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Adhesion Properties of Free-Standing Hydrophobin Bilayers

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Abstract

Hydrophobins are a family of small-sized proteins featuring a distinct hydrophobic patch on the protein's surface rendering them amphiphilic. This particularity allows hydrophobins to self-assemble into monolayers at any hydrophilic/hydrophobic interface. Moreover, stable pure protein bilayers can be created from two interfacial hydrophobin monolayers by contacting either their hydrophobic or their hydrophilic sides. In this study, this is achieved via a microfluidic approach, in which also the bilayers' adhesion energy can be determined. This enables us to study the origin of the adhesion of hydrophobic and hydrophilic core bilayers made from the class II hydrophobins HFBI and HFBII. Using different fluid media in this setup and introducing genetically modified variants of the HFBI molecule, the different force contributions to the adhesion of the bilayer sheets are studied. It was found that in the hydrophilic contact situation, the adhesive interaction was higher than in the hydrophobic contact situation and could be even enhanced by reducing the contributions of electrostatic interactions. This effect indicates that the van der Waals interaction is the dominant

contribution that explains the stability of the observed bilayers.

Introduction

Compartmentalization is the fundament for the functioning of living systems, keeping important molecules in a confined space and thus inhibiting uncontrolled diffusion and dilution. Also in industrial applications, e.g. to produce pharmaceuticals, the separation of media or solutes is an important aspect. In living organisms, this function is performed by lipid bilayers. In bio-mimicking applications, also other building blocks are used: Studies report of membranes and compartments made from, e.g., polymers¹, polymer-protein conjugates, engineered proteins²⁻⁴, or, recently, even naturally occurring amphiphilic proteins called hydrophobins⁵. Especially about membranes formed by hydrophobins and their stabilizing forces, little is known.

Hydrophobins, are small, globular proteins expressed by filamentous fungi⁶. Their name refers to their strong amphiphilicity, which results from a relatively large water-repellant surface area, which is called the 'hydrophobic patch'⁷. As the rest of their surface area is hydrophilic, hydrophobins are strongly

amphiphilic molecules. Like surfactants, they are therefore able to cover interfaces or adsorb to solid substrate surfaces and alter their wettability^{8–10}. This surface-covering function is employed by fungi in biological processes that involve physical interactions with interfaces, e.g. penetration of the air-water interface by aerial hyphae and during attachment to hydrophobic surfaces¹¹. Their high surfaceactivity together with their native origin renders hydrophobins also interesting for technical applications; food industry for instance is interested in hydrophobins to stabilize emulsions, e.g. in beer^{12,13} and ice-cream as well as other food foams¹⁴. Moreover, hydrophobins are also envisaged to enhance the acceptance of medical implants^{15,16} as well as to promote controllable cell growth for biodevices^{17,18}.

The possibility of forming bilayers and vesicles made purely from hydrophobins in aqueous as well as oily phases may give rise to an even larger variety of applications. The reason for their stability and the contributing forces are, however, not studied in detail, yet, and are largely unclear.

For this study, the class II hydrophobins HFBI and HFBII were used. When adsorbed to fluid interfaces, these proteins form monolayers, like a surfactant. Almost independently from the protein bulk concentration, they cover the surface completely in a very dense monolayer^{19,20}. In these layers, the individual proteins remain mostly in their solute conformation²¹ and are oriented such that the hydrophobic patch faces away from the aqueous phase^{22,23}. Moreover, they are ordered in honeycomb lattices (instead of rodlets as class I hydrophobins do)⁷ giving rise to an unusual high dilatational and shear elasticity of the layer^{24–26}. Upon contacting two hydrophobin interfacial layers, it is therefore possible to form stable double-layers, similar to lipid bilayers^{5,27}. In the focus of this study are the contributing forces that stabilize these unusual bilayers. Therefore, a microfluidic approach able to produce free-standing

bilayers is employed. Introducing the hydrophobins to this setup allows for the formation of protein bilayers with either a hydrophobic or a hydrophilic core, in contrast to what is possible with lipids. The adhesion properties of the protein bilayers are then measured *in situ* by optical microscopy. This permits an estimation of the average pair interaction energy of a single protein pair across the bilayer sheets. To study the relevant forces for the bilayer formation, in particular the role of the electrostatic contribution on the measured adhesion energy, variants of the HFBI molecule exhibiting mutations of the charged amino acids of the protein are employed.

Materials and Methods

Proteins

Hydrophobin HFBI and its variants The HFBI molecule is a small (ca. 7.5kDa) and compact protein of which one pole is made up from purely hydrophobic amino acids that is called the hydrophobic patch. The remaining protein surface is more polar and contains six charged amino acid residues. These are located on the pole opposing the hydrophobic patch (Asp40, Asp43, Arg45 and Lys50), which we'll refer to as the 'hydrophilic pole', and in a region between the poles (Asp30 and Lys32) (see figure 1a) that is predicted to be oriented towards neighbouring HFBI molecules within an assembled HFBI monolayer⁷. The proteins used in this study are the wild type (WT) form of HFBI, produced by the filamentous fungus *Trichoderma reesei*, and four genetically modified variants of HFBI (production process described by Lienemann et al.³¹), cf. fig. 1. These mutants were created by changing the charged residues to similar sized but charge-neutral asparagine (Asn) or glutamine (Glu) residues. The latter was chosen in the case of Asp30 and Asp43 to avoid additional structural changes through N-glycosylation.

The HFBI variants were designed to study the effect of four groups of charged amino acids on the interfacial HFBI assembly. These groups are the counter charge pair close to the hydrophobic patch and, at the hydrophilic pole, the two positive charges, the two negative charges, and all four charged residues (cf. Lin-

a) wild type (HFBI-WT)

Figure 1: a) Protein structure of wild type HFBI (PDB entry 2FZ6²⁸). The six charged side groups are marked and colored in blue (basic) and magenta (acidic); the hydrophobic patch (red) is only partly visible. b)–d) Using again the wild type’s structure for the respective mutant, only the remaining charged side groups are marked: b) HFBI-DK, c) HFBI-RK, d) HFBI-DD, and e) HFBI-DDRK. f) Protein structure of HFBIII (PDB entry 2B97²⁹) shown from a similar perspective as the structures above. Charged groups and hydrophobic patch are colored as above. Additionally, the five hydrophobic groups opposite to the hydrophobic patch, which are replaced by hydrophilic groups in HFBI, are colored red.

(The figures were produced using UCSF Chimera³⁰.)

der et al.⁶). Exchanging these groups results in the variants HFBI-DK (see fig. 1b), HFBI-RK (fig. 1c), HFBI-DD (fig. 1d), and HFBI-DDRK (fig. 1e), respectively. It was shown previously that these mutations affect the interactions of the proteins laterally within an interfacial layer as well as with the water phase but do not alter the folding state of the proteins^{31,32}.

HFBIII Further experiments were performed with the protein HFBIII, which is also a class II hydrophobin produced by the same organism as HFBI. Although HFBI and HFBIII are very similar in size, structure, and function^{14,28}, they show small differences in their amino acid sequence that may affect their behavior in the experiments of this study: At the hydrophilic pole, HFBIII contains five surface-exposed, hydrophobic amino acids (Ile31, Ala32, Ala37, Ile38, and Ala41) in its crystal structure³³. Even in its dissolved form, these amino acids are very likely not buried inside the protein core. These groups are substituted by more hydrophilic or even charged groups in the case of HFBI. Moreover, HFBIII has eight charged amino acids (four positive, four negative) as opposed to six in the case of HFBI. Only two of them are positioned at the hydrophilic pole, the rest at the side of the protein (similar to the ‘DK’ group in HFBI) with the acidic amino acids closer to the hydrophobic patch.

Preparation of the protein solution

The lyophilized proteins were dissolved in acetate buffer (pH 5, buffer concentration 10mM) at a protein concentration of 100μM. These stock solutions contain a calculated ion concentration (ionic strength) of 6mM. Prior to usage, the solutions were sonicated in order to dissolve possible protein aggregates and diluted with the same buffer to the final concentration. For the two types of bilayers

studied here (with hydrophilic or hydrophobic core), minimum protein concentrations were used that ensured the fast formation of a stable interfacial layer: This concentration was 20 μ M in the case of a bilayer with hydrophilic core and 5 μ M for a hydrophobic core bilayer. Within this concentration range we can safely assume that the proteins fully cover the interface since the saturation value for the amount of proteins adsorbed to the interface and hence the interfacial tension do therefore not depend on the bulk concentration.

Figure 2: Temporal evolution of the interfacial tension γ of HFBI wild type (WT), and the genetic variants DK, RK, and DDRK at a hexadecane/buffer interface measured using the pendant drop technique. The values given in table 1 correspond to the plateau value of each trace.

Protein solutions with higher ionic strength were prepared by mixing the stock solutions with buffers supplemented with NaCl. The ionic strengths of these buffers were adjusted to receive final protein solutions with ionic strength of 100, 200, 500, or 1000mM.

Interfacial Tension

interfacial tensions γ of the various HFBI protein monolayers at oil/water and air/water interfaces were measured with the pendant drop method using a commercial measurement device (OCA 20, DataPhysics Instruments

GmbH, Filderstadt, Germany). Droplets of protein solution with a concentration of 5 M were produced by introducing droplets of the protein solution from a steel needle into the surrounding air or oil phase. As oils, hexadecane ($C_{16}H_{34}$, prod. no. A10322, Alfa Aesar, Karlsruhe, Germany), and FC70 (3M, St. Paul, MN, USA) were used. Since FC70 is denser than water, a hooked needle was used to produce buoyant droplets of protein solution in FC70 surrounding. The shape of all droplets was fitted with the Young-Laplace equation to obtain the interfacial tension. After the initial formation of a droplet, proteins adsorb to the Table 1: interfacial tension of the employed proteins at the interfaces buffer/air, buffer/hexadecane, and buffer/FC70 as measured by the pendant drop method. Buffer ionic strength: 6mM. Error values are standard deviations.

interface	protein	interfacial tension γ / mN/m
buffer/...		
air	HFBI	16.2 ± 0.6
	HFBI	17.6 ± 0.9
hexadecane	HFBI	13.9 ± 1.5
	HFBI	22.2 ± 0.8
	HFBI-DK	11.5 ± 1.1
	HFBI-RK	11.4 ± 1.4
	HFBI-DD	10 ± 2
FC70	HFBI-DDRK	11 ± 1
	HFBI	29 ± 1
	HFBI	25 ± 1
	HFBI-DK	28 ± 0.9
	HFBI-RK	28 ± 1.2
	HFBI-DD	30 ± 4
	HFBI-DDRK	31 ± 1.5

Table 2: Interfacial tension of HFBI wild type at hexadecane/buffer interfaces for different ionic strengths of the buffer as measured by the pendant drop method. Error values are standard deviations.

ionic strength	interfacial tension
I / mM	$\gamma / \text{mN/m}$
6	13.9 ± 1.5
100	14.2 ± 1.8
500	17.5 ± 2.3
1000	20.3 ± 1.8

interface leading to a reduction in interfacial tension. This decrease was recorded over several minutes until a plateau was reached (see fig. 2).

During the course of an experiment, the produced droplets may decrease in volume due to water evaporation in the case of air as surrounding medium, dissolution into oil, or due to fluctuations in the experimental setup. A continuing volume reduction after the formation of

in a ‘NOA chip’ using hexadecane as finger and buffer as continuous phase. The protein double layers are sketched showing their orientation in the layers. Red represents the hydrophobic poles and white the hydrophilic poles.

a complete interfacial protein layer leads, however, to a wrinkled surface. This wrinkling is a sign for an elastic behavior of the formed layer. For elastic behavior, however, the Laplace model is not applicable anymore^{34,35} and will yield large errors²⁶. Hence, the droplet’s shape cannot be analyzed by the standard droplet analysis method. In order to avoid this difficulty and to mimic the situation in the microfluidic channels, the interfacial tension evolution was recorded with a constant or slightly increasing drop volume to prevent a solidification of the protein layer (see SI for a more detailed discussion). The obtained values have to be regarded, however, as upper approximation of the surface energy of the respective interface.

Microfluidic set-up

To form a free-standing protein bilayer, a microfluidic chip with a cross-channel geometry was used, which was already successfully applied to to produce lipid bilayers³⁶. In this geometry, a continuous phase (e.g. oil) separates two liquid fingers of another phase (e.g. aqueous buffer solution containing the surface active proteins), see fig. 3. The distance of the two fingers can be controlled by syringe pumps. Thus, the two layers of the surface active molecules formed at each fluid interface can be brought into contact while being monitored by optical microscopy.

Since it is necessary that the continuous phase wets the walls of the microfluidic device, two different chip materials were used to generate hydrophobin bilayers with hydrophilic and hydrophobic core (see ref.⁵ for details of the device fabrication): PDMS (sylgard 184 – Dow Corning) served as hydrophobic material (in the following called ‘PDMS’ chip) for air, hexadecane, and FC70 as

Figure 3: a) Sketch of the cross-channel geometry of the microfluidic chip. Finger (blue) and continuous phase (green arrow, fluid not shown) can be oil, air or buffer. The channel are 300 μm in width and 100 μm in height. b) Hydrophobic core HFBI bilayer in a PDMS chip using buffer solution as finger and hexadecane as cont. phase. The angle θ gives the bilayer contact angle. c) Hydrophilic core HFBI bilayer

continuous phase. For aqueous solution as continuous phase, a UV-curable polymer (Norland Optical Adhesive, NOA83H, Edmund Optics, USA) was utilized as hydrophilic chip material (called 'NOA chip').

Production of protein bilayers with controllable protein orientation

To create protein bilayers, two liquid fingers were injected face-to-face into the cross geometry of the microfluidic chip previously filled with the continuous phase (see fig. 3a). One of the two phases is the protein containing buffer solution. The two fingers were left to rest for 5–10 minutes, to reach a protein saturation at each fluid/fluid interface. That time span was taken as an upper approximation from ellipsometric measurements of the protein coverage at a flat water/air interface, where a saturation was reached after 5 min for similar concentrations³⁷. Subsequently, the two fingers were slowly inflated again and brought into contact (interface velocity 5–10 m/min), and finally the system was left to rest for 30–40 minutes in order to confirm the bilayer stability. Upon contact, a straight interface is formed that increases until, after several seconds, a constant bilayer contact angle is reached and measured. This effect and the absence of coalescence indicates already the presence of a free-standing bilayer. For bilayers with a hydrophobic core (where the finger phase is aqueous and thus conducting), capacitance measurements as reported before⁵ confirmed the formation of a solvent-free protein bilayer.

Bilayer tension and adhesion energy

From the values of the interfacial tension γ and the bilayer contact angle θ , which were obtained from pendant drop measurements or optical micrographs (see fig. 3b), respectively, the bilayer tension Γ can be calculated using Young's equation^{38,39}

$$\Gamma = 2\gamma \cos\theta. \quad (1)$$

Additionally to the bilayer tension, also the corresponding adhesion energy per unit area between the bilayer sheets, ΔW , can be derived from interfacial tension and contact angle using the Young-Dupre relation

$$\Delta W = 2\gamma(1 - \cos\theta). \quad (2)$$

Results and Discussion

Adhesive properties of HFB bilayers at different interfaces

In order to study the adhesive properties of HFBI and HFBII bilayers, the tension of the single interfacial layers as well as the contact angle between two contacted interfaces need to be known. Therefore, the interfacial tensions of the three interfaces air/buffer, hexadecane/buffer, and FC70/buffer covered with a protein layer were measured using the pendant drop method. The results are displayed in table 1. The obtained values are consistent with interfacial tensions reported for similar systems^{40,41}. For HFBI at the air/buffer interface, pendant buffer droplets in air as well as buoyant air bubbles in buffer phase were used. For both cases, nearly identical interfacial tension values were recorded, which shows that neither evaporation nor interface curvature affect the tension measurements.

For the measurement of the contact angle θ , HFBI and HFBII bilayers with hydrophilic as well as hydrophobic cores were formed in the microfluidic setup using the same combinations of liquids and hydrophobins as in the interfacial tension measurements. The contact angle θ of a stable bilayer was determined from optical micrographs. From these values, the bilayer tension Γ and the adhesion energy ΔW of the six different types of bilayers are calculated using equations 1 and 2. The resulting values are compared in figure 4.

The bilayer tension Γ was found for each type of molecule to be mainly dependent on the phase combination used (see fig. 4a). Thereby, the comparison of the different tensions shows that the bilayer tension follows the trends given by the interfacial tensions of the individual interfaces. The highest tension of $55 \pm 3 \text{ mN/m}$ was measured for HFBI bilayers in combination with the fluorinated oil FC70. Compared to the typical lysis tension for lipid vesicles, which ranges about 10 mN/m ⁴², this value is impressively large. That the hydrophobin bilayer can sustain such a tension without immediate rupture and droplet fusion reflects the very high lateral cohesion of the hydrophobin molecules in such a layer.

The bilayer orientation, in contrast, has only little influence on the bilayer tension. The difference in Γ between hydrophilic and hydrophobic core bilayers at the same media combination is due to the different adhesion strengths of the respective bilayer cores. That this difference is much smaller than the absolute bilayer tension reflects the notion, that the free interfacial energy of the individual interfaces is much larger than the adhesion energy between them.

Indeed, we determined, in comparison to the individual interfacial energies, rather small adhesion energies ΔW in the range of several mJ/m^2 (see figure 4b)). This finding is

consistent with the observation of an independent experiment described in the SI. For HFBI, we find that the interaction energy between the hydrophobic sides of the proteins is about 50% lower than between the hydrophilic sides. Moreover, for the bilayers with a hydrophobic core, we cannot observe differences in the interaction energy for different media: For all of these interfaces, nearly the same value for ΔW , ca. 1.2 mJ/m^2 , is obtained. With the area occupied by one protein, taken as 3.2 nm^2 from Lienemann et al.³¹, the energy per molecule pair for the interaction between their hydrophobic sides can be estimated as ca. $0.9 k_B T$. This low interaction energy gives rise to the fact that the two sheets forming the bilayer can be easily removed from each other without much disturbance of the single interface⁵ since the adsorption energy of a HFBI molecule to the alkane/water interface is about two orders of magnitude higher⁴³.

The adhesion energy of the hydrophilic contact varies more for different media combinations than the energy for the hydrophobic contact situations. Moreover, the adhesion energy of the hydrophilic contact of HFBII molecules varies much more upon change of the hydrophobic medium than the respective energy for HFBI does. A possible explanation for this behavior might be that the

Figure 4: a) Bilayer tension and b) adhesion energy of HFBI and HFBII bilayers for the different finger phases defining the protein orientation. Different colors mark different “hydrophobic” phases: air (blue), hexadecane (purple), and FC70 (orange). Hashed bars represent values from hydrophobic core bilayers and non-hashed bars from hydrophilic core bilayers.

affinity of HFBI and HFBII for the different phases is slightly different⁴³. Thus, the exact position or height of both proteins in the interface differs, which might also hint at slightly different orientations or heights of one protein type at different interfaces.

The adhesion energy of HFBII bilayers is in most measured cases higher than the respective energy for HFBI, with the exception of the hydrophilic contact in air. The most prominent difference is found for the adhesion energy of the hydrophilic contact in combination with hexadecane. Here, we recorded the highest value throughout our measurements: 5.9 mJ/m². The higher adhesion energy of HFBII bilayers compared to the respective HFBI bilayers might be explained by the presence of hydrophobic amino acids on the hydrophilic pole of HFBII which was also concluded by Basheva et al.²⁷. In aqueous surrounding, these groups are subject to the hydrophobic effect, when brought into contact. The strength of this effect is demonstrated in the experiments of Goldian et al.¹⁰ more dramatically: For two HFBI layers contacted with their hydrophobic sides in pure water as medium, they measured an adhesion energy of 56 mJ/m². This is more than one order of magnitude larger than the values we obtained for the hydrophobic contact of HFBI layers in oil or air, where no hydrophobic effect is present.

In the case of the hydrophilic contact of HFBI layers, we don't expect a contribution of the hydrophobic effect. The hydrophilic sides interact, however, more strongly than the hydropho-

of the HFBI mutants at the interfaces buffer/hexadecane (h'phobic core, red bars), hexadecane/buffer (h'philic core, blue bars), and FC70/buffer (h'philic core, green bars).

b) Bilayer adhesion energy of hydrophilic core HFBI bilayers at the hexadecane/buffer interface with varied salt concentration in the buffer phase.

bic sides. Therefore, the questions arise, which interactions dominate the attraction in this situation and which role the molecule's charges play. Since the charges at the hydrophilic pole of the HFBI molecule are well separated and are equal in number, a patch-charge attraction might be possible⁴⁴. On the other hand, the charges might also just obscure and counter an attraction between deeper hydrophobic groups as it is e.g. the case with charge stabilized polystyrene latex beads⁴⁵. This will be addressed with the employment of the HFBI mutants and the variation of the ionic strength below.

Van der Waals is the dominant contribution of the adhesion energy

To study the influence of the protein's charges on the adhesion energy of the HFBI bilayers, measurements in the identical way as presented above with HFBI-WT were conducted with the four HFBI mutants and the media hexadecane and FC70/buffer (see Fig.

5a). Although the mutations involving the charged residues alter the mutual protein interactions as well as the interaction with the surrounding water phase^{31,32}, stable bilayers were able to be produced from all variants. Like in the case of wild type HFBI, the determined bilayer tension values Γ (see fig. S1a) in the SI) for all mutants follow the trends given by the interfacial tension of the protein covered single interfaces (see table 1). Thereby, the bilayer tensions do not vary much between the different mutants and reveal a slightly lower bilayer tension than the wild type at the respective interface, with the exception of HFBI-DD and -DDRK at the FC70/buffer interface.

The adhesion energy ΔW of the hydrophobic core bilayers shows no significant differences for the different mutants. This is expected, since the hydrophobic patch is preserved for all mutants. It shows, however, that the adhesion energy is mainly determined by shortranged interactions while presence or absence of charges at the outside of the bilayer is irrelevant. For the hydrophilic core bilayers, however, a clear influence of the protein's charge distribution on the adhesion energy was found. While the wild type's adhesion energy is the lowest, the DDRK mutant's is the highest. This indicates that a reduction of the absolute number of charged amino acids (WT: 6, DK, RK, and DD: 4, DDRK: 2) leads to a higher adhesion energy, whereas the net charge ($-2e$ for RK, $+2e$ for DD, 0 for all others) seems to have little influence. Thus, the electrostatic interaction between the bilayer sheets can be assumed as to be repulsive.

While the employment of mutants offers a defined way to study the effect of the individual charges on the adhesion energy of the hydrophilic core bilayers, it is only possible to compare between an "on" and "off" state. Therefore, we performed also a second set of experiments using buffers with higher ionic strength to gradually screen the charges of the WT molecule. For this purpose, we used buffers with $I = 100, 500$, and 1000mM additionally to

the already probed ion concentration of 6mM . According to the Debye-Huckel theory, these ion concentrations correspond to Debye screening lengths λ_D of 4, 1, 0.4, and 0.3nm , respectively. As finger phase, hexadecane was used in all of these experiments.

The interfacial tension γ of the HFBI-WT monolayer at hexadecane/buffer interfaces was found to increase with increasing salt concentration, see table 2, which is a similar behavior but stronger than in a pure hexadecane/buffer system⁴⁶. Consequently, the bilayer tension of the hydrophilic core bilayers increases from $25 \pm 3\text{mN/m}$ at $I = 6\text{mM}$ to $35 \pm 3\text{mN/m}$ at 1M (figure S1b). Moreover, the screening induces also an increase in the adhesion energy ΔW (see figure 5b). For the highest ion concentration used ($I = 1\text{M}$ corresponding to $\lambda_D \approx 0.3\text{nm}$), the energy is roughly doubled compared to the lowest ion concentration. Although by screening charges, one cannot discriminate between effects of net charge or absolute number of charges, the results reflect again the repulsive nature of the electrostatic interaction between the protein layers.

Based on the above presented results for the HFBI bilayers, we can therefore conclude, that neither the hydrophobic effect nor the electrostatic force are responsible for the attractive interaction between the two sheets of a bilayer. Especially in the hydrophobic core configuration, the only possible attractive force seems to be the dispersion, or van der Waals (vdW), interaction. In a continuum approximation and neglecting the special structure of the protein, the strength of the vdW interaction between two protein layers was calculated to be of the order of some mJ/m^2 (see Fig. 6 and SI for details of the calculation), which seems sufficient to explain the experimentally observed adhesion energy. Moreover, these calculations also give an approximately doubled interaction strength for the hydrophilic bilayer situation with the ionic strength having only a minor influence. Thus, the experimentally observed adhesion energies

for different orientations and ionic strengths can at least be described qualitatively.

A question that might arise is how these calculations might explain the difference between the "special interfacial behavior" of hydrophobins as compared to other proteins, as e.g. BSA. The calculation of the vdW inter-

Figure 6: Van der Waals energy for two infinite half spaces of protein (1 and 2) interacting across a medium m (depicted in the upper right corner) versus the distance l between the half spaces. Medium m is either oil (dashed red line) or water with different Debye screening lengths λ_D (solid blue lines).

action uses continuum theory where a continuous layer is assumed. For the very dense hydrophobin layers (ca. 3.5 mg/m^2 with film thickness of ca. 3 nm ^{19,37,47}), this is in our opinion a good approximation. BSA (and also other proteins) forms less dense monolayers (ca. 1.6 mg/m^2 and 3 nm film thickness for BSA⁴⁸). This explains already a part of the smaller adhesion. Moreover, BSA and many other large proteins change their conformation quite drastically at the air/water interface or other hydrophobic interfaces, exposing the polar and charged groups to the water phase^{48–50}). This increases again the coulombic repulsion between two BSA layers. Thus, the simplifications used in the calculation might not be easily applicable to other systems. Additionally, hydrophobin layers are characterized by an unusual high lateral

cohesion and very high surface affinity, which both make a film rupture very unlikely. This effect is not captured by the model.

Conclusions

In this study, the class II hydrophobins HFBI and HFBII as well as four genetically modified variants of HFBI were employed to generate bilayers with hydrophobic and also hydrophilic cores, i.e. separating two aqueous or oily compartments, in a microfluidic approach in order to determine their adhesion energy and explore the origin of this adhesion energy. All bilayers showed a high stability and exceptional resistance against lateral tension owing to the high lateral cohesion of the molecules. The measured adhesion energies range between 1 and 6 mJ/m^2 , which is comparable to lipid bilayers. Thereby, the hydrophilic core bilayers exhibit in most cases higher values than their respective inverted equivalents with hydrophobic core. The experiments with varied charge distribution on the proteins and charge screening demonstrate that the main attractive force between the bilayer sheets results from van der Waals interactions. The higher attractive force in the case of hydrophilic core bilayers compared to the hydrophobic core ones is thus explained by the higher polarizability of the proteins' side groups which are in contact. The attractive van der Waals interaction is in the hydrophilic core case, however, opposed by electrostatic repulsion acting between the charged side groups. Thus, by screening this repulsion, the strength of the overall interaction can be adjusted.

Although showing the same trends in most experiments, HFBII, shows much larger variation in the recorded adhesion energy values than HFBI. An explanation might be found in the different distribution of hydrophobic and hydrophilic groups on the surface of both molecules very likely owing to their presumed slightly different natural function. The less clear polarization of HFBII,

manifested in the presence of more hydrophobic groups at the hydrophilic pole and more charged groups between the poles, might cause different positions of HFBII molecules at interfaces compared to HFBI molecules and a higher orientational variability. Moreover, the comparison of HFBI and HFBII bilayers reveals that the hydrophobic effect may enhance the interaction between the sheets in a hydrophilic core bilayer; however, even without this effect, pair interaction energies of at least $1k_B T$ are reached, sufficient to form stable bilayers.

In sum, the presented results allow for a distinction of the effects of the involved interactions. These results may be used to optimize hydrophobin bilayers and vesicles for their application in different environments as it was shown that the adhesion strength can be tuned by the choice of media, hydrophobin species, and, in the case of the hydrophilic contact, by the salt concentration. An extension of the study to other media combinations and other suitable hydrophobins or proteins that also feature well defined hydrophilic/hydrophobic regions might increase the number of possible application scenarios.

Supporting Information

Figure showing bilayer tensions corresponding to Fig.5, details to the calculation of the vdW interactions, additional information and discussion of the experimental methods, additional experiment corroborating the interaction energy in the hydrophilic core bilayer case.

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Notes The authors declare not competing financial interest.

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Graphical TOC Entry