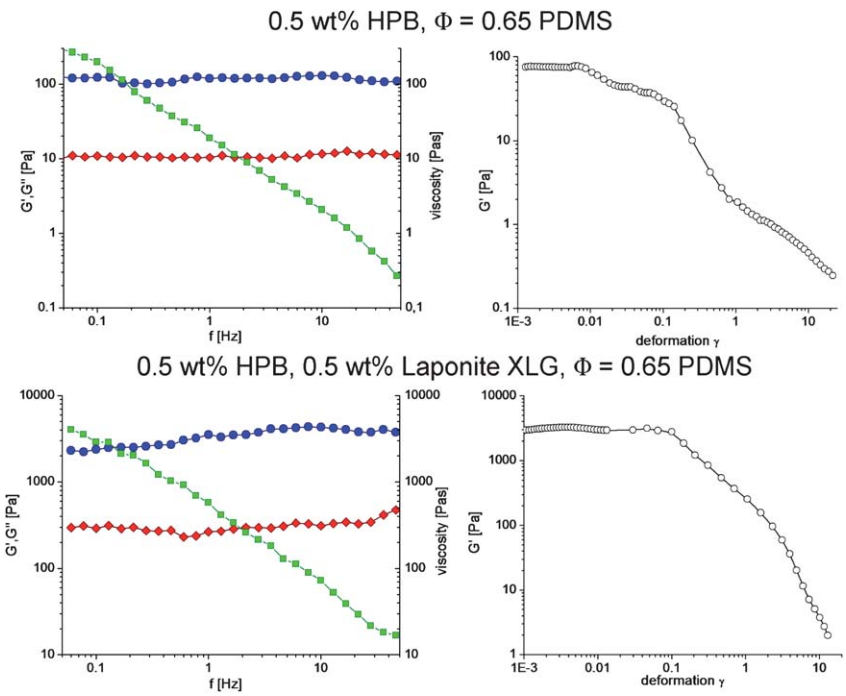

Fig. 6 Comparison of the rheological properties of emulsion layers prepared from 0.5 wt% HPB (upper row) and from a combination of 0.5 wt% HPB and 0.5 wt% Laponite XLG (lower row) acting as emulsifiers. Shown are, on the left side, rheograms ($\tau = 0.05$ Pa) with the color code: blue: storage modulus 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.

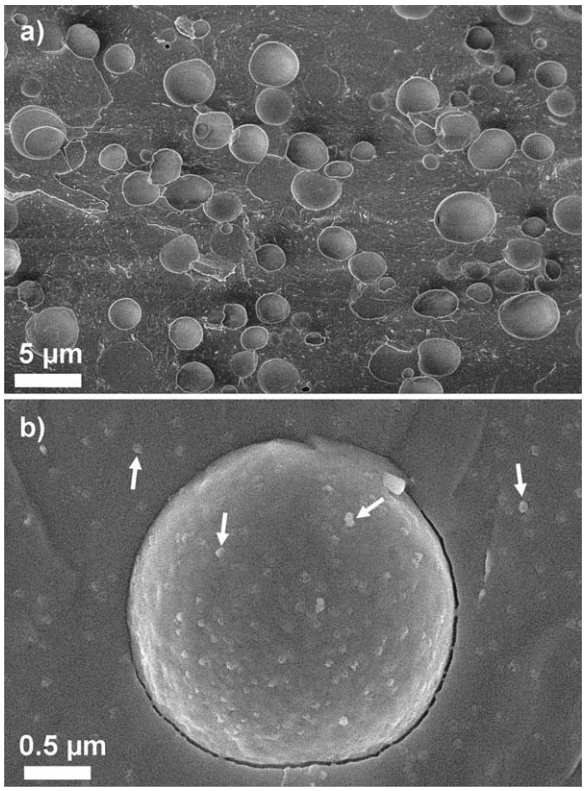

Fig. 7 Cryo-SEM micrographs of a hydrophobin–clay synergism based emulsion. An overview of the emulsion topology is provided in (a). White arrows in (b) indicate clay particles to be located at the interface and in the water layer. Sample composition: 0.5 wt% HPB, 0.5 wt% XLG, $\Phi =$ 0.65 PDMS, 1000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

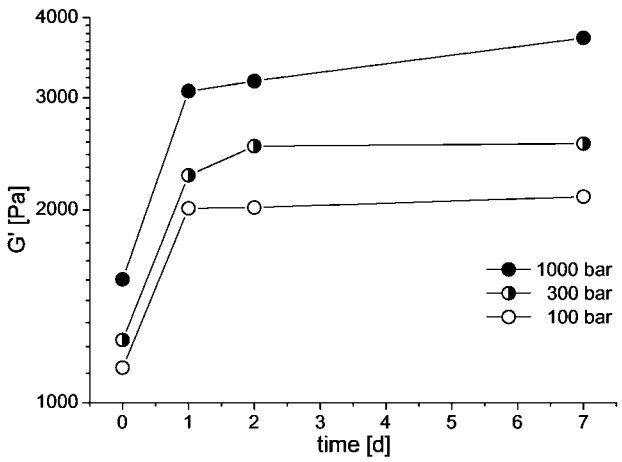

Fig. 8 Storage moduli G' (Pa) measured at $\tau = 0.5$ Pa and $f = 1$ Hz at different time points. Sample composition: 0.5 wt% HPB and 0.5 wt% Laponite XLG and oil mass fraction $\Phi = 0.65$ PDMS; emulsions prepared with the high pressure emulsifier.

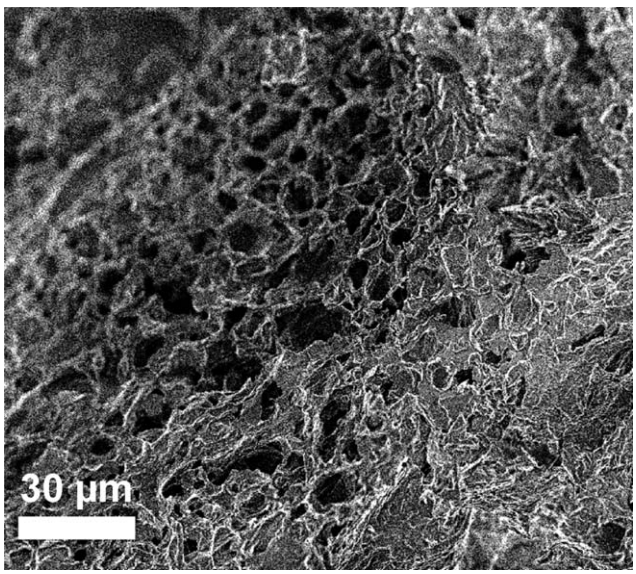

Fig. 9 SEM micrograph of the freeze-dried residue of an emulsion containing 0.5 wt% HPB, 0.5 wt% Laponite XLG and an oil mass fraction $\Phi = 0.65$ decane, prepared with the high pressure emulsifier at 300 bar.

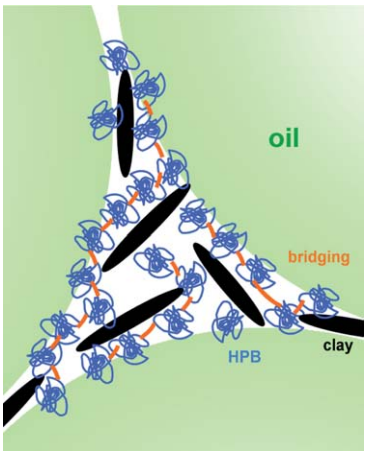

Fig. 10 Conceptual diagram of the self-supporting, three-dimensional network existing formed by hydrophobin covered clay particles. The HPB molecules (blue) absorbed on the clay (black) interact (orange) with each other. The clay particles serve as strengtheners of the hydrophobin network.

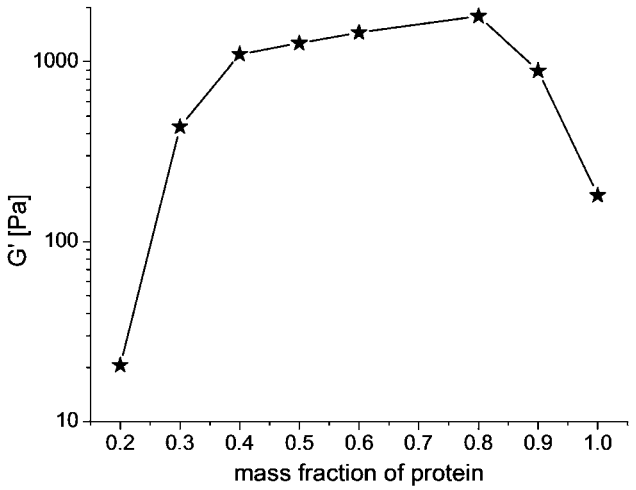

Fig. 12 Comparison of G' values ($\tau = 0.5$ Pa, $f = 1$ Hz) measured directly after emulsification (300 bar) for emulsions containing different protein/clay mass fractions. The emulsions contained 1 wt% of protein/clay as well as a PDMS mass fraction $\Phi = 0.65$.

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

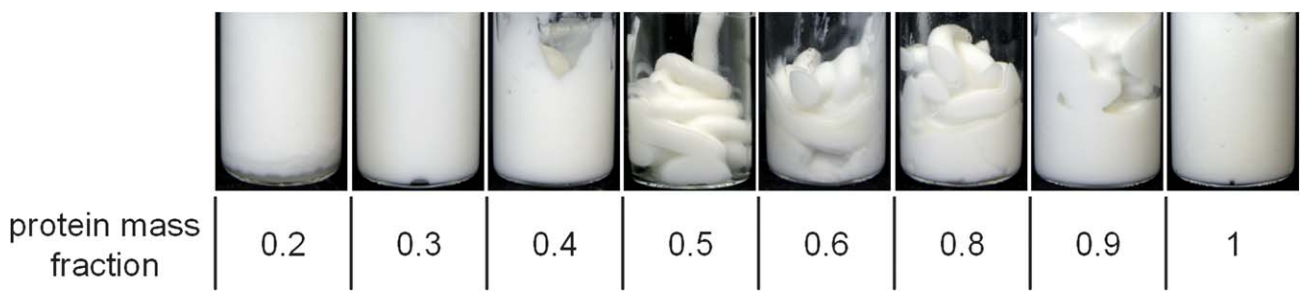

Fig. 11 High pressure emulsions (300 bar) containing different hydrophobin/clay mass fractions. The emulsions contained 1 wt% HPB/Laponite XLG as well as a PDMS mass fraction $\Phi = 0.65$.

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 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.

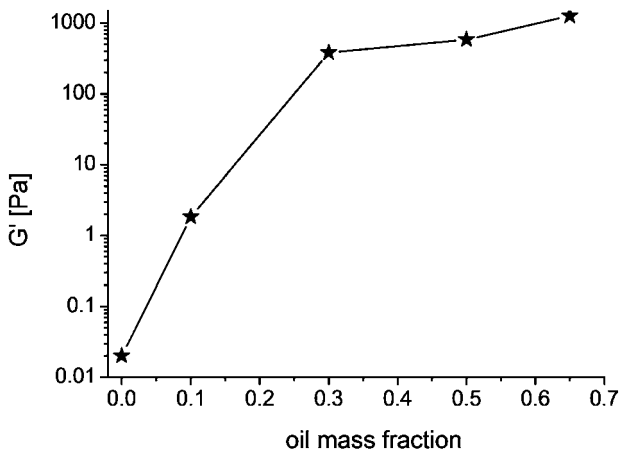

Fig. 13 Storage moduli G' (τ = 0.5 Pa, f = 1 Hz) measured directly after emulsification at 300 bar for emulsions containing different PDMS oil mass fractions Φ. The emulsions contained 0.5 wt% HPB and 0.5 wt% Laponite XLG as well as PDMS mass fractions Φ between 0.1 and 0.65.

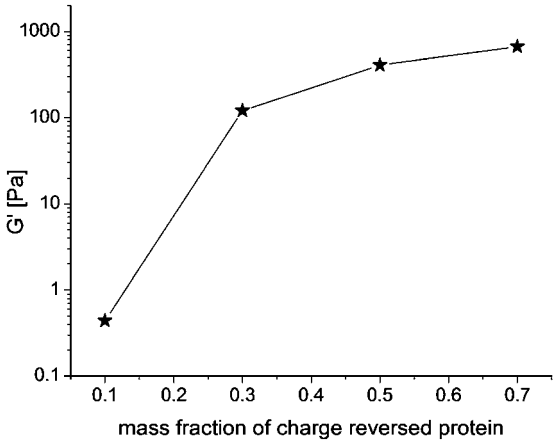

Fig. 14 Storage moduli G' (τ = 0.5 Pa, f = 1 Hz) measured directly after emulsification at 300 bar for emulsions prepared with charge reversed protein. The emulsions contained 0.1–0.7 wt% charge reversed HPB, 0.5 wt% Laponite XLG and an oil mass fraction Φ of 0.65 PDMS.

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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Notes and references

1 Y. Jeyachandran, E. Mielczarski and J. Mielczarski, *Langmuir*, 2009, **25**, 11614–11620.
2 K. Ralla, U. Sohling and T. Scheper, *Bioprocess Biosyst. Eng.*, 2010, **33**, 847–861.
3 J.-M. Séquaris, A. Hild and M. J. Schwuger, *J. Colloid Interface Sci.*, 2000, **230**, 73–83.
4 K. Esumi, Y. Takeda and Y. Koide, *Langmuir*, 1997, **13**, 2585–2587.
5 S. Holzheu and H. Hoffmann, *Prog. Colloid Polym. Sci.*, 2000, **115**, 265–269.
6 P. E. Levitz, *Colloids Surf., A*, 2002, **205**, 31–38.
7 G. Sposito, N. Skipper and J. Greathouse, *Proc. Natl. Acad. Sci. U. S. A.*, 1999, **96**, 3358–3364.
8 A. Meleshyn and C. Bunnenberg, *J. Phys. Chem. B*, 2006, **110**, 2271–2277.
9 A. Shalkevich, A. Stradner and P. Schurtenberger, *Langmuir*, 2007, **23**, 3570–3580.
10 H. van Olphen, *Discuss. Faraday Soc.*, 1951, **11**, 82–84.
11 H. van Olphen, *An Introduction to Clay Colloid Chemistry*, Wiley and Sons, New York, 1997.
12 J. Zou and A. C. Pierre, *J. Mater. Sci. Lett.*, 1992, **11**, 664–665.
13 M. Dijkstra, J.-P. Hansen and P. A. Madden, *Phys. Rev. E: Stat. Phys., Plasmas, Fluids, Relat. Interdiscip. Top.*, 1997, **55**, 3044–3053.
14 B. Jolanun and S. Towprayoon, *Bioresour. Technol.*, 2010, **10**, 4484–4490.
15 Y. Yamaguchi and H. Hoffmann, *Colloids Surf., A*, 1997, **121**, 67–80.
16 G. Lagaly, M. Reese and S. Abend, *Appl. Clay Sci.*, 1999, **14**, 83–103.
17 W. Wohlleben, T. Subkowski and U. Baus, *Eur. Biophys. J.*, 2009, **39**, 457–468.
18 H. Wösten and M. de Vocht, *Biochim. Biophys. Acta*, 2000, **1469**, 79–86.
19 X. Wang, M. de Vocht and G. Robillard, *Protein Sci.*, 2002, **11**, 1172–1181.
20 X. Wang, J. Graveland-Bikker and G. Robillard, *Protein Sci.*, 2004, **13**, 810–821.
21 K. Kisko, G. Szilvay and R. Serimaa, *Langmuir*, 2009, **25**, 1612–1619.

22 R. De Lisi, G. Lazzara and N. Muratore, *Langmuir*, 2006, **22**, 8056–8062.
23 L. Jing and H. Hoffmann, *Colloid Polym. Sci.*, 2004, **283**, 24–32.
24 M. Reger, T. Sekine and H. Hoffmann, *Soft Matter*, 2011, **7**, 8248–8257.
25 H. Hoffmann, *ACS Symp. Ser.*, 1994, **578**, 1–31.
26 A. Fischer, M. Meyer and H. Hoffmann, *J. Phys. Chem. B*, 2002, **106**, 1528–1533.
27 T. G. Mason, J. N. Wilking and S. M. Graves, *J. Phys.: Condens. Matter*, 2006, **9**, 193–199.
28 D. G. Dalgleish, *Trends Food Sci. Technol.*, 1997, **8**, 1–6.
29 E. S. Basheva, P. A. Kralchevsky and A. Lips, *Langmuir*, 2011, **27**, 2382–2392.
30 W. Ramsden, *Proc. R. Soc. London*, 1903, **72**, 156–164.
31 S. U. Pickering, *J. Chem. Soc. Trans.*, 1907, **91**, 307–314.
32 R. Aveyard, B. P. Binks and J. H. Clint, *Adv. Colloid Interface Sci.*, 2003, **100–102**, 503–546.
33 H. Hassander, B. Johansson and B. Törnell, *Colloids Surf.*, 1989, **40**, 93–105.
34 V. Ikem, A. Menner and A. Bismarck, *Angew. Chem.*, 2008, **120**, 8401–8403.
35 S. Guillot, F. Bergaya and J.-F. Tranchant, *J. Colloid Interface Sci.*, 2009, **333**, 563–569.
36 Y.-C. Chang, C.-C. Chou and J.-J. Lin, *Langmuir*, 2005, **21**, 7023–7028.
37 C.-C. Chou and J.-J. Lin, *Macromolecules*, 2005, **38**, 230–233.
38 A. Salonen, F. Muller and O. Glatter, *Langmuir*, 2008, **24**, 5306–5314.
39 F. Muller, A. Salonen and O. Glatter, *J. Colloid Interface Sci.*, 2010, **342**, 392–398.
40 L. Thompson, S. P. Armes and D. W. York, *Langmuir*, 2011, **27**, 2357–2363.
41 B. Tigges, T. Dederichs and O. Weichold, *Langmuir*, 2010, **26**, 17913–17918.
42 G. Lagaly, M. Reese and S. Abend, *Appl. Clay Sci.*, 1999, **14**, 279–298.
43 B. P. Binks, J. A. Rodrigues and W. Firth, *Langmuir*, 2007, **23**, 3626–3636.