Evaluating hydrophobins for emulsion stabilisation

In the last few years an increased consumer demand for natural ingredients in cosmetic products has been continuously developed. Consequently the cosmetic industry launched a lot of products containing substances with a biological background, like palm oil or hyaluronic acid, on the market. Moreover another effect of this raised customer pressure is an enhanced demand for exclusive, innovative and surfactant-free systems or products from the cosmetic industry.

Of course, proteins are interesting polymers that can be used in personal care products. They are of natural origin, non toxic and easily biodegradable. Therefore proteins are well-accepted by customers. Proteins are natural polymers. Due to their building blocks of hydrophilic and hydrophobic amino acids, proteins are amphiphilic molecules and lower the surface tension of water. Moreover many scientific studies have been carried out on the use of proteins as emulsifying agents. However the obtained emulsions often provided an insufficient tolerance against typical emulsion instabilities, like coalescence or creaming. Furthermore a lot of proteins are weak emulsifiers or the used protein concentrations were quite high, consequently potential industrial applications suffered from the huge cost of the raw materials.

A special class of fungal proteins, called hydrophobins, raised the interest of scientists in the early 1980s.[1] Hydrophobins are small proteins of around 100 amino acids and are believed to be the most surface active proteins.[2] Considering the unique architecture of hydrophobins, their distinct amphiphilic nature becomes visible (Fig. 1). A pronounced, local accumulation of hydrophobic amino acids is the so-called 'hydrophobic patch'. Moreover, hydrophobins are rigid, globular proteins. The rigidity is provided by the formation of four disulphide bridges in the tertiary protein structure. As a result, hydrophobins are very stable against external stress, like thermal heating. Due to these unique structural features hydrophobins can be considered as rigid bio-surfactants. Hydrophobins are well known for their strong tendency to adsorb on interfaces and their tendency of self-assembly. Hydrophobin films provide a remarkable mechanical stability.[4]

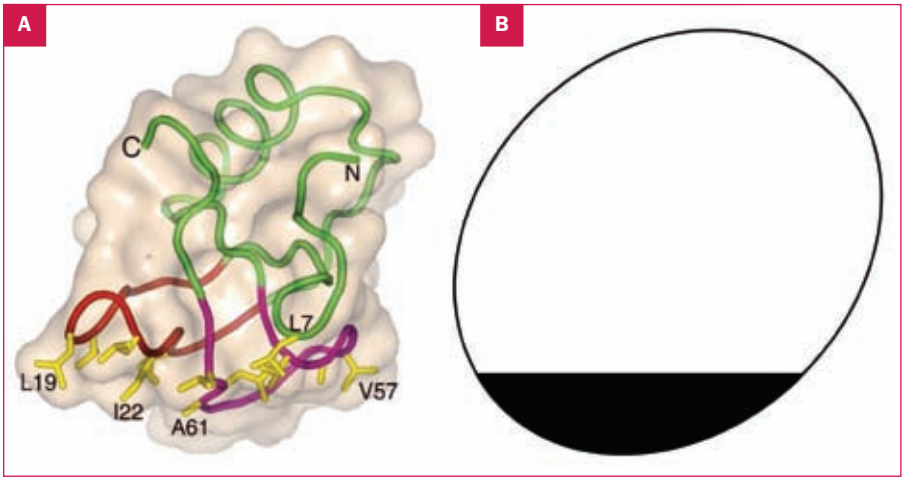

Figure 1: *Illustration of the position of the hydrophobic surface patch in the tertiary structure of HFBII from* Trichoderma reesei *(A).3 The amino acid residues that are part of the hydrophobic patch are shown in yellow. A schematic abstract figure (B) points out more clearly the distinct amphiphilic character of hydrophobins. The hydrophobic patch is drawn in black.*

Their natural origin combined with these unique properties make hydrophobins very interesting for industrial applications. However their use was restricted by their low availability. The yield of protein purification from mushrooms was unsatisfactory. Recently, due to the use of white biotechnology, BASF succeeded in the high-scale production of recombinant hydrophobins, called H Star Proteins A and B.[5] Hydrophobins are now available in sufficient amounts and ready for industrial applications, for instance as emulsifiers. Of course, there is still undoubtedly a huge interest in using natural emulsifiers instead of surfactants. Therefore one part of this study will evaluate the emulsifying performance of these recombinant hydrophobins.

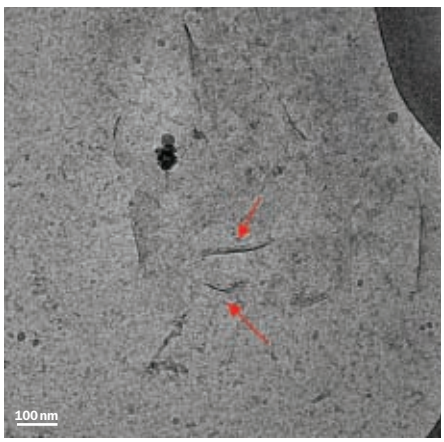

Figure 2: *Cyro-TEM micrograph of an aqueous solution of 0.1 wt% H Star Protein B. The red arrows show membrane fragments of more than 100 nm that are formed within the sample.*

Moreover, clay particles are used in cosmetic applications as rheological additives. Clays are known to undergo a sol-gel transition after crossing a critical concentration of about 2-3 wt% in the aqueous phase depending on the used clay. Clays, for example, are mixed into cosmetic emulsions in order to increase the viscosity of the aqueous phase. An increased viscosity is desirable as it slows down the Brownian motion within the sample. A decreased motion of the emulsion droplets is correlated with higher emulsion stability, because the rate of coalescence or creaming events is reduced.

Clays are disc-like particles with a

thickness of 1 nm. Synthetic and natural clays are colloidal building blocks with a well-defined structure.[6] Clays possess an excess negative charge due to special ion-substitutions, like Si by Al, Al by Mg and Mg by Li. As clays are solid particles, they can be used as stabilisers in Pickering emulsions. Emulsions only stabilised by clay particles are not very stable. Interestingly, in combination with surfactants, the stability of emulsions based on surfactant-covered clays can be increased.[7] Nevertheless, it seems that nobody has yet investigated the emulsion performance of protein covered clays. In this study, the synergistic use of low concentrations of hydrophobin and the clay Laponite as emulsifying agent resulted in toothpaste-like, homogenous and very stable Pickering emulsions.[8] These systems provide an extraordinary, fascinating and novel way to achieve emulsion stability.

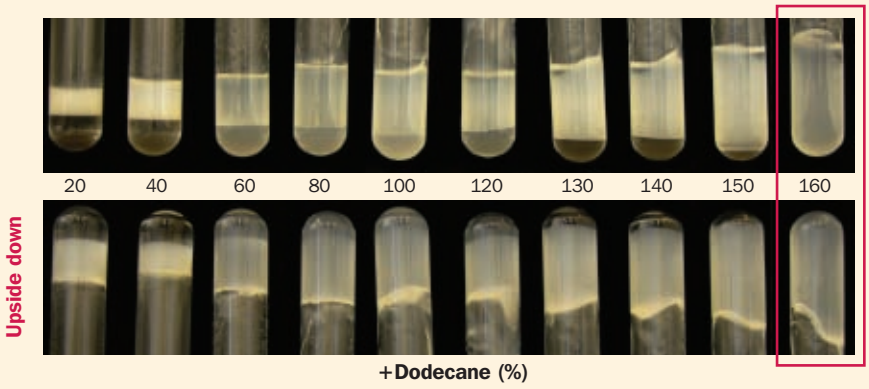

Figure 3: *Three days old emulsions prepared from 0.5 wt% HPB at pH 6; the aqueous phase contained 60 wt% glycerole (for refractive index matching). The emulsions were prepared by vortexing the samples for 30 min with the highest shear. The content of the apolar oil dodecane was successively increased from 20 wt% to 160 wt%. Homogenous emulsions could be obtained by adding 160 wt% oil. The upside down photograph confirms the gel-like properties of the obtained emulsions.*

Materials and methods

H Star Protein B, from now on abbreviated to HPB (19 kDa; IEP: 6.15; Zeta Potential – 31 mV for 1 wt% solution), is a recombinant hydrophobin from BASF, Ludwigshafen. The clay, Laponite XLG, was purchased from Rockwood Clay Additives GmbH, Moosburg. The silicone oil polydimethylsiloxane (PDMS) was obtained from Shinetsu Kagaku, Tokyo. Its general formulation is (CH3)3SiO[(CH3)2SiO]nSi(CH3)3. The polymerisation degree Ë ranges from 5 to 19 (>98 %) and the viscosity is approximately 6 mPas. Merck, Darmstadt, supplied the nonpolar oil Dodecane, as well as the polar oil Octylmethoxycinnamate (OMC, brand name: Eusolex 2292). All other chemicals not further specified were of analytical grade or equivalent.

Cryo-Transmission Electron Microscopy (Cryo-TEM): A drop of the sample was transferred to a TEM-grid (200 mesh, Science Services, Munich). 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 were examined with a Zeiss EM922 Omega EFTEM operating at 200 kV. All images were digitalized with the CCD camera system from Ultrascan 1000, Gatan.

All emulsions were prepared from aqueous solutions of the desired emulsifier. Additionally all emulsions contained 0.5 wt% phenoxyethanole as antimicrobial agent. It was only possible to produce high oil content emulsions by stepwise oil addition, as one step oil addition to the aqueous phase led to the breakdown of the emulsions. Emulsions were prepared by using the vortex device, the Ultra Turrax or the High Pressure Emulsifier (APV 1000, Albertslund). The high pressure emulsifying procedure 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).

Light microscopy was used in order to evaluate the average emulsion droplet size of the emulsions. The samples were trapped between a microscope slide and a cover glass and were investigated with a Zeiss light microscope (model: 47 60 05 – 9901). The micrographs were digitalised with the DFK 41F02 camera and analysed with 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 was analysed with the Haake RheoWin Data Manager, Version 3.3.

For scanning electron microscopy (SEM) the emulsion sample was stored for one day at room temperature and afterwards freeze dried for two days. The freeze dried sample was investigated using a Zeiss 1530 Scanning Electron Microscope with a field emission cathode.

For Cryo-SEM, a drop of the emulsion was trapped between aluminium specimens. Afterwards it was 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 a defined thickness. Finally the Ultra Plus Zeiss SEM harbouring a third generation Gemini electron optical column was charged with the coated specimens. The integrated Thermo Scientific MagnaRay WDS spectrometer automatically handled alignment and analysis.

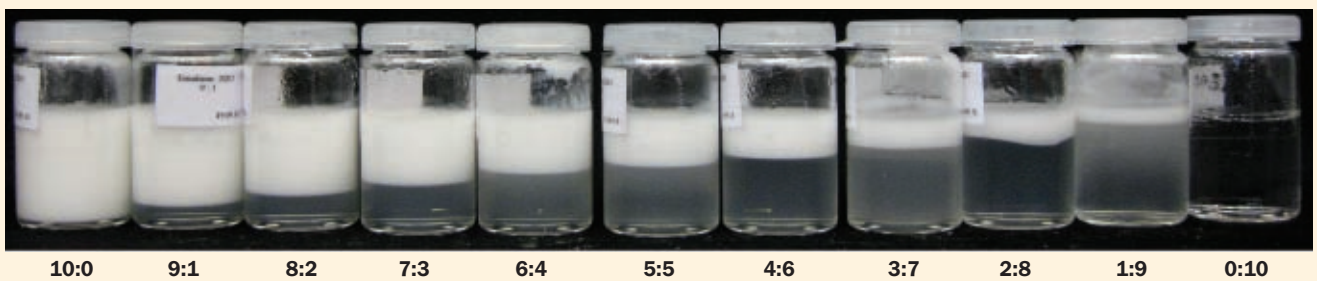

Figure 4: *Stepwise dilution of a homogenous emulsion with water. The original emulsion contained 0.5 wt% HPB and 65 wt% of the oil PDMS and was prepared with the high pressure emulsifier at 300 bar. The picture was taken two days after mixing with water.*

Results and discussion

A preliminary physico-chemical characterisation of the recombinant hydrophobins confirmed that they indeed maintain the same properties as natural proteins.[9] Time dependent surface tension measurements confirmed that that the interface adsorption of hydrophobins can be considered as a two step process. The first step – the initial adsorption – is fast. In a second step – the surface or interface induced reorientation – the hydrophobins undergo conformational rearrangements in order to establish a better interaction with the new environment. For example hydrophobic regions that have been hidden from the aqueous milieu in the core of the protein, can now interact with the new interface. A consequence of adsorption and conformational rearrangements is the availability of alternative interaction sides for the hydrophobins. Due to their distinct ability to self-assemble, the hydrophobins can entangle more effectively with each other in a time dependent process. Also Cryo-TEM investigations resolved membrane fragments and film formation of the recombinant hydrophobins (Fig. 2).

Both hydrophobins HPA and HPB can be solubilised in water up to a concentration of 5 wt%. The solutions with 1 wt% hydrophobin have a pH of 7.95 (HPA) and 7.54 (HPB).

First, emulsification tests using the recombinant hydrophobins were performed. In order to establish a simple system, the hydrophobin was used as received without any further treatment, like adding buffers. One first goal was to achieve a homogenous emulsion layer stabilised by hydrophobins. Interestingly, a simultaneous increase of the hydrophobin and the oil concentration did not result in homogenous emulsions. Nevertheless, only increasing the oil content at a fixed protein concentration led to single layer emulsions (Fig. 3). Adding a 160 wt% dodecane to the aqueous solution of 0.5 wt% HPB finally resulted in a homogenous emulsion. Turning the samples upside down confirmed the gel-like properties of the emulsions. The origin of these elastic properties can be explained by the time-dependent developing of a self-supporting three dimensional network formed by the hydrophobin. Obviously the hydrophobins do not lose their tendency to self-assemble upon adsorbing at an interface. Instead of the fact that the recombinant hydrophobins possess an excess negative charge, they obviously attract each other in the short range distance. Two experiments were designed in order to confirm the existence of an attractive energy between the hydrophobin covered oil droplets and of the assumed hydrophobin network.

Figure 5: *Obtained light material after removing the oil and water of an emulsion. The emulsion was incubated for two weeks at 65˚C in a cabinet dryer.*

As hydrophobin is negatively charged, the hydrophobin-coated oil droplets should repel each other. Therefore a homogenous emulsion should stay in this situation after being diluted with water. The situation of an emulsion being stepwise diluted with water after two days is shown in Figure 4. Apparently the emulsion droplets attract each other indicating by the distinct phase boundary between the top emulsion layer and the lower aqueous layer. The attractive energy is obviously bigger than the repulsive electrostatic energy. Recently the origin of this attractive energy was clarified.[10] An adhesion energy, acting in the short range distance, is responsible for the aggregation of hydrophobins. One can conclude that the hydrophobin-coated oil droplets also attract each other. Upon stepwise interpenetration of hydrophobins located at different droplets, a network evolves within the emulsion. The gel-like properties are a consequence of this process. Moreover it has already been shown that this network strengthens with time due to the progressed hydrophobin entanglement.[9]

Furthermore after removing the oil and the water by drying, a light material remains (Fig. 5). A REM-micrograph of the material showed that the emulsion droplet size was identical to the one determined by light microscopy. The structure had not collapsed during the removal of the oil and water. This seems to have been only possible if the individual films around the oil droplets were cross-linked to a network structure.[9]

In conclusion the emulsion droplets attract each other. These introduced a novel way of emulsion stability that is actually contradictory. Normally an attraction of oil droplets is linked to emulsion instability mechanisms like coalescence or flocculation. Time dependent studies, however, confirmed that the average droplet size as well as the visual appearance of the emulsions based on hydrophobins did not change.[9] This confirms the emulsion to be stable.

Another point of this study evaluated the influence of external stress, like pH shift or salt addition, on the emulsions. It turned out that tuning the pH from 8 to 3 by adding HCl did not destabilise the emulsions. Moreover, emulsions prepared at pH values near to the isoelectric point of the hydrophobins turned out to be more gel-like (Fig. 6). This can be explained by the decrease of the electrostatic repulsion as the negative excess charge becomes smaller by adding HCl. Moreover the emulsion stability is also not much affected by the addition of salts, like NaCl or $CaCl_2$. In the case of adding $CaCl_2$ the emulsion did not show any ageing effects. Addition of 100 mM NaCl also did not influence the emulsion's properties. Furthermore it turned out that thermal treatment supports the evolution of the gel-like properties.

An interesting point was the

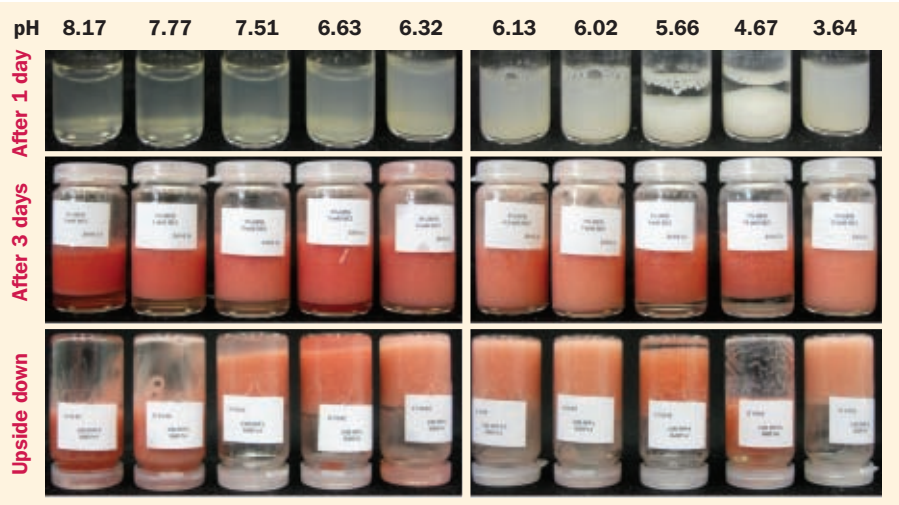

Figure 6: *Influence of pH shifting towards the gel-like and phase behaviour of the emulsions. The emulsions contained finally 0.5 wt% HPB and 50 wt% PDMS and have been prepared with the Ultra Turrax. The oil phase has been coloured with the dye Sudan III.*

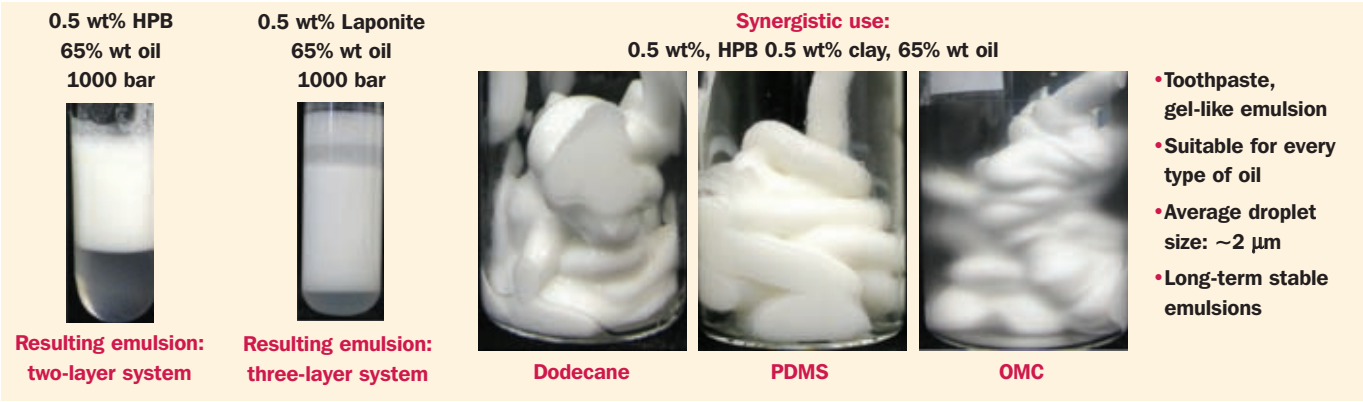

Figure 7: *Demonstration of the synergistic use of hydrophobin and clay for emulsion stabilisation. The resulting Pickering emulsions are toothpaste like and long-term stable. All types of oil, like the apolar dodecane, the silicone oil PDMS and the polar octyl-methoxycinnamate, can be emulsified.*

determination of the minimum amount of hydrophobin that is needed to provide sufficient emulsion stability. Homogeneous, gel-like emulsions could be obtained with a protein concentration between 0.02 wt% and 1 wt% and an oil mass fraction of more than 0.65. More details are given in Reference 9.

It is well known in emulsion science that the applied shear stress during emulsion preparation correlates with the final obtained average emulsion droplet size and the polydispersity. Using the high pressure emulsifier at high shear rates of 1000 bar did decrease the emulsion droplet size of emulsions prepared on hydrophobin significantly. However, the emulsions lost their homogenous character and the phase separated. In order to stop phase separation at high shear rates, an increased yield stress in the emulsion was introduced. Therefore the clay particles, respectively Laponite XLG, were modified with hydrophobin and used as emulsifying agents. The resulting Pickering emulsions had toothpaste, homogenous and gel-like properties (Fig. 7). The shown Pickering emulsions have been stored at room temperature and are now already stable for one year. The average emulsion droplet size as well as the rheological properties are demonstrated in Figure 8. Detailed studies about the synergistic use of clay and hydrophobin can be looked up in Reference 8.

Conclusion

Hydrophobins are able to stabilise emulsions by forming a time-dependent, three-dimensional network after adsorbing at the oil-water interface. Homogenous emulsions can already be obtained by using only 0.02 wt% of hydrophobin. Nevertheless, in order to achieve homogenous emulsions, it is important to use high concentrations of oil. Otherwise the emulsion will phase separate. This effect is also a strong confirmation of the attractive force acting between the hydrophobin covered oil droplets. The emulsion stability, as well as the gel-like character can be achieved, by using clay and hydrophobin synergistically. The obtained Pickering emulsions provide a long-term stability due to the formation of the hydrophobin network that is stabilised by the disc-like clay particles. PC

Acknowledgement

The author is a 28-year-old scientist with a biochemical background from Germany. He will complete his PhD in the spring of 2012. His supervisor is Prof. Dr. em. Heinz Hoffmann. The author thanks the Shiseido Company, Yokohama, Japan, for financial support. BASF SE, Ludwigshafen, Germany, provided the recombinant hydrophobins.

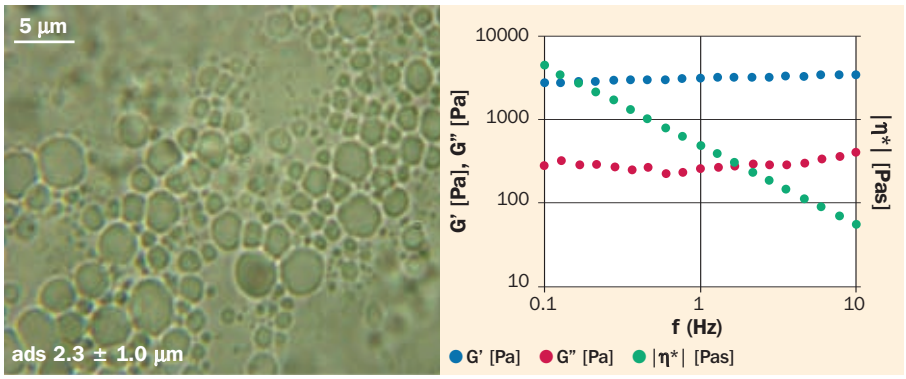

Figure 8: *Light microscopy (left) and rheogram (τ=0.5 Pa; right) of the Pickering emulsion prepared from 0.5 wt% HPB, 0.5 wt% Laponite XLG and 65 wt% PDMS. The high storage moduli G' indicates the high gel-like properties of the emulsions.*

References

1 Dons JJM, Springer I, de Vries S, Wessels JGH. Molecular cloning of a gene abundantly expressed during fruiting body initiation in *Schizophyllum commune*. *J Bacteriol* 1984; **157**: 802-8.

2 Wösten HA, de Vocht ML. Hydrophobins, the fungal coat unraveled. *Biochim Biophys Acta* 2000; **1469**: 79-86.

3 Hakanpää J, Paananen A, Rouvinen J. Atomic resolution structure of the HFBII Hydrophobin, a self-assembling amphiphile. *J Bio Chem* 2004; **279**: 534-9.

4 Basheva ES, Kralchevsky PA, Christov NC, Danov KD, Stoyanov SD, Blijdenstein TBJ, Kim HJ, Pelan EG, Lips A. Unique Properties of bubbles and films stabilized by HFBII hydrophobin. *Langmuir* 2011; **27**: 2382-92.

5 Wohlleben W, Subkowski T, Bollschweiler C, van Vacano B, Liu Y, Schrepp W, Baus U. Recombinantly produced hydrophobins from fungal analogues as highly surface-active performance proteins. *Eur Biophys J* 2010; **39**: 457-68.

6 Sposito G, Skipper N, Greathouse J. Surface geochemistry of the clay minerals. *Proc Natl Acad Sci USA* 1999; **96**: 3358-64.

7 Lagaly G, Reese M and Abend S. Smectites as colloidal stabilizers of emulsions I. Preparation and properties of emulsions with smectites and nonionic surfactants. *Applied Clay Science* 1999; **14**: 83-103.

8 Reger M, Sekine T, Okamoto T, Watanabe K, Hoffmann H. Pickering Emulsions stabilized by novel clay-hydrophobin synergism. *Soft Matter* 2011; **7**: 11021-30.

9 Reger M, Sekine T, Okamoto T, Hoffmann H. Unique emulsions based on biotechnically produced hydrophobins. *Soft Matter* 2011; **7**: 8248-57.

10 Basheva ES, Kralchevsky PA, Danov KD, Stoyanov SD, Blijdenstein TBJ, Pelan EG, Lips A. Self-assembled bilayers from the protein HFBII-hydrophobin: Nature of the adhesion energy. *Langmuir* 2011; **27**: 4481-8.