Engineering Principles of Hydrophobin Fusion Proteins

Katri Kurppa
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A doctoral dissertation completed for the degree of Doctor of Science (Technology) to be defended, with the permission of the Aalto University School of Chemical Engineering, at a public examination held at the lecture hall Ke2 of the school on 2nd June 2017 at 12 noon.

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School of Chemical Engineering
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Abstract

The research presented in this thesis focuses on the design and use of hydrophobin fusion proteins for technological applications. Hydrophobins are small fungal proteins with interfacial function. This characteristic arises from a unique, bipolar structure. Hydrophobins also partition effectively in liquid two-phase systems. The aim of the work presented in this thesis was to connect the molecular function of the hydrophobin HFBI to other operational functionalities by methods of protein engineering.

Proteins have become a central focus of research in the fields of biotechnology and material development. The vast interest is due to the inherently detailed structure of proteins, forming complex functionalities that build up to great application potential. Nature has created detailed and precise function to these molecules, which can be harnessed to build new materials. The art of protein engineering may be used to join and modify elements in new combinations.

A central theme throughout this research was to evaluate aspects such as protein component stoichiometry, material geometry and charge effects, as well as holistic factors influencing application design. Firstly, suitable model hydrophobin fusion proteins were designed and produced, and their functionality at liquid-liquid and solid-liquid interfaces was studied. In the following segment of this study, the functionality of the fusion proteins was assessed in model applications as a hybrid material with carbon nanoparticles. The results presented in this thesis demonstrate the design and use of protein functionalities for creation of biomolecular assemblies based on the self-assembly of hydrophobin HFBI.

The solution equilibrium of class II hydrophobins plays a crucial role in the usability of its fusion derivatives, alongside with the mechanistic details of the interfacial assembly. The results were evaluated in the frame of the design process of hydrophobin fusion proteins. This process consists of an engineering step, a formulation step and a final application step. Thereby, this thesis highlights the importance of considering protein architecture and stoichiometry throughout the process.

Keywords hydrophobin, fusion protein, surface-active, amphiphilic, QCM-D

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Tulokset arvioitiin tarkastellen hydrofobiinifuusioon suunnitteluprosessia, johon kuuluu muokkausvaihe, valmistevaihe sekä sovellusvaihe. Tämä tutkimus osoittaa erityisesti, että proteiinirakentehtävä ja molekyylikomponenttien stoikiometria on huomioitava tarkasti suunnitteluprosessin joka vaiheessa.
Preface

As often happens in life, the realization of this thesis did not follow the expected route. It has been a long way since I began working in the fascinating world of proteins. The work in this thesis spans the years 2007-2017, and was carried out at the Technical Research Institute of Finland, VTT Ltd. Of these ten years, I spent a good part at home taking care of my children. Thinking backward, those times played a significant role in the outcome of this work.

Life. There were days when I thought this thesis might never see its final form. Surely, I am not the only one who thought this. At times, the whole concept seemed distant, with my hands full of life that did not easily coincide with academic goals. More importantly, there were the days when I felt confident in life and its timespans. That is, the recurring fact that it all usually works out in the end. Today I am happy to find myself at this checkpoint. Of course, none of this would have succeeded were I in this alone. Luckily enough I am not - inspired scientists, good friends and loving family have supported me throughout.

Gratitude. Foremost, I want to express my sincere gratitude to Professor Markus Linder, my thesis supervisor, professor and former team leader. Thank you for your encouragement, superior guidance and utmost patience during these past ten years. Your profound work on the hydrophobins has laid the ground for this thesis among many others. Just as much, I warmly thank Docent Jussi Joensuu, my thesis supervisor and colleague, for devoted supervision of my thesis work. You always miraculously found the time. Thank you for many fruitful discussions and shared experiments. Where there is a swamp and hoe, there is Jussi (and the hydrophobins).

Conditions. I thank former and current managers of VTT Biotechnology and SONE for providing the excellent research environment, and for the possibility of dividing time between projects and PhD work. I cordially thank Tekes and VTT Ltd. for funding. I also thankfully acknowledge the National Doctoral Programme in Informational and Structural Biology ISB for support and the opportunity to participate in its activities.

Teamwork. I want to thank all my co-authors for valuable discussions and sharing work on these interesting subjects. Research across disciplines is difficult as it is; thank you for the open-minded atmosphere and great collaboration. A special thank you goes to Lauri Reuter, Miika Soikkeli, Sanna Arpiainen and Vesa Hytönen for your easy-going cooperation.

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My warmest thank you also to members of former teams, especially team leader Riitta Partanen for her care and attention. A special thank you to the good old Nanobiomaterial team for the inspired scientific attitude and all the fun times, especially you Päivi Laaksonen, Jani-Markus Malho and Suvi Arola. Thank you to all you VTT colleagues, with whom I have shared labs and coffee over many years.

Driving force. I want to thank all my family members and dear friends for believing in me, for sharing and caring. I especially thank my loving and wise parents Leena and Esa for always being there. I am immensely grateful for the world of creativity, people, and intellect in which I have had the privilege to grow. My beloved siblings Heikki, Hanna and Kalle form a major part of that world. Thank you for existing, thank you for your devotion. My warmest thank you also to my parents in-law Sirpa and Pauli for your unconditional help and support.

Love and inspiration. I dearly thank my husband Jani for his sturdy support and for always standing by me, lightening up difficult days during the lengthy making of this thesis. Thank you for seeing the wood behind the trees when I miss it, and for sharing our life with me.

I dedicate this thesis to my three children Klaara, Iiris and Eero, whom make my each day an adventure and are an endless source of inspiration. I love you all to pieces. You wrote this book.

Espoo, May 2017

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Academic dissertation

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List of publications

This thesis is based on the following original publications, which are referred to in the text as I–V. The publications are reproduced with kind permission from the publishers.


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Author’s contributions

I. (*KK and LR share equal contributions as first author).

KK took part in expression experiments in *N.benthamiana*, characterized the proteins and performed mass spectrometry measurements. KK performed all QCM-D experiments and analyzed the results. KK screened for suitable detergents for two-phase extraction and optimized the experimental conditions for ATPS. KK determined experimental and analytical methods for antibody purification in buffer solution. KK and LR performed final quantitative experiments on chosen model antibody together and LR studied the two-phase separation of fusion proteins from plant leaf extract. LR generated and characterized the BY-2 cell lines, performed the pilot scale experiments and conducted the microscopy experiments. KK wrote the first version of the manuscript and edited the final version together with LR.

II. The author conducted experimental work, interpreted the results and had the main responsibility for writing of the publication. HJ conducted TEM imaging and GS was in charge of engineering the HFBI cysteine mutant.

III. The author conducted all protein characterization experiments, performed all QCM-D experiments, interpreted the results and had the main responsibility for writing the article. ML and VH performed construction of the recombinant production strains of HFBI-Avd.

IV. The author conducted all protein purification and characterization work, QCM-D experiments and interpreted the results. The author was in charge of planning the biomolecular work. The author wrote the article from the biochemically relevant parts.
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<th>Description</th>
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<tbody>
<tr>
<td>ATPS</td>
<td>Aqueous two-phase separation</td>
</tr>
<tr>
<td>QCM-D</td>
<td>Quartz-crystal microscopy with dissipation monitoring</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Polyacrylamide gel electrophoresis using sodium dodecyl sulphate</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>FET</td>
<td>Field-effect transistor</td>
</tr>
<tr>
<td>GFET</td>
<td>Graphene field-effect transistor</td>
</tr>
<tr>
<td>CNT</td>
<td>Carbon nanotube</td>
</tr>
<tr>
<td>SWCNT</td>
<td>Single-walled carbon nanotube</td>
</tr>
<tr>
<td>MWCNT</td>
<td>Multi-walled carbon nanotube</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic force microscopy</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
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</tbody>
</table>
1. Introduction

A group of proteins called the hydrophobins forms the core of this work. Proteins have become a central focus of research in the fields of biotechnology and material development. The vast interest is due to the inherently complex structure of proteins, forming complex functionalities that build up to great application potential. Nature has created detailed and precise function to these molecules, which can be harnessed to build novel hybrid materials.

At the same time, developments in nanotechnology have led to the need for solutions to integrate and control biophysical systems and interfaces. A cross-disciplinary effort is needed to create smart materials or sensing applications for, e.g., healthcare. Especially, biofunctionalization of surfaces is relevant in adapting physical devices to biological environments (Niemeyer 2007). Control of interfaces at material borders is a prerequisite for efficient communication – signal transduction, creating structural order in materials or molecular transport.

The art of protein engineering is used to combine and modify biomolecular elements in new combinations. Proteins can be seen as building blocks. This work describes the process of joining different functional proteins in an effort to expand their ways of use in nano- and biotechnological applications. The tailored proteins have been employed in model applications in an attempt to holistically understand the influential factors in protein design.

1.1 Hydrophobins

The fusion proteins described in this study are all based on a surface-active protein, a hydrophobin named HFBI. Hydrophobins are a group of small (10 kDa) proteins produced by fungi, for example the mushrooms commonly used as a food ingredient. Hydrophobins have evolved in nature to operate at different interfaces during the growth of the fungus (van der Vegt et al. 1996; Wösten & de Vocht 2000; Linder 2009). In nature, hydrophobins mediate attachment of the growing fungal hyphae to solid, hydrophobic substrates. Hydrophobins also form protective layers on parts of the fungi and are involved in the formation of appressoria cells (Khalesi et al. 2015). All hydrophobins contain eight Cys residues which bond intramolecularly to form four disulphide bridges. This special cross-linked structure of hydrophobins allows a group of aliphatic side chains to be faced outwards, forming a hydrophobic patch.
on one side of the protein (Figure 1). The resulting protein structure is robust. Generally hydrophobins are classified as class I or class II depending on the pattern of amino acid side chains and resulting solubility characteristics (Wessels 1994). Structurally, the amino acid sequence of class II hydrophobins is more conserved as compared to class I hydrophobins.

Figure 1. Class II hydrophobin HFBI was connected to fusion proteins via an N-terminal linker. The hydrophobic patch is marked in turquoise. The positively charged, surface-exposed amino acids are shown in blue and negative residues in red. The crystal structure was retrieved from the Protein data bank (ID 2FZ6; DOI: 10.2210/pdb2fz6/pdb) and the image was produced using Chimera.

The X-ray structure of HFBI shows that HFBI consists of four beta sheets and one alpha-helix with lattice parameters of a=108.9 Å, b=49.6 Å and c=85.8 Å (Hakanpää et al. 2006). Hydrophobic amino acids Leu12, Val23, Leu26, Ile27, Leu29, Val59, Ala60, Val62, Ala63, Ala66, Leu67 and Leu68 form a surface-exposed hydrophobic patch with an area of 738 Å² (Hakanpää et al. 2006). Four disulphide bridges tie the structure from within, and allow the exposure of the hydrophobic amino acids. The hydrophilic part of class II hydrophobins consists of an α-helix. Other prominent features of the hydrophilic part include a group of charged amino acids Asp30, Lys32, Asp40, Asp43, Arg45 and Lys50. The charged residues situated opposite of the hydrophobic patch, namely Asp40, Asp43, Arg45 and Lys50, have also been referred to as the charged patch (Lienemann et al. 2013; Hakanpää et al. 2006).

The crystal structure of HFBI has also been produced in a tetrameric assembly consisting of four HFBI molecules (Figure 2a; Hakanpää et al. 2006). The structure of the tetramer is twisted and slightly curved, with more hydrophobic area exposed
on the flat side (Figure 2b). The x-ray structures have also shown detergent-associated forms of the tetramer assembly where the HFBI oligomer was bound to with detergent molecules at the outside of the curved tetramer assembly. Two tetramers were found to form a detergent-bound octamer.

Figure 2. a) Crystal structure of HFBI showing tetrameric assembly. Chains A, B, C and D are indicated by green, blue, pink and orange colouring, respectively. b) Crystal structure of a detergent-associated HFBI octamer, formed by two interacting tetrameric assemblies. Detergent molecules are denoted by red colouring. The crystal structure was retrieved from the protein data bank (ID 2GVM; DOI: 10.2210/pdb2gvm/pdb) and the images produced using Chimera.

At the air-water interface class II hydrophobins self-assemble to form a monolayer, in which molecules have arranged in a distinct hexagonal pattern (Figure 3; (Paananen et al. 2003). This feature is due to the unique amphiphilic structure as well as favorable intermolecular amino acid interactions, based on three-dimensional structure. Functionally the most prominent feature of the hydrophobins is the hydrophobic patch. However, hydrophobin assembly is finally a matter of balance between the surrounding hydropathy environments; also self-assembly on polar surfaces has been demonstrated (Grunér et al. 2012).
The main roles of the hydrophobins include reducing the surface tension at the air-water interface, mediating adhesion to a solid substrate or formation of protective layers. These events are crucial for many organisms and several groups of surfactant type proteins exist to serve this purpose (Schor et al. 2016). These include small amphiphilic peptides, lipid-associated amphiphiles and independently acting globular proteins, such as the hydrophobins. They all have a common amphipathic structure, meaning that hydrophilic and hydrophobic side chains are clearly separated in distinctive areas. This structure is comparable to the bipolar structure of a surfactant, i.e., soap. Surfactant molecules assemble at interfaces by directing the hydrophobic tail of the molecule towards the hydrophobic phase (air or solid substrate) whilst retaining the hydrophilic head-groups in water. By doing so, the energy barrier at the interface is lowered as the water molecules can seek more favorable interactions within the aqueous phase. Functionally, the hydrophobins have many similarities with surfactant molecules. However, the structural complexity of the hydrophobins results in more complicated behavior and functionality.
Surfactants are amphiphilic molecules with a hydrophobic carbon tail and a hydrophilic head group (gray sphere). Surfactants form micellar structures in an aqueous solution, with hydrophilic groups arranged on the outside of a disordered oily inner phase formed by the hydrophobic tails.

To date, several different fusion proteins of class II hydrophobins HFBI and HFBII have been constructed (Table 1). The fusion partner is typically fused to either the C- or N-terminal of the hydrophobin via a linker. The linker is typically a PEG-linker or peptide chain. The N-terminal of the hydrophobin HFBI is situated across from the hydrophobic patch (Figure 1), while the C-terminus is found lower, near the hydrophobic patch. The N-terminus is also more available for modifications, as it elongates more out of the protein core than the C-terminus (Figures 1 and 2a).

This thesis describes the use of class II hydrophobin HFBI and its N-terminal fusion derivatives, which are discussed in detail later (Section 2).

Table 1. Fusion proteins of HFBI

<table>
<thead>
<tr>
<th>Fusion partner</th>
<th>Terminal Production organism</th>
<th>Functionality</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dual-chain avidin</td>
<td>N</td>
<td>T. reesei</td>
<td>Affinity binding</td>
</tr>
<tr>
<td>Glucose oxidase</td>
<td>C</td>
<td>N. benthamiana</td>
<td>Enzymatic</td>
</tr>
<tr>
<td>GFP</td>
<td>C</td>
<td>N. benthamiana</td>
<td>Fluorescence</td>
</tr>
<tr>
<td>Leucine zipper ZE</td>
<td>N</td>
<td>T. reesei</td>
<td>Dimerization</td>
</tr>
<tr>
<td>ProteinA</td>
<td>N</td>
<td>N. benthamiana</td>
<td>Affinity binding</td>
</tr>
<tr>
<td>Cellulose binding module</td>
<td>N</td>
<td>T. reesei</td>
<td>Affinity binding</td>
</tr>
<tr>
<td>Cellulase endoglucanase</td>
<td>C</td>
<td>T. reesei</td>
<td>Enzymatic</td>
</tr>
<tr>
<td>Thiol group</td>
<td>N</td>
<td>T. reesei</td>
<td>Chemical</td>
</tr>
</tbody>
</table>

1.2 The hydrophobic effect

The mechanism of noncovalent association of hydrophobic moieties is a consequence of the surrounding aqueous environment (Chandler 2005; Israelachvili 1991). The tendency of the nonpolar groups to escape water contact is a consequence of disrupting the hydrogen bonded structure of the surrounding water molecules. When the tetrahedral water molecules come in contact with a nonpolar sur-
face, they cannot form hydrogen bonds in their most energetically favorable configuration. The water molecules are forced to reorient themselves, creating a more ordered structure in the vicinity of the nonpolar surface. The result is decreased entropy and, consequently, increased free energy. As the water molecules do not find pairs for hydrogen bonding, they are forced out of their most energetically favorable configuration, thereby causing the hydrophobic effect. The hydrophobic effect is a key driving force in many biophysical events, such as protein folding or protein-DNA binding (Kuriyan et al. 2012).

To minimize energy, hydrophobic entities tend to face away from water, leaving more hydrogen bonding sites for the water molecules to interact with. As a consequence, a phenomenon called dewetting is observed in the void of the water molecules (Chandler 2005; Meyer et al. 2006). The structure of water is sensitive to local solute structure, such as shape and chemical structure of the nonpolar group. Understandably, the size of the hydrophobic surface, i.e., the area over which the water molecules are strained, is relevant for the strength of the hydrophobic effect. It has been shown that for larger areas of low curvature, water molecules are not capable of maintaining the hydrogen bonded network. Simulations showed that when a spherical hydrophobic particle resembling the space-filling size of a methane molecule was placed in water, surrounding water molecules were capable of maintaining the tetrahedral hydrogen-bonding structure (Chandler 2005). As a comparison, the study showed a simulation of a cluster of 135 methane molecules, filling a spherical volume with a radius of 1 nm. In this case, the structure of surrounding water molecules was no longer maintained. For large hydrophobic areas, the solvation free energy is thus enthalpically dominated.

In conclusion, when a particle of low curvature and an area larger than 1 nm² was placed in water, the interfacing water molecules were not capable of maintaining the hydrogen-bonded network, resulting in depletion strong enough to cause dewetting (Chandler 2005). Comparing this value to the area of the hydrophobic patch of HFBI (7.4 nm²) it is evident, that the hydrophobic effect must be a major factor dictating the behavior of the hydrophobin molecules, as well as connected fusion partners.

1.3 Hydrophobins at interfaces

The assembly of class II hydrophobins at the air-water interface is well described in literature. The hydrophobins HFBI and HFBII have been shown to form viscoelastic, skin-like films at the air-water interface (Szilvay et al. 2007). The surface of a drop of hydrophobin solution has been shown to flatten due to structural ordering of the proteins at the air-water interface. These features, alongside the distinctive hexagonal nanostructure of self-assembled air-water interfacial monolayer (Figure 3), are descriptive manifestations of the unique nature of the hydrophobins. The phenomena related to the air-water interfacial films of the class II hydrophobins also illustrate how vastly the hydrophobins differ from small-molecule surfactants, to which they are often compared in literature.
Another central task of the hydrophobins in nature is to mediate adhesion to solid substrates or to form protective layers. Class II hydrophobins bind to solid, hydrophobic surfaces by the hydrophobic patch to form rigid, reproducible and ordered monolayers (Linder et al. 2002; Linder 2009; Laaksonen et al. 2010). The driving-force for surface adsorption of hydrophobins is thus the hydrophobic effect. In other words, the solvation energy of the hydrophobin monomer is minimized by binding to the solid surface, thereby hiding the hydrophobic patch from water.

Interfacial self-assembly of HFBI is likely closely related to the solution behavior of the hydrophobin. For surface-active functionality to be efficient, interfacial self-assembly must be favored over the formation of solution oligomers. Indeed, crystallization studies imply that interaction with a hydrophobic surface (detergent molecule) is favored over multimer formation (Hakanpää et al. 2006). Solvent-accessible areas of the hydrophobic patches in HFBI crystal structures can be compared to examine how effectively the hydrophobic patches are buried from water contact in each assembly. The solvent accessible area of the hydrophobic patch in the monomeric, detergent-associated form was clearly smaller than in the multimeric assemblies. Thus, interaction of the hydrophobic patch with a hydrophobic surface (as detergent molecules) seems to be energetically more favorable than multimer formation.

Class II hydrophobins self-assemble at the solid-liquid interface of water and hydrophobic surfaces (Linder et al. 2002). The adsorbed layers are stable and resist forces exerted by ambient fluid flow. Due to these features, the hydrophobin platform has been applied to the biofunctionalization of various surfaces. Moreover, the hydrophobin molecule is on a size scale that is well compatible with components used in nanotechnology. This sets a basis for using engineered hydrophobins in the development of hybrid materials and bioadaptable nanodevices. Hydrophobin assemblies at the solid-liquid interface have been reported to be advantageous in interfacial applications concerning for example adhesion of matrices (Laaksonen et al. 2011; Malho et al. 2015), lubrication (Hakala et al. 2012), drug-particle protection (Valo et al. 2013), reducing nanotoxicity (Yang et al. 2013) and biosensor functionalization (X. S. Wang et al. 2010). In addition, self-assembled hydrophobin surfaces can be used as an immobilization base layer for more delicate biomolecules (Lienemann et al. 2015; Z. F. Wang et al. 2010).

1.3.1 Detailed understanding of hydrophobin interfacial assembly

Understanding of the molecular mechanisms related to hydrophobin function is crucial in designing functional fusion proteins, because disturbance of most relevant interaction sites may then be avoided. The most recent investigations of HFBI and HFBII self-assembly show, that the hexagonal assembly at the air-water interface originates from an arrangement of hydrophobin hexamers (Magarkar et al. 2014). This was shown by a computational study consisting of protein-protein docking and molecular dynamics simulations, in combination with cryo-EM and diffraction measurements.
The surface film of hydrophobin HFBI clearly exhibits distinctive plane symmetry. A plane symmetry group is a mathematical representation of a geometrical, two-dimensional pattern (Figure 5). Different symmetry groups are based on the number of rotation centres and possible reflection planes that can be identified in the structure. A P3 symmetry group has three rotation centres of 120 ° and no reflection planes. The P6 symmetry group has one rotation centre of 60°, and in addition three 120 ° rotations, but no reflection planes. Both P3 and P6 symmetry groups show a hexagonal lattice type with equal lattice constants.

Figure 5. Geometrical symmetry groups P3 and P6 show hexagonal lattice type. The P3 symmetry group includes third order rotations of 120° (triangle). The P6 group shows sixth order rotations of 60° (hexagon) as well as second and third degree rotations (oval and triangle). Neither of these has reflection planes or lines.

Geometrical analysis suggests that the hydrophobin film structure belongs to the P6 symmetry plane group. However, cryo-EM measurements of HFBI as well as protein-protein docking experiments of HFBI and HBII revealed a structure with lattice dimensions smaller than those of the hexamer, only with P3 symmetry (Magarkar et al. 2014). Also the Monte Carlo simulations of the formation of the self-assembled hydrophobin surface demonstrated a possible role of a P3 symmetric transition state. The rate of formation of the final hexamer-based assembly was accelerated when the metastable trimer assembly was allowed. It was also noted that self-assembly exhibited two kinetically different regions; an initial phase of increasing growth rate, followed by a steady-state region. While these observations reveal the critical molecular interactions involved in hydrophobin self-assembly, present knowledge is not sufficient to draw a direct connection between solution oligomers and interfacial structures.

The rigidity of the surface film in itself poses the requirement that all protein molecules at the surface are connected with a neighboring molecule. Such structures are all based on a trimer unit (Magarkar et al. 2014). A trimer unit would require the hydrophobin molecule to have three-fold symmetry, which is not the case. Protein-
protein docking experiments ruled out the option of a pentamer structure, thus narrowing molecular arrangements to hexamer structures consisting of two identical trimers, α-HFBI and β-HFBI. The structures could be matched to previously reported experimental results via the convergence of geometrical parameters and positioning of the trimer structures (Kisko et al. 2009).

Cryo-EM measurements for HFBII showed hexagonal lattices with 56 Å dimensions, and similar lattice vectors were found for both HFBI and HFBII (Magarkar et al. 2014). The hexamers were found to form a ring structure. The air interface of the hexamer was stated to be both electrostatically neutral and apolar. However, in contrast to the computational results, Fourier analysis of the cryo-EM images showed hexamers with P3 rather than P6 symmetry. Further analysis of the possible molecular arrangements revealed, that the α-HFBI and HFBII hexamer structures are able to convert from P3 to P6 symmetry through simple rotation and with only minor adjustments to the structure. The existence of this P3 structure is not supported by experimental findings, as its lattice parameters are lower than those in the observed hexamer unit. This P3 is thus considered to be a temporary structure, which possibly formed during hydrophobin assembly at the air-water interface.

Monte Carlo simulations were performed to investigate the role of the temporary trimer in hydrophobin assembly, taking the surface-active nature of the hydrophobins as a governing energetic factor (Magarkar et al. 2014). Because the viscoelastic properties of the hydrophobin film are a consequence of attractive interactions forming across a protein network, the rate of formation of the film was considered to be directly proportional to the properties. The results showed, that the formation of the hydrophobin film was significantly accelerated when the metastable P3 trimer structure was allowed. Moreover, the simulations showed that formation of the hydrophobin surface film takes place in two steps. Firstly, the surface is filled in at an increasing growth rate. Following, the film grows in steady-state, where the growth rate is independent of the initial conditions and finite size effects. Upon closer examination of the proposed trimer structures α-HFBI and β-HFBI, a dominant saltbridge between Lys32 and Asp30 was found for the structure β-HFBI. This implies that the β structure may be more stable than the α-HFBI structure.

1.4 Hydrophobins in a liquid environment

Class II hydrophobins are highly soluble in water, despite the large, exposed hydrophobic patch. This can be explained by formation of multimers, where the hydrophobins interact by their hydrophobic patches to hide them from water thus lowering solvation energy. Several sets of experimental data have provided evidence of multimer formation in solution. Hydrophobins HFBI and HFBII were first observed to form tetramers in X-ray scattering measurements (Torkkeli et al. 2002). The existence of dimer and tetramer assemblies was also shown by crystallization studies of HFBI (Hakanpää et al. 2006).
The solution behavior of HFBI was studied by size-exclusion chromatography, small-angle x-ray scattering and fluorometric measurements of a specifically labelled hydrophobin. (Szilvay et al. 2006) This NCys mutant of HFBI was labelled specifically at the single available N-terminal thiol group. The presented SAXS, FRET and SEC data showed that HFBI forms solution tetramers in a concentration dependent manner, and that all HFBI is in tetramer state at concentrations >20 \( \mu \text{M} \) (Szilvay et al. 2006). This translates to 0.15 mg/ml using the molecular mass of HFBI \( M=7540 \text{ Da} \). The results suggest cooperative multimerization, possibly from monomers to dimers and eventually to tetramers. The concentration range for oligomer formation was later confirmed for HFBI and an HFBI fusion protein (Lienemann et al. 2013; Lienemann et al. 2015).

The details of solution behavior and the possible mechanistic connection to surface adsorption still lack explicit understanding. Nonetheless, recent work in the area has produced important results to clarify solution characteristics (Figure 7). Self-assembly at the air-water interface lowers the surface tension of the interface.

![Figure 7](image)

Figure 7. Illustration of hydrophobin behavior and different probable equilibrium states. The arrows indicating equilibrium transitions are indicative. The different states are marked by letters: M = monomer, C = monomer in crystalline film, A= site at air-interface, S= site at solid surface, O = site at oil interphase, D = detergent interaction.

Lienemann et al. have studied the role of the hydrophilic surface of a fusion derivative of HFBI to its function (Lienemann et al. 2013). They engineered a series of fusion proteins of green fluorescent protein and HFBI (GFP-HFBI), in which the charged residues of the hydrophilic end of HFBI were changed to a glutamine or asparagine residues. Oligomerization habits of the produced mutants were studied by flow fractionation (AF4). The conclusion of the work was, that the charged residues have a role in the functionality of GFP-HFBI. The mutant displayed different oligomerization tendency, and an increased oligomer-monomer ratio was found to correlate negatively with the mass of the surface-adsorbed layer. These observations were interpreted to mean that, increasingly, stabilization of solution oligomers
is related to enhanced protection of the hydrophobic patch, thereby decreasing the driving force for surface adsorption. QCM-D experiments showed that mutations near the hydrophobic patch decreased the mass of the layer to less than half of that of the wild-type (Lienemann et al. 2013).

The charges on amino acids Lys32 and Asp30 were found to be crucial for the function of the GFP-HFBI fusion protein (Lienemann 2013). This is in accordance with the computational study describing hydrophobin assembly at the air-water interface (Magarkar et al. 2014).

Interestingly, while the effects of charge mutations had marked effects on the oligomerization behavior of the HFBI fusion protein, only slight effects on the functionality of the non-fused HFBI were observed (Lienemann et al. 2015). Changes in elastic properties of the air-water interface film were minor when the charged amino acid residues of wild-type HFBI were replaced. Results of the size-exclusion chromatography experiments showed, that all of the charged variants as well as wild-type HFBI exist in monomeric state at concentrations <30 μM. Larger complexes were observed to form at concentrations >100 μM. The concentration range for multimer association is in agreement with results presented by Szilvay et al. (Szilvay et al. 2006).

1.4.1 Detailed understanding of solution behavior

The crystal structure of HFBI shows a tetrameric assembly, where the HFBI monomers exist in two different conformations (Figure 2) (Hakanpää et al. 2006). Two of the monomers in the tetramer assembly (A and C) have the β-hairpin motif in a closed conformation (c), while the other two (B and D) show an opened confirmation (o) of this loop (Figure 6a). A recent simulation study predicts, that the movement of this β-hairpin motif is related to the stability of the monomers, dimers or tetramers in solution (Riccardi & Mereghetti 2016). Movement in the hairpin region of amino acids 60-66 was suggested to be driven by the formation of the HFBI tetramer. Moreover, the formation of dimer assemblies was found to take place only from the closed conformation (c) of the hairpin region.

The theoretical findings were concluded to support two possible mechanisms for HFBI multimerization. The most probable route includes tetramerization via dimerization of two monomers in closed conformation (cc; Figure 6b). After dimerization, the hairpin on one of the monomers in the dimer changes to open conformation (co). Assembly of these dimers (co) could then produce the tetramer (coco), which would correspond to the reported crystal structure. The small energy difference between the dimer co and tetramer coco implies that conformational change of the hairpin motif occurs rather easily. Arrangement of the hairpin region modifies the hydrophobic interaction area of HFBI as well as lateral intermolecular interactions. Thus, this function was proposed to be connected to fine-tuning of hydrophobin interfacial assembly (Riccardi & Mereghetti 2016).
Figure 6. a) Side-view image of the HFBI tetramer showing conformational changes in loops in the β-hairpin loops (magenta). The hairpin loop on monomer chain A (green) is in closed conformation (c) and in open conformation (o) on chain B (blue). The loop is not designated on chains C (pink) and D (orange) for clarity. b) Schematic presentation of probable mechanism for tetramer assembly. The steps illustrate: 1. dimer assembly of monomers in closed conformation, 2. conformational change (c → o) in dimer and 3. final conformational adjustment induced by tetramer assembly.

1.4.2 Electrical behavior of hydrophobin monolayers

The electrical behavior of the hydrophobin monolayer becomes a relevant issue in many nanotechnological applications. The structure and chemical composition of the immobilized layer dictate electrical characteristics of a protein monolayer. Self-assembled monolayers of HFBI labelled with gold nanoparticles on graphene have been investigated by conducting AFM (Kivioja et al. 2009). The results showed that HFBI acted as an insulator. This observation lead to the conclusion that electrical transport in the HFBI layer occurs mainly via tunnelling. In other words, the electron wave arriving at the dielectrical protein interface does not decrease instantly but is slowly tapered off, and the probability function may be transferred through the thin molecular layer to some extent.

When dealing with charged species in electrolyte solutions and electric applications, the effect of charge interactions has significant effects. Charged species, such as proteins or ions, exert an effect on charges within a certain distance. This distance is called the Debye length $\kappa^{-1}$, and it describes the net electrostatic effect of a charge carrier. In electrolyte solutions, Debye length is defined as
\[ \kappa^{-1} = \sqrt{\frac{\varepsilon_r \varepsilon_0 k_B T}{2 N_A e^2 I}} \]  

Equation 1.

where \( \varepsilon_r \) is the dielectric constant, \( \varepsilon_0 \) is the permittivity of free space, \( k_B \) is the Boltzmann constant, \( T \) is temperature, \( N_A \) is Avogadro’s constant, \( e \) is the elementary charge and \( I \) is the ionic strength of the electrolyte.

However, protein layers have been shown to exhibit atypical behavior in respect to the concepts of electrical screening and Debye length (Stigliano et al. 2013). The nanoscale topography and chemical composition of the protein layer cause structural defects in the surrounding water, for example by local deprivation of hydrogen bonds. The resulting confinement of water around the interfacial protein layers causes changes in the polarizability of the molecular structures. In addition, organization of water molecules inside the protein layer becomes localized according to the supramolecular protein structure and its water content. Interfacial layers of hydrophobins HFBI and HFBII have been shown to have low liquid content, in the range of 10-30 % (Krivosheeva et al. 2013).

1.4.3 Aqueous two-phase separation

Liquid interfaces of hydrophilic and hydrophobic matter do not occur only at phase boundaries, but also in molecular structures, for example, in surfactant molecules. These interfaces are in a central role in a process referred to as aqueous two-phase separation (ATPS), which is based on phase separation of detergent molecules in aqueous solution (Figure 7). A group of non-ionic detergents exhibit reverse solubility vs. temperature behavior in water solutions (Holmberg et al. 2002). Aqueous mixtures of these surfactants phase separate as temperature is risen above the so called cloud point temperature (cp). The strength of hydrogen bonds is weakened effectively with increasing temperature due to more rapid exchange. As a consequence, the amount of water surrounding the hydrophilic EO groups is reduced.

A two-step process of extraction and recovery (ATPS) is routinely used to purify class II hydrophobins from host cell proteins. Hydrophobins partition effectively in two-phase systems formed by non-ionic ethoxyalcohol surfactants (Figure 7; Linder et al. 2001; Collén et al. 2002; Linder et al. 2004). In a second step, a long-chain alcohol (\( i \)-butanol) is added. It displaces the hydrophobin molecules back to the aqueous phase. This is likely based on the lack of hydrophilic bulk on the long-chain alcohol, driving the hydrophobin back to the aqueous phase to minimize solvation energy of the hydrophilic surface.
Figure 7. Schematic presentation of the principle of aqueous two-phase separation. Green particles represent impurities in starting sample.

Ethoxyalcohol surfactants have either an aliphatic or aromatic carbon tail, which can also contain branched configurations or unsaturated functional groups (Table 2). The hydrophilic end is an ethoxyalcohol chain of varying length. The cloud point temperature of a surfactant depends on both the length of the carbon tail as well as the number of polyethoxylene units on the hydrophilic end, and finally the concentration of the surfactant solution. The cloud point is also affected by solution composition.

Table 2. Common surfactants used in ATPS for hydrophobins

<table>
<thead>
<tr>
<th>Product name</th>
<th>Chemical composition</th>
<th>Chemical structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Berol 532</td>
<td>C11E2</td>
<td><img src="image" alt="Berol 532" /></td>
</tr>
<tr>
<td>Triton X-114</td>
<td>C10E8</td>
<td><img src="image" alt="Triton X-114" /></td>
</tr>
<tr>
<td>Agrimul NRE1205</td>
<td>C12-18E5</td>
<td><img src="image" alt="Agrimul NRE1205" /></td>
</tr>
</tbody>
</table>

Aqueous mixtures of these surfactants phase separate as temperature is raised above the so called cloud point temperature (CP; Holmberg et al. 2002) Hydro-
phobins interact with the amphiphilic surfactant molecules and are partitioned between the aqueous phase and the detergent phase by an unknown mechanism, presumably based on hydrophobic interactions. (Linder et al. 2001) Interestingly, the molecular interaction of HFBI and a detergent has also been observed in crystallographic studies (Figure 2b; Hakanpää et al. 2006). The crystal structure revealed the cysteine variant NCysHFBI bound to surfactant molecules by the hydrophobic patch. The monomers of NCysHFBI were not in contact with each other.

Phase partition of molecules in the ATPS system is described by the partition factor \( k \), determined by concentrations of the upper (T) and lower (B) phases (Equation 2).

\[
k = \frac{C_T}{C_B} \tag{Equation 2}
\]

One of the most important individual factors which affects the partition factor of a molecule in an ATPS system is the electrochemical component (Collén 2001). It is governed by the net charge of the proteins in question. If solution ions have different affinity for the separated phases, a potential difference across the phase boundary can occur. To minimize this effect, it is recommendable to work at a pH near protein pI or otherwise account for recognized salt effects.

Protein hydrophobicity determines the suitability of the proteins in question to separate in the phase system. Formations of higher-order oligomers, denaturation of proteins or possible conformational changes all affect the final hydrophobicity of the extracted molecules. Also the size of the proteins is naturally affected by accompanying molecules, which have to be located in the micellar structure following the surface-active hydrophobin molecule. The k-value is separate from the yield of the ATPS process. In practice, the optimal conditions may be those which increase the recovery of the protein in question, and at the same time ensure protein stability and feasible processability.

Phase separation of HFBI and HFBI fusion proteins in ATPS system was studied systematically for a range of pure detergents with increasing hydrophilicity, C10E2-C10E5 (Linder et al. 2004). The most determining factor that influenced the outcome of the ATPS process was the hydrophobicities of the molecules involved. Firstly, the hydrophobicity of the detergent, moreover the balance of the hydrophobic and hydrophilic ends (the hydrophilic-lipophilic balance, HLB), governs the structure and water content of the micellar phase. The denser, more hydrophobic surfactants (such as Berol 532) contain less water in the micelle structure, and also the hydrophilic head group is rather small in size. Consequently, the wild-type hydrophobins are very efficiently purified in ATPS systems of Berol532 (Linder et al. 2004). For hydrophobin fusion proteins it was observed, that when the volume of the micellar phase is larger, e.g., contains more water, hydrophilic fusion proteins are more easily incorporated in the detergent phases (Linder et al. 2004). Hydrophobin fusion proteins could be purified by Berol532, when the fusion partner was small and hydrophobic. The surfactant C12-18E5 (Table 2) was shown to be efficient for more hydrophilic fusion proteins (Linder et al. 2004).
Different surfactants can be used successfully in two-phase separation of hydrophobins. In addition to the hydrophilic-lipophilic balance, the surfactants have features, which cause them to function differently. Triton-X 114 (Table 2) is also efficient for purification of hydrophobin fusion proteins (Joensuu et al. 2010). Triton X-114 is an effective all-around surfactant and was used in purification of the HFBI fusion proteins that were produced in tobacco plants in Publication I.

For some non-ionic surfactants, micelle behavior is labile and easily affected by temperature or solution composition. Typically, micelle size increases with temperature, concentration and decreased length of the ethoxyalcohol (EO) tail (Holmberg et al. 2002). The shape and structure of the micelles are equally important in determining the final properties of the surfactant solution. The packing parameter \( \text{cpp} \) (Equation 3) is used to quantify these factors and determine the shape of the micelle (Goddard 1989).

\[
\text{cpp} = \frac{V_H}{l_c a_0}, \quad \text{Equation 3.}
\]

where \( V_H \) is the volume occupied by the hydrophobic groups in the micellar core, \( l_c \) is the length of the hydrophobic group in the core and \( a_0 \) the cross-sectional area occupied by the hydrophilic group at the micelle-solution interface. The micellar shape changes from spheroidal to cylindrical and lamellar going from \( \text{cpp}=0 \) to \( \text{cpp}=1 \). In the application of the concept of \( \text{cpp} \), it must be noted that the values of \( V, l_c \) and \( a_0 \) are likely affected by conditions, temperature, salt, etc. For example, additives, such as medium-chain alcohols that are solubilized near the polar head groups, increase the value of \( a_0 \). This factor may cause differences in performance between different batches of technical surfactants.

Despite functional similarities between hydrophobins and small-molecule surfactants, drawing comparison between the two neglects the fine-tuned molecular details of the hydrophobins. It was shown by Lienemann et al. that certain charged amino acid side chains have an effect on the ATPS process of a hydrophobin fusion protein (Lienemann et al. 2013). Mutations were shown to disrupt the extraction behavior of the fusion protein GFP-HFBI, implying that charges have an effect on the behavior of hydrophobin fusion proteins in ATPS systems. However, the final outcome can be overrun by the other chemical and physical forces, which are also likely related to the characteristics of the fusion partner.

### 1.5 Industrial application of hydrophobins

Due to their distinctive properties and robust demeanor, hydrophobins show potential for various technological applications (Khalesi et al. 2015). The worldwide patent search (Espace.net) shows 408 hits for a patent search using the keyword ‘hydrophobin’. Interfacial self-assembly behavior makes the hydrophobins promising candidates for example for foam stabilization, analyte binding, or specialized coatings.
Accordingly, the focus areas of hydrophobin-related patents fall into three main categories: surface modifications, food and beverage, and cosmetics.

Availability and price have set a borderline for large-scale applications of biosurfactants in industry (Makkar et al. 2011). This applies also to hydrophobins. Large multinational companies associated with hydrophobin research include Unilever, BASF and Danisco. Currently, only the class I hydrophobin SC3 is commercially available at Sigma Aldrich at a price of 625 eur/mg. Class II hydrophobins HFBI and HFBII and their fusion proteins have been produced in gram per liter levels in T. reesei (Askolin et al. 2001) and tobacco BY-2 suspension cells (Reuter et al. 2014).

Down-stream processing can form a crucial bottle-neck and a major cost in the production of biotechnological molecules (Mukherjee et al. 2006). The down-stream process of class II hydrophobin production generally includes ATPS, which can be readily up-scaled (Linder et al. 2004; Reuter et al. 2014).

1.6 Fusion protein partners

The fusion proteins applied in this thesis are HFBI-ProteinA, HFBI-dcAvd and HFBI-ZE (Figure 8). These fusion partners were chosen to incorporate either structural modularity, biorecognition or affinity binding capability to the final applications. The supramolecular assembly properties and the comparative dimensions of these fusion proteins vary. These aspects are discussed in detail in Section 3. The individual properties of the fusion partners are presented in the following subchapters.

Figure 8. Fusion protein constructs used in this research exhibit varying supramolecular behavior and dimensions (increment of scale bar is 1 nm).
1.6.1 Avidin

Avidin was used to add a binding function to the hydrophobin core of HFBI (Publication II). Chicken avidin is a tetrameric protein found in chicken egg-white (Figure 9). (Green 1963; Green 1990) Avidin binds the small molecule biotin with high affinity ($K_d \sim 10^{-15} \text{ M}$). Due to this interaction the avidin-biotin pair is commonly used in biotechnological applications involving bioconjugation steps (Laitinen 2007).

Wild-type avidin consists of four identical chains which form the functional tetrameric assembly. Each of the four monomers binds one biotin molecule. (Livnah et al. 1993) A dual-chain form of avidin has been engineered to allow differentiation of the binding sites for biotechnological purposes (Nordlund et al. 2004; Hytonen et al. 2006). In dual-chain avidin (dcAvd) two of the monomer chains of wild-type avidin have been joined as one (Figure 9). Hence, the active form of dcAvd is a dimer, of which both halves bind two biotin molecules.

Figure 9. Crystal structure of dual-chain avidin. The crystal structure was retrieved from the Protein Data Bank (ID 2C4I; DOI: 10.2210/pdb2c4i/pdb) and the image was produced using Chimera. The $\beta$-barrels (green and blue ribbon) belong to the avidin monomer chains, which are connected as one peptide chain in dcAvd. Biotin molecules are bound to the binding sites (wire model).

1.6.2 ProteinA

A fusion protein compiled of HFBI (or HFBII) and ProteinA is presented in Publication I of this thesis. ProteinA is an antibody binding protein, which binds monoclonal antibodies (mAb) of the IgG class (Figure 10; Forsgren & Sjöqvist 1966; Sjödahl 1977; Deisenhofer 1981) ProteinA contains five binding domains denoted as A, B, C, D and E. Therefore, theoretically one ProteinA molecule binds up to five antibodies, but experimental data has shown the actual ratio to be approximately 1:2 (Uhlen et al. 1984; Yang et al. 2003; Moks et al. 1986).
There is one site for ProteinA binding on both heavy-chains of the antibody, meaning that each ProteinA molecule can bind two antibodies in a pH-dependent manner. The antibodies can be released from the binding sites on ProteinA by lowering the pH below 2.5. ProteinA is typically used for chromatographic purification of antibodies, where ProteinA is immobilized in column matrix (Hober et al. 2007). ProteinA based technology can also be used for immobilization of antibodies (Byrne et al. 2009).

An increasing demand for antibodies has set a need to develop more efficient downstream processing methods (Elvin et al. 2013; Low et al. 2007). ProteinA-based chromatographic purification suffers from high cost, leakage of ProteinA and low throughput, which has accelerated the development of new purification strategies more suitable for large-scale use (Shukla & Thömmes 2010). For example, a ProteinA-oleosin fusion protein was designed for separation of antibodies based on intrinsic phase separation into an oil bodies by McLean et al. (McLean et al. 2012). Development of a liquid antibody-harvesting process based on a fusion protein of HFBI and ProteinA is described in Publication I.

1.6.3 Leucine zippers

The interfacial acitivity of the hydrophobin was combined with a pairing function in an aim to create a switchable, self-assembling molecule unit. A peptide dimer called the leucine zipper (Figure 11) was chosen as the switching module (Moll et al. 2001).

In nature, the leucine zipper peptides are found in cells, where they are involved in DNA binding (O’Shea et al. 1989). The leucine zipper peptides are coiled coil structures (Lupas & Bassler 2016), which consist of two alpha-helical peptides, both 40 amino acids in length (Figure 12). The leucine zipper peptide structure is characterized by a repeating heptad unit.
Two turns of the α-helix comprise the heptad arrangement of seven amino acid residues (Figure 11a). In the heptad unit, the a and d positions denote hydrophobic amino acid residues, such as leucine. Positions e and g belong to charged amino acid residues. Repetition of the heptad units forms an amphiphilic structure, with a band of hydrophobic leucine residues aligned along the length of the α-helix. Hydrophobic interactions of countered leucine residues on the helixes form a heterodimer, assisted by four attractive salt bridges g → e’. The homodimer has correspondingly two attractive and two repulsive salt bridges. The peptide dimer eventually forms a superhelical quaternary structure (Figure 11b).

Figure 11. a) Presentation of helical wheel model of the leucine zipper dimer and binding interactions in the heptad repeating unit denoted by letters a-g. b) Crystal structure of a leucine zipper domain in a dimeric state. The crystal structure was retrieved from the protein data bank (ID 4DMD, DOI: 10.2210/pdb4dmd/pdb) and the images produced using Chimera

A pair of designed parallel heterodimerizing leucine zippers with a stability of 10^{-15} M were applied by Zhang et al. to create self-assembling protein scaffolds (Zhang et al. 2005). This zipper peptide sequence ZE was slightly modified and engineered to form a hydrophobin fusion protein (Laaksonen et al. 2010).
Aims of the study

This research focuses on the use of tailored hydrophobin fusion proteins in model applications. Hydrophobins are small fungal proteins with interfacial function. This characteristic arises from a unique, bipolar structure. Hydrophobins also partition effectively in liquid two-phase systems. The aim of the work presented in this thesis was to connect the molecular function of the hydrophobins to other operational functionalities by methods of genetic engineering.

Firstly, suitable model hydrophobin fusion proteins were designed and produced and their functionality at liquid-liquid and solid-liquid interfaces was studied. In the following segment of this study, the functionality of the fusion proteins was assessed in model applications as a hybrid material. A central theme throughout this thesis is to evaluate aspects such as protein component stoichiometry, material geometry and charge effects, as well as holistic factors influencing application design using hydrophobin proteins.

Figure 12. Schematic presentation of the aims of this research.
2. Materials and methods

This section will provide reference to the fusion proteins used in this research (Table 3). Central techniques used for characterization and application development are also introduced.

2.1 Proteins

Fusion proteins used in the research included in this thesis are listed in Table 3.

<table>
<thead>
<tr>
<th>Fusion protein or conjugate</th>
<th>Production organism</th>
<th>Functionality</th>
<th>Publication</th>
</tr>
</thead>
<tbody>
<tr>
<td>HFBI-ProteinA</td>
<td><em>N. benthamiana</em></td>
<td>Affinity binding</td>
<td>I</td>
</tr>
<tr>
<td>HFBIII-ProteinA</td>
<td><em>N. benthamiana</em></td>
<td>Affinity binding</td>
<td>I</td>
</tr>
<tr>
<td>Au-NCysHFBI</td>
<td><em>T. reesei</em></td>
<td>Nanoparticle</td>
<td>II</td>
</tr>
<tr>
<td>HFBI-Avd</td>
<td><em>T. reesei</em></td>
<td>Affinity binding</td>
<td>III</td>
</tr>
<tr>
<td>HFBI-dcAvd</td>
<td><em>T. reesei</em></td>
<td>Affinity binding</td>
<td>III</td>
</tr>
<tr>
<td>HFBI-ZE</td>
<td><em>T. reesei</em></td>
<td>Dimerization</td>
<td>IV</td>
</tr>
</tbody>
</table>

2.2 Aqueous two-phase separation

Aqueous two-phase separation (ATPS) was used to purify the produced hydrophobin fusion proteins (Figure 8). Two technical grade detergents are commonly used in the purification routine of class II hydrophobins and their fusions, known by tradenames Berol532 and Agrimul NRE1205 (Table 2).

To purify hydrophobin fusion proteins, ATPS was performed starting from crude leaf extract or supernatant of *T. reesei*. For *T. reesei* supernatants the ATPS was conducted at pH 5 using Berol 532 (HFBI-dcAvd). The back-extracted fractions were finally purified by high-pressure liquid chromatography and recovered yields were calculated by integration of the chromatograms. HFBI-ProteinA was purified from crude *N. benthamiana* leaf extract at pH 7, and final purification was done by affinity chromatography. The recovered yields were analysed by SDS-PAGE.

HFBI-ProteinA was first allowed to bind to the target antibodies in buffer solution. The experiments were conducted at room-temperature and neutral pH to avoid antibody fouling. Possibilities to influence the two-phase system were thus restricted to the choice of surfactant, buffer composition and possible additives. The solution was then added to a batch of pre-weighed detergent and mixed carefully. In the
case of Triton X-114 the detergent is the lower phase and, hence, the upper phase (residue) was collected. To selectively release the antibodies from HFBI-ProteinA, the detergent phase was washed with acidic buffer. After phase separation had re-occurred, the acidic top phase was collected and neutralized to avoid antibody denaturation.

The functionality of the HFBI-ProteinA fusion protein was tested also in the presence of host cell background. For this, samples of plant-leaf extract were spiked with the target antibody Rituximab and the HFBI-ProteinA fusion protein. Finally, the whole solution was subjected to the two-phase system described above.

2.3 QCM-D

A quartz-crystal microbalance with dissipation monitoring was used in this research to study the assembly of the hydrophobin fusion proteins at liquid-solid interfaces. The QCM-D technique uses an oscillating piezoelectric quartz crystal which is set to vibrate at its resonance frequency (Figure 13; Rodahl et al. 1997) The crystal surface can be spin-coated with various materials.

![Figure 13. Operating principle of QCM-D technology. a) When mass is adsorbed to the surface of the sensor crystal, the resonance frequency of the crystal is changed. The change in frequency $\Delta f$ is proportional to the adsorbed mass $\Delta m$ for rigid molecular layers. b) The adsorbed molecules dampen the oscillation of a freely oscillating crystal, yielding the dissipation factor $D$. Dissipation describes the viscoelastic properties of the adsorbed layer.]
During measurement, the crystal is firmly placed in a closed fluid chamber and surrounded by the chosen measurement fluid. The measurement chamber is connected to a temperature-stabilized flow channel and the fluid flow is operated by a peristaltic pump. As molecules reach the surface of the measurement crystal and adsorb to the vibrating surface, the oscillation frequency of the crystal is decreased by the increased mass. The change in resonance frequency $\Delta f$ and overtones 3, 5, 7, 9 and 11 are monitored. The measured $\Delta f$ can be translated to mass $\Delta m$ using the Sauerbrey equation (Equation 4).

$$\Delta m = -\frac{C \cdot \Delta f}{n_3}$$  \hspace{1cm} \text{(Equation 4)}$$

where $C = 17.7 \, \text{ngHz}^{-1}\text{cm}^{-2}$ for a 5 MHz quartz crystal and $n_3 = 3$, the overtone number.

Dissipation describes the viscoelastic properties of the mass bound to the crystal surface. Once the electric circuit driving the crystal is cut, the sensor crystal is left to oscillate freely. As a result, the oscillation starts to gradually dampen down as oscillation energy is lost. This change is measured as the dissipation factor, $D$ (Equation 5).

$$D = \frac{E_{\text{lost}}}{2\pi E_{\text{stored}}}$$  \hspace{1cm} \text{(Equation 5)}$$

A large, uncoupled mass bound to the surface will cause the crystal to pause faster. On the contrary, a firm molecular layer is more coupled with the oscillation of the crystal and will dissipate the oscillation energy more slowly. The Sauerbrey relation (Equation 4) is valid only for firm molecular layers, i.e., when mass is adequately coupled to the oscillation of the crystal. For soft, viscoelastic layers the contribution to $\Delta f$ is unsensed by the sensor crystal and viscoelastic models must be applied to examine the properties of the bound mass.

### 2.4 Carbon nanomaterials

This section presents an overview on the carbon nanomaterials used in this research (Figure 14; Publications II and IV)
2.4.1 Graphene

Graphene (Figure 14a) is a two-dimensional, one-atom thick layer of sp²-bonded carbon atoms (Novoselov 2004; Novoselov et al. 2012). Carbon atoms in graphene form a hexagonal lattice structure (Figure 14a). The graphene monolayer is a building block for other carbon allotropes. A stack of graphene layers forms graphite, the more commonly known form of carbon. Graphene is outstanding in terms of its mechanical and conductive properties; graphene is over 100 times stronger than steel (Lee et al. 2008; Mayorov et al. 2011; Geim & Novoselov 2007).

Electronically, graphene is a zero-gap semiconductor material, meaning there is no gap between the energy states of the conductance and valence band electrons (Castro Neto et al. 2009; Geim & Novoselov 2007; Novoselov 2007). The conductance and valence bands of graphene meet at the Dirac point (Figure 15). Graphene exhibits a low density of energy states near the Dirac point, which makes its electronic properties very sensitive to the surroundings at this point (Novoselov et al. 2005). Electrons interact with the honeycomb lattice to produce massless Dirac fermions, electrons and holes as they move from the valence band (highest occupied molecular orbital, HOMO) to the conductance band (lowest occupied molecular orbital, LUMO).
2.4.2 Carbon nanotubes

Carbon nanotubes (CNT) can be described as rolled-up sheets of graphene (Figure 14b) (Iijima 1991; Baughman et al. 2002). They are long, cylindrical particles which are hollow inside. Carbon nanotubes are categorized as single-walled (SWNT) or multi-walled (MWNT), depending on their wall structure. They exist as bundles, which are stacked together longitudinally due to π-stacking of the C-C sp² bonds. Carbon nanotubes are highly hydrophobic, but can be solubilized in water similarly to graphene, i.e. by chemical modification and ultrasonication, or by using organic solvents (Tasis et al. 2006; Hirsch 2002). Also biomolecules can be used for solubilisation of carbon nanotubes (Asuri et al. 2006; Zheng et al. 2003).

Carbon nanotubes are extremely strong and stiff, with a tensile strength 10-50 times that of stainless steel. Electronically carbon nanotubes are different from graphene, CNT’s are always either metallic or semiconducting. Carbon nanotubes also exhibit very good thermal conductivity as well as unique optical properties (Baughman et al. 2002).

2.4.3 Exfoliation and adsorbent interactions

Separation of individual graphene sheets or carbon nanotubes in solution is called exfoliation. Exfoliation in liquid phase is traditionally achieved by using aromatic organic solvents such as toluene. Water solubility can be attained by chemical modifications (Loh et al. 2010). Covalent modification of the carbon surface by for example oxidation increases water solubility of the carbon material, but disrupts the valuable sp² structure of the particle surface and thereby deteriorates material performance.
To ensure material performance, noncovalent functionalization methods can be used. These include modification with hydrophobins (Laaksonen et al. 2010). Noncovalent exfoliation of carbon nanotubes or graphene proceeds via interaction with surfactants or suitable biomolecules, such as proteins (Marchesan & Prato 2015). In addition to the solubilizing effect, the adsorbed surface-active molecules can further interact with graphene: transfer electrons, induce holes, scatter carrier transport, or create localized spots with high gate voltage. The nature of the induced effect is surfactant dependent, and is a consequence of the amphiphilic structure and surfactant dipole strength.

Engineering of nanoscaled surfaces requires control over surface chemistry and molecular orientation is affected by van der Waals forces (Novoselov et al. 2016). In nanoscale, the final realization of forces and interactions involved depends greatly on particle size and geometry (Autumn et al. 2002). In addition to physical surface forces, the interfacial arrangement of ions in water solution influences the electric characteristic of a surface. In the case of biomolecular surfaces, cavities or variation of chemical structure may confine water molecules and ions into unpredictable orientations, causing dewetting, but also polarization effects (Stigliano et al. 2013).

2.4.4 Graphene field-effect transistor

A field-effect transistor (FET) is a semiconductor device which is composed of three electrodes, the source, the drain and the gate (Young & Freeman 1996). The source and drain are connected by a gate electrode. The current between the source and drain is controlled by their respective potential difference, and by the charge on the gate. No current runs through the gate. In the case of a graphene field-effect transistor (GFET), a graphene sheet connects the source and drain, forming the channel (Stine et al. 2013).

Hydrophobin fusion proteins were used to functionalize the graphene surface in a field-effect transistor based biosensor (BioFET) (Figure 16; Riikonen et al. 2013). The graphene channel was connected to four electrodes: source, drain, gate ($V_{gate}$) and reference ($V_L$). The graphene channel was immersed in a fluid chamber filled with measurement buffer. The conductivity of the graphene channel was monitored at the Dirac point.
Figure 16. Schematic presentation of a G-FET device in biosensor setup.
3. Results

3.1 Hydrophobin fusions in 3D systems

The interfacial activity of the hydrophobin was connected to the antibody binding capability of ProteinA (I). The ATPS technique was applied to create a liquid-based method for molecular handling using the hydrophobin as a tag in the two-phase system (Figure 17).

Figure 17. Schematic representation of HFBI-ProteinA antibody harvesting process. The green shapes denote impurities.

A fusion protein of HFBI or HFBII and ProteinA was designed and produced in the tobacco plant *N. benthamiana* with good yield. The fusion protein was successfully purified by ATPS using Triton X-114. The recovered yield of HFBI-ProteinA was 62±5%. The partition coefficients $k$ for HFBI-ProteinA were 4.8±0.9 and for HFBII-ProteinA 2.4±0.6. Native ProteinA did not partition to the surfactant phase in the same ATPS conditions, as shown by a $k$-value of 0.4±0.1.

Functional characterization of HFBI-ProteinA and HFBII-ProteinA was completed by QCM-D measurements. Both fusion proteins were observed to tightly bind to the hydrophobic polystyrene substrate. Both of the fusion proteins bound the Rituximab antibody in an amount comparable to physisorbed native ProteinA. HFBI-ProteinA bound 1.5 moles of Rituximab per mole of HFBI-ProteinA. The corresponding value for both HFBII-ProteinA and native ProteinA was 1.2. Control measurements showed that no antibody bound to layers of wild-type HFBI or bovine serum albumin.
The formed molecular surfaces were reproducible and regenerable (Figure 18). The bound antibodies could be released by rinsing the surface with acidic glycine buffer. The underlying HFB-ProteinA layer was left intact. After neutralization of the solution pH, a new round of antibodies could be bound to the HBF-ProteinA layer without a significant loss in binding capacity.

Figure 18. a) Preliminary QCM-D measurements demonstrate the regenerability of the surface layer of HFBII-ProteinA. The measurement shows the initial adsorption of HFBII-ProteinA, followed by two rounds of IgG binding (IgG on) and release (IgG off). The basal HFBII-ProteinA layer is not affected by the sequential process of analyte binding and release.

A lower areal density of functional groups can increase the effective analyte response through reducing steric hindrance. This can be advantageous, for example, in the case of large analyte molecules, such as antibodies. Diluting hydrophobin fusion proteins with wild-type hydrophobin has been previously observed to enhance surface packing by reducing steric hindrance at the surface (personal discussion). In this research, preliminary QCM-D measurements indicated, that there was no significant difference in the amount of bound IgG whether the hydrophobin was surface was formed by HFBII-ProteinA alone or by a 5:1 molar mixture of HFBII-ProteinA/HFBI (Figure 19).
Figure 19. Preliminary QCM-D measurements indicate, that the amount of IgG bound is not significantly improved by co-assembly of HFBII-ProteinA with HFBI.

QCM-D measurements allowed examination of the molecular layers by viscoelastic modelling (Figure 20). The QCM-D signals were followed at several overtones $f_n$ of the resonance frequency $f$ ($n = 3, 5, 7, 9$ and $11$) and the viscoelastic Kelvin-Voigt model was used to examine the thicknesses of the bound protein layers. The model was applied to both layers separately. The density of the layer was assumed as $1000 \text{ kg/m}^3$ and viscosity $0.001 \text{ kg/ms}$. Ordering of the basal hydrophobin monolayer places chemical functionalities of connected groups (ProteinA and IgG) in defined locations causing elevated shear force.

Figure 20. a) Thicknesses of layers by estimated by viscoelastic modelling (Kelvin-Voigt). b) Modelled values of shear force in HFBI-ProteinA/IgG layers. Wild-type ProteinA is shown as a reference.
On the basis of the characterization results, the hydrophobin was harnessed in use in a surfactant-buffer two-phase extraction system. The molecular complex of HFBI-ProteinA and an IgG antibody Rituximab were mixed with the two-phase detergent/buffer system of Triton X-114 and phosphate buffer. Initial experiments immediately showed that HFBI indeed was able to guide the fusion protein/antibody complex to the detergent phase. SDS-PAGE gel imaging verified a decreased concentration of antibodies in the residual buffer phase (Figure 21). Correspondingly, enrichment of antibodies was observed in the buffer after release from the detergent. However, the partitioning of the antibody in initial experiments was much poorer than was expected on the basis of HFBI-ProteinA behavior in ATPS. Phase separation was also unstable.

![Figure 21. SDS-PAGE gel image demonstrating proof of concept for the HFBI-ProteinA-based antibody harvesting process.](image)

In an effort to enhance partitioning of the HFBI-ProteinA/IgG complex to the detergent phase, addition of a crowding agent (PEG, MW 6000, 10 w-%) was tested (unpublished data). The added polymeric component was expected to increase pressure for movement of the HFBI-ProteinA/antibody–complex to the detergent phase (Collén et al. 2002). However, although the addition of PEG supported the phase separation event in the system, the crowding agent interfered with subsequent SDS-PAGE analyses. This could be avoided by precipitation of the samples prior to loading. However, the additional step was concluded to add uncertainty in terms of antibody stability and possibly lowered yield. The use of polymeric additives was thus omitted from the process and development of the harvesting routine was continued with the buffer/Triton X-114 system.
Hydrophobin-based harvesting of antibodies was demonstrated finally using samples spiked with plant leaf extract (I). This sample background was chosen to model the function of the HFBI-ProteinA in more realistic complex fluids. Using HFBI-ProteinA, a recovered yield of 28±1% of Rituximab was measured (I). This was clearly higher than the recovery of bare Rituximab (12±2 %).

3.2 Fusion proteins for adsorption to solid surfaces

The interfacial function of HFBI was connected to a different type of binding function in the fusion protein HFBI-dcAvd (Publication III). The connection of avidin and hydrophobin was initially constructed in two different stoichiometries (Figure 8), leading either to either a 1:1 ratio of hydrophobin/biotin-binding site in HFBI-Avd and 1:2 hydrophobins/biotin-binding site in HFBI-dcAvd.

Both fusion proteins were produced successfully in T.reesei. However, the different assemblies displayed very different solubility. The HFBI-Avd fusion was tightly bound to the mycelium after cultivation, while HFBI-dcAvd was found to be soluble in the culture medium. The same tendency was noted during protein purification. HFBI-Avd was released from the mycelium by SDS and could not be retained in solution without SDS or ethanol. In contrast, HFBI-dcAvd was secreted during production and could be easily handled in the solution. HFBI-dcAvd was purified in normal manner by ATPS using Berol 532. The concentration of the extract after two-phase separation was 0.65 mg/l. Cation exchange chromatography was used for final purification.

To allow good comparison, the biotin-binding capability of the HFBI-dcAvd fusion was studied by the same techniques that were used priorly to characterize dcAvd (Nordlund et al. 2005). Measurement of the fluorescence of tryptophan residues in the biotin binding sites during titration with free biotin was used to determine the effective number of biotin-binding sites. The obtained value for HFBI-dcAvd was 2.4, which was slightly lower than has been reported previously for dcAvd (3.9).(Nordlund et al. 2005) The fluorometric measurement was compared to results of a 3H-assay, which yielded a value of 2.9 as the number of biotin-binding sites for HFBI-dcAvd.

The biotin affinity of HFBI-dcAvd was investigated by studying the dissociation of radiolabelled 8,9[^3H]-biotin. These measurements produced a dissociation constant $k_{\text{diss}}$ of $7.6\pm0.8 \cdot 10^{-6} \text{ s}^{-1}$ for HFBI-dcAvd. A value of $k_{\text{diss}} =3.0 \cdot 10^{-6} \text{ s}^{-1}$ has been previously reported for dcAvd (Nordlund et al. 2005).

The HFBI-dcAvd fusion protein was observed to self-assemble reproducibly on a polystyrene substrate in QCM-D measurements. The Sauerbrey mass of the surface layer was 825 ng/cm². Considering the molecular masses based on the protein sequence, the result indicated the formation of a molecular monolayer. The dissipation values were typically low, $<1\cdot10^{-6}$, confirming again the rigidity of hydrophobin-mediated surface-adhesion.

The equilibrium binding isotherms of HFBI and HFBI-dcAvd were measured by QCM-D using protein solutions of different concentrations. The Sauerbrey mass
(Equation 4) of the saturated HFBI surface layer was measured to be 290±17 ng/cm² and 825 ng/cm² for HFBI-dcAvd. The dissociation constants for both HFBI and HFBI-dcAvd were in the order of 10⁻⁷ to 10⁻⁸ M. The dissociation constant for HFBI-dcAvd was slightly lower, the 95% confidence interval for HFBI being 0.02-0.2 μM and for HFBI-dcAvd 0.1-0.5 μM. QCM-D measurements show that the dissipation (Equation 5) of the adsorbed HFBI layer is very low, < 1·10⁻⁶. This indicates that the formed layer is rigid and well-coupled with the oscillation of the crystal.

Biotinylated substrates were bound to the self-assembled HFBI-dcAvd layer to confirm the operational functionality of the dual-chain avidin module. The binding of biotinylated fusion protein of green fluorescent ProteinAnd hevein (bGH) was measured by QCM-D. The HFBI-dcAvd was found to readily bind the biotinylated protein in a 1:1 ratio. The molecular ratio was estimated on the basis of Sauerbrey masses (Equation 4) and molecular masses of bGH and HFBI-dcAvd. When bGH was immobilized via HFBI-dcAvd the layer structure was rigid, yielding a low dissipation factor D (Equation 5) in the order of 10⁻⁶. Control experiments showed, that bGH did not bind to a surface of wild-type HFBI in comparable amounts. The Sauerbrey mass of bGH on the HFBIdcAvd substrate was 530 ng/cm², whereas on wt HFBI only a layer of 70 ng/cm² was adsorbed.

The HFBI-dcAvd monolayer was produced using solutions of 0.01 mg/ml concentration. In the same conditions, avidin did not bind to the surface in a mentionable amount. In subsequent control measurements, layers of mass equivalent to a monolayer of avidin could be produced when avidin concentration was increased to 1 mg/ml. This avidin layer was observed to bind high amounts of the biotinylated substrate, clearly higher than possible for an ordered monolayer. Indeed, dissipation in the layer was elevated after binding of the substrate, indicating a loose and unordered molecular layer. In addition, reproducibility of the physisorbed avidin surfaces was poor compared to the HFBI-dcAvd layers.

3.3 Hydrophobin functionalized surfaces in 3D systems

Hydrophobin assembly at solid-liquid interfaces was demonstrated also in 3D assemblies of HFBI and carbon nanotubes (II). Carbon nanotubes could be water-solubilized and exfoliated in solutions of class II hydrophobins at room temperature using ultrasonication. The interaction of HFBI and carbon nanotubes was first verified by studying the solubilisation of dry carbon nanotubes in aqueous solutions of HFBI, assisted by sonication. HFBI was observed to effectively solubilize carbon nanotubes. Single-walled carbon nanotubes (SWNT’s) could be solubilized to 200 μg/ml by HFBI solutions of 0.25 mg/ml (0.03 mM). The amount of carbon nanotubes solubilized by BSA in the same conditions was found to be negligible. The solutions were produced by ultrasonication and were found to be stable to handling and months of storage at room temperature.

UV/Vis spectroscopy was used for initial characterization of the HFBI-SWNT solutions. Transmittance values of the solutions at 550 nm were 28 % for HFBI-SWNT and 96% for BSA-SWNT. The absorbance spectrograms displayed poorly resolved
Van Hove peaks that are characteristic to solubilized SWNTs. Circular dichroism was measured to examine possible changes caused to the protein structure. No changes to the HFBI structure were detected.

Transmission electron microscopy was used to examine the assembly of carbon nanotubes with hydrophobin molecules. Initial experiments confirmed that individual carbon nanotubes were solubilized in the HFBI solution. The carbon nanotubes were trapped in a film of hydrophobin, which stretched across the holey carbon grid. However, it was impossible to separate the CNT-bound hydrophobins from the extending film.

To get a closer view on the molecular assembly of the HFBI-CNT hybrids, the hydrophobin was labelled with gold nanoparticles. This was achieved by using an engineered hydrophobin mutant, the NCysHFBI (Szilvay et al. 2006). In NCysHFBI, the N-terminus is continued with a peptide linker of 13 amino acids, ending at a cysteine residue. NCysHFBI is produced and purified as a dimer (NCysHFBI)$_2$, bound by a disulphide bond of the thiol groups. The thiol groups were generated by reduction with dithiotreitol and subsequently used to couple the protein to maleimide functionalized gold nanoparticles. This bioconjugation step ensured a determined 1:1 stoichiometry of the HFBI and Au nanoparticles, allowing structural distinction in transmission electron micrographs.

The positioning of the HFBI molecules was verified by visualization of the gold nanoparticles in the micrographs. TEM images of SWCNT’s solubilized by Au-NCysHFBI confirmed the assembly of carbon nanotubes with specifically bound hydrophobin molecules. The gold nanoparticles were arranged regularly by the SWNT wall at a 2.6±0.4 nm interdistance. As a control, HFBI was mixed with the Au nanoparticles and the mixture was used for CNT solubilisation. Evaluation of these samples by TEM showed a random distribution of gold nanoparticles evenly spread across the whole film.

The reduced form (NCysHFBI)$_2$ was also tested for used in solubilisation experiments, but did not show any enhanced performance. However, the films of (NCysHFBI)$_2$ were observed to be more resistant to damage caused by the microscope beam.

### 3.4 Hydrophobin fusion proteins for electronic applications

To study the feasibility of nanocarbon modification with hydrophobin fusion proteins, biofunctionalization of graphene surfaces was pursued. The suitability of hydrophobin for use in bioelectronics applications was demonstrated in Appendix IV.

Two different HFBI fusion proteins were used to biofunctionalize the graphene channel of a G-FET biosensor. The conductive graphene surface was connected to the surrounding aqueous environment via a self-assembled layer of the HFBI-fusion protein. Changes in the vicinity of the graphene surface affect electrical conductivity and could be directly measured. Formation of the monolayers at the interface of CVD graphene and buffer was examined by AFM in both wet and dry states. Both proteins HFBI-ZE and HFBI-ProteinA were observed to form dense monolayers of...
4-5 nm thickness immediately upon introduction of the protein solutions to the graphene surface. Drying was observed to cause cracks in the protein layers, but the monolayer was resumed within 5 minutes after rewetting with the original buffer.

Functionality of the surface layers of HFBI-ZE and HFBI-ProteinA were examined by QCM-D measurements. HFBI-ZE formed a monolayer on polystyrene, yielding a Sauerbrey mass (Equation 4) of 270 ng/cm², corresponding to a monolayer as estimated using the molecular mass and hydrophobin dimensions (12 kDa). The dissipation value (Equation 5) for surface-adsorbed HFBI-ZE at pH 7 was 2-2.5·10⁻⁶. This is somewhat higher than for monolayers of wild-type HFBI (D<1·10⁻⁶). When the peptide ZR was added to the HFBI-ZE surface, the dissipation value decreased, but no change in frequency was observed. At pH 5 the dissipation value of HFBI-ZE was measured to be <<1·10⁻⁶. Surface plasmon resonance measurements verified the binding of the HFBI-ZE to ZR in approximately 1:1 ratio.

QCM-D measurements showed that HFBI-ProteinA bound to the polystyrene surface forming a layer with a Sauerbrey mass of 752 ng/cm², corresponding to a molecular area of 9 nm² using a 44 kDa molecular mass. Molecular dimensions of 2.5 nm x 4 m were used for the estimation of molecular area of HFBI-ProteinA. The mass was thereby estimated to correlate with an average 3.2±0.2 nm spacing between the proteins. These values were estimated on the basis of the ProteinA subunit B (Protein Data bank ID 1BDC). Addition of the IgG1λ antibody increased the Sauerbrey mass by 1331 ng/cm², corresponding to an IgG/HFBI-ProteinA ratio of roughly 1.9:1.

To study the absorption of HFBI-ProteinA to the CVD graphene surface used in the final application, a piece of CVD graphene was attached to a SiO₂ sensor crystal. When the binding of HFBI-ProteinA was measured, the protein was observed to bind to the SiO₂ surface partly covered with CVD graphene control, as well as the SiO₂ surface used as a control. The Sauerbrey mass of HFBI-ProteinA was similar in both cases, although clearly lower than on polystyrene. However, the amount of IgG bound to the graphene covered surface was five times greater than to the reference SiO₂ surface.

Functionality of the developed sensor was demonstrated by measuring analyte conditions in model solutions by using two different HFBI functionalized surfaces, HFBI-ZE and HFBI-ProteinA. The binding of the negatively charged HFBI-ZE to the graphene surface caused the Dirac peak to shift to more positive voltage. When the positively charged peptide ZR was bound to the HFBI-ZE layer from a 10 μM solution, the Dirac peak was observed to return to the clean sensor state, indicative of neutralization of the zipper charges upon dimerization. The detection range for the HFBI-ZE-ZR system was found to be between 10 fM - 10 μM and detector response occurred in less than 1 s.

The corresponding measurements were conducted using the HFBI-ProteinA functionalized graphene surface. Binding of both HFBI-ProteinA and IgG induced a negative shift of the Dirac peak. The detection range was found to range from 80 fM to 80 nM, also displaying a fast initial response followed by slower saturation.

Selectivity of the hydrophobin functionalized surface was studied by crossing the used analytes. When IgG1 was added to the sensor surface functionalized with
HFBI-ZE, a less than 1% response in channel resistance was measured. Control measurements of ZR binding to a clean graphene surface and a monolayer of wild-type HFBI were also performed. In both cases the measured shift of the Dirac peak was an order of magnitude lower than for the specific interaction of ZR on the HFBI-ZE surface.
4. Discussion

Construction of recombinant fusion proteins allows straightforward access to functional biomolecules. However, connecting different proteins together does not necessarily result in a sum of the different functionalities. The as-created fusion protein is a completely new molecule and may exhibit unpredicted behavior. The combined protein functionalities may not operate as they would if they were secluded. Effects of protein fusioning finally depend on the created protein construct as well as the chemical and physical microenvironment in which the fusion protein is to be applied. The results presented in Section 3 allow identifying certain general circumstances, where the presence of the fusion protein may challenge hydrophobin functionality. (Figure 22).

![Figure 22](image-url)

Figure 22. Schematic presentation of actors compromising functionality of hydrophobin fusion proteins.

The task of constructing and applying functional fusion proteins can be presented as a stepwise process (Figure 23). Final technological application of biomolecules includes many cross-disciplinary landmarks, in which the fusion proteins must be examined in diverse settings. The value of a recombinant protein is finally dictated by its suitability for use in a target application. This sets a demand for smart protein engineering, but also requires skill in protein formulation and biophysical application. Recognizing main issues related to each step of the development process will thus facilitates actual industrial implementation.
Figure 23. Design process of hydrophobin fusion protein technology.

Access to the final functional proteins follows a route involving three steps (Figure 23). The results presented in Section 3 will be examined in this context to gain a more general view of factors associated with hydrophobin fusion technology.

The engineering step proceeds from selection of functional fusion partners to engineering a line of fusion constructs with varying properties and composition. This step continues with production of the designed constructs and finally, protein purification. The engineering step is governed by the choice of production organism, which dictates the overall flexibility of the whole design process in terms scale-up and timeline. The production organism may also impose restrictions on structural elements of the proteins.

The formulation step entails experimental efforts aimed at verifying the functionality of both all protein parts individually, and establishing optimal conditions for final use of the fusion protein. In other words, formulation entails characterization of the whole molecular system. Steps aiming at stabilization or activation for final application should also be considered at this stage.

Finally, the fusion protein is harnessed to use in the application step. This step includes examination of the final setup from multiple aspects, defined by the end-technology. The application step can bring out unexpected behavior related to additive interactions in the final system. To tune the system, the engineering process must return to characterization step to optimize conditions or, ultimately, to the engineering step.

**Engineering step**

The main factors contributing to hydrophobin fusion protein behavior are related to protein stoichiometry and multimerization habits. Molecule stoichiometry was found
to be a critical factor for the functionalities of HFBI-Avd and HFBI-dcAvd, where both of the fusion partners respectively form multimers in solution. HFBI-Avd was not operational, because of challenges in solubility after release from the mycelium. HFBI-dcAvd on the other hand could be handled in a feasible manner. The high multimerization affinity of avidin monomers in HFBI-Avd hindered independent multimerization and solubility of the fusion protein. Within the limits of this imposed geometrical restriction, the solvation energy of the hydrophobic patches could be sufficiently lowered only by adsorption to mycelial structures.

Also HFBI-ProteinA performed well at the liquid-solid surface. The amount of bound IgG was found to be insensitive to whether the surface consisted of only HFBI-ProteinA or had been co-assembled with HFBI. This observation indicates that the wild-type hydrophobin and fusion protein do not compete equally for surface adsorption sites, perhaps due to different stabilities in multimer equilibria. It is also possible, that the amount of bound analyte on co-assembled surfaces would differ more for analytes smaller than the IgG molecule.

Transition of the HFBI-ProteinA fusion to a 3D system revealed a more complicated situation. The recovered ATPS yields were low and phase separation was unstable. The degree of association in the HFBI-ProteinA/IgG interaction leads to a large molecular complex and may hinder hydrophobin multimerization, thereby weakening the driving force for hydrophobin interaction with detergent. One ProteinA molecule can bind up to five antibodies, each of which have two possible binding sites for ProteinA. Considering this, it is clear that a random compilation of large, interconnected proteins would result. Thus, reducing the size of the complexes by adjusting the ratio of associated HFBI:IgG in the protein engineering step may be beneficial and should be studied in the future.

Considering the area of the hydrophobic patch and the hydrophilic area of the adjacent hydrophilic end containing ProteinA and bound antibody, it would be plausible to assume, that the large hydrophilic molecules might hinder the surface-activity of the hydrophobin. The hydrated molecular surface area exceeds the area of the hydrophobic patch by orders of magnitude and could possibly surmount the driving-force of hydrophobin assembly. However, even when the hydrophobin was connected to a large hydrophilic complex, association with the detergent phase was not completely sacrificed. This may be understood through a loose correlation to micellar geometries, in which the hydrophilic bulk of the head group does not affect micelle geometry, but only the cross-sectional area of the hydrophilic moiety is relevant (Equation 3). It is impossible to determine, whether hydrophobin association with the detergent phase occurs via the monomeric form of the protein, or rather the multimeric assemblies become incorporated in detergent micelles of corresponding geometry.

Another possible explanation for the low recovery yields in the antibody harvesting system are problems in the binding of the antibodies to the ProteinA module. Necessary geometrical arrangements may be restricted if hydrophobin multimerization prevails solution behavior. This problem could be especially prominent in the case of the HFBI-ProteinA fusion protein, because of the large size of the binding antibodies. Steric clashing of vicinal molecules may disturb binding of the antibodies
to the ProteinA binding sites even in a 3D system. Indeed, entrapment of water molecules due to localized restrictions in molecular assemblies has been presented to lead to decreased entropy and thus energetically unfavoured states (Chandler 2005; Stigliano et al. 2013).

Regarding adsorption at the solid-liquid interface, the main advantages of hydrophobin fusion technology are 1) structural order and 2) high-affinity for surface binding. These features result in defined orientation of the fusion partners and reproducible surface coverage. Self-assembly of hydrophobin at the liquid-solid interface was not observed to be disturbed by the fusion partners. The resulting molecular complexes were not observed to loosen from the substrate despite increased solvated surface area. This was the case for all fusion proteins, and it is indication of the strength of the interaction of HFBI and the solid hydrophobic surface.

For certain applications a high number of bound analyte is desirable. However, possible steric effects of the bound molecules may decrease the degree of surface coverage. This should be taken into account in construct design. Although the two-step mechanism of self-assembly has been proposed for only the air-water interface, the natural function of the hydrophobin implies, that a two-step process is required for efficient surface coverage. The initial occupancy of hydrophobin at an interface is random and the molecules are rapidly expelled from aqueous solution to the surface due to the dewetted hydrophobic patch. As more monomers keep approaching the surface, the previous molecules have to be able to diffuse laterally to adjust the incoming monomers. The mode of contact between the surface and the hydrophobic patch, would be in a major role in this event. Friction between the surface and the hydrophobic patch, as well as steric clashes of bound fusion partners may create an energetic barrier for the lateral movement of the surface-bound monomers. The effect of the fusion partner may be more pronounced at the solid-liquid interface, as the solid surface does not allow protrusion of molecular parts during a possible reorientation.

In addition to stoichiometry, molecular geometry determines solution behavior and functionality of hydrophobin fusion proteins. Wild-type hydrophobins form a variety of multimeric solution states, and experimental findings suggest that the same is true for hydrophobin fusion proteins. The shape of the oligomers and positioning of fusion proteins is determined by molecular contacts between hydrophobin monomers. Multimerization habits and challenging geometries may be controlled by genetic engineering of fusion partners. This was found to be beneficial in the case of avidin fusion proteins. For surface adsorption, it is important that the molecular orientation of the fusion partners does not hinder contact with the surface. This may be solved by avoiding multimerizing fusion protein partners as well as by considering linker length and rigidity.

Considering the tetramerization mechanism proposed by Riccardi et al (Riccardi & Mereghetti 2016), it is clear that any fusion protein or function connected to HFBI should not disturb movement of the β-hairpin loop. The amino acids 60-66 of this region are in direct connection to the N-terminal, separated by a peptide chain of 10 amino acids. In the wild-type HFBI crystal structure the N-terminal is fixed to the protein core by hydrogen bonding. However, conformational changes in the loop
from which the N-terminal extends may be hindered if a strong opposing force is present at the N-terminus. Such may be the case for example for large multimeric fusion partners displaying strong binding interactions, such as avidin or ProteinA.

The results described in this research underline the fact, that molecular mechanisms of hydrophobin assembly should not be understood simply in view of the amphiphilic structure. On the contrary, it is the amphiphilic structure which gives rise to diverse interactions with the surrounding environment. It seems clear that the energetical penalty of exposing the dewetted hydrophobic patch cannot be avoided; an increased degree of solvation at the hydrophilic end of the molecule does not diminish the driving force for the hydrophobic interaction.

However, although the surface-activity of the hydrophobin is seemingly unaffected by fusion partners, the existence of connected large molecules has other effects on the self-assembled structures. Hydrophobic molecules interact with water also in other ways than merely expelling water from the hydrophobic surface. Theory supports the presented observations on behavior of hydrophobin fusions. In the case of amphiphilic molecules, solvation of the hydrophobic area is not an excluded phenomenon (Chandler 2005). Hydrophilic parts of the molecule are involved in strong interactions with water molecules. On the other hand, orientation of the hydrophilic parts is dictated by positioning of the hydrophobic parts which are buried from water contact. These interactions cause restrictions to the free movement of surrounding water molecules. As a result, water molecules become locally entrapped.

The free energy of transferring an amphiphile to water is the same as the energy for transferring the hydrophobic part to water, because the hydrophilic part will always stay solvated (Chandler 2005). Formation of a micelle is thus opposed by two free-energy contributions: the formation of a stable interface and, secondly, the reduction of available configurations of water molecules, which entails entropic loss. These factors govern micelle size. As the number of molecules in a micelle grows, there is no space to maintain a dense interior while simultaneously placing head groups on the exterior.

According to theory, the factors associated with micelle formation are the width of the hydrophobic tail (α) and the length over which hydrophilic parts and hydrophobic parts are separated (δ; Chandler 2005) These factors determine micelle radius $L$ in terms of number of associated molecules, $n$:

$$L = (\alpha^2 \delta)^{1/3} n^{1/3}$$  \hspace{1cm} \text{Equation 6.}

The number of associated molecules in a micelle is thus given by

$$n = \beta \gamma \delta^2$$  \hspace{1cm} \text{Equation 7.}
where $\beta=1/k_B T$, $\gamma$ is the oil-water surface tension, $k_B$ is the Boltzmann constant and $T$ is temperature. Applying this theory to a hydrophobin tetramer ($n=4$) gives a micelle radius $L$ of 2.8 nm. The diagonal length of the HFBI tetramer is roughly 6.5 nm, as estimated by measuring from the crystal structure. This is in compliance with the theoretical presentation (Equations 6 and 7).

The main points to be considered in the engineering step are thus narrowed to geometry and steric hindrance. The presence of large fusion partners may disrupt the stability of solution oligomers due to entropic loss. This factor may be even more prominent in the case of multimeric tertiary structures. Disruption of solution assemblies may result in poor solubility of the molecules which is problematic during production and purification. In addition, control over molecular positioning in applications is lost due to energetically unstable solution assemblies and suboptimal packing efficiency at solid-liquid surface.

**Formulation step**

The formulation step of the design process includes examination of solution composition, molecule concentrations and optimization of the molecular composition at the interface. This step, although often self-evident and therefore disregarded, is actually a crucial step for successful implementation of final applications.

The binding affinity of hydrophobins to the polystyrene surface is low if compared for example to the exceptionally high biotin binding affinity of avidin ($10^{-15}$ M) and slightly lower for the fusion protein HFBI-dcAvd than for wild-type HFBI. The observed difference may be interpreted to be caused by the large, hydrated bulk of dcAvd, which poses a counteracting force for hydrophobin surface adsorption. A fusion partner may thus destabilize the surface assembly in similar manner as for oligomerization. The Langmuir isotherm fails to take into account molecular interactions between vicinal, adsorbed molecules. This may diminish the differences in surface affinity of the wild-type hydrophobin and its fusion protein. However, a crystalline surface structure has not been shown for hydrophobins at the solid-liquid surface, which suggests that there are probably mechanistic differences in adsorption at solid-liquid interface and the air-water interface.

The pH dependent dissipation values measured for HFBI-ZE at the solid-liquid interface point out the fact, that the fusion partner may significantly affect the behavior of the interfacial molecular layer with respect to solution composition. Structural rearrangements and changes in the viscoelasticity of the protein layer also likely affect the electrical behaviour of the protein surface. Thus, careful analysis of interfacial structures in the final application conditions is essential to be able to distinguish target signals, i.e., analyte binding.

Preliminary observations on difficulties in co-assembly of HFBI-ProtA and HFBI at the solid-liquid interface imply, that wild-type hydrophobin and hydrophobin fusion proteins possess different dissociation constants of multimer assembly. This in turn would affect the proportion of monomers available for surface adsorption. In the case of co-assembly, parallel events of solution multimerization and surface adsorption of the fused and unfused hydrophobins compete (Figure 24). The multimeric
forms of the fusion proteins may be less stable in comparison to those of the native hydrophobin, thus presenting more monomeric form available for surface adsorption. The formation of co-assembled surfaces would thus be related to the concentration range of solution multimerization.

Figure 24. Biofunctionalization of surfaces with hydrophobin fusion. a) Surface coverage may be governed by steric effects of fusion partner. b) Different solution dynamics of fused and non-fused hydrophobins may cause unequal competition for available surface adsorption sites.

When aiming at biofunctionalization of solid surfaces, the geometry of the surface should be complimentary with the hydrophobic patch. The rather rigid nature of the hydrophobin molecule likely requires a suitable geometric counterpart. The relationship between the diameter of carbon nanotubes and protein interactions has been recognized and it has been suggested that CNT diameter needs to be >10 nm for long-lasting contact with interacting macromolecules (Marchesan & Prato 2015).

Results regarding carbon nanotube functionalization indicated that carbon nanotubes were fully covered with Au-NCysHFBI at regular interspacing. The observed measure corresponded well to the diameter of the HFBI molecule. The radius of curvature of the smallest individual carbon nanotubes is possibly too large to fully bind to the hydrophobic patch of HFBI.

The carbon nanotube surface has been observed to induce conformational changes in the peptide backbone of certain interacting proteins, to adjust to the radius of curvature of the CNT (Marchesan & Prato 2015). In the case of the hydrophobins such deformation is not likely due to the rigid sulphide bridged structure. However, the hydrophobins were able to assemble at the water-carbon interface and the resulting solutions were very stable. The hydrophobic force exerted by the carbon surface could possibly cause effective distortion, i.e. closer packing, in the hydrophobic side chains, even though large structural rearrangements did not occur based on CD measurements (II).
According to general understanding, the interaction between the carbon nanotube surface and the hydrophobin occurs mainly via hydrophobic interactions of aliphatic residues in the hydrophobic patch and the aromatic carbon surface. However, aromatic rings can also act as acceptors to hydrogen bonds or cationic charge. Cationic charge is present near the hydrophobic patch of HFB1 in residues Lys32 and Asp30. Participation of these amino acid residues would require a suitable geometric orientation due to the orientation of the nitrogen orbitals (90° angle; Figure 25). This additional mechanistic detail may be involved in positioning of hydrophobins at aromatic carbon surfaces. If the low curvature of the carbon nanotube hinders intermolecular interactions between hydrophobin monomers, the proteins may be anchored via cation donation of the positively charged groups, explaining thus the regular arrangement of hydrophobins even in the void of optimal binding surface.

Figure 25. The interaction between hydrophobins and carbon nanotubes is challenged at small nanotube diameters due to high curvature. The positively charged amino acids near the hydrophobic patch may act as cation donors and participate in positioning of the hydrophobins on the carbon.

Hydrophobins show diverse, oligomeric solution structures based on interaction of the hydrophobic patches. Solution structures may be stabilized by additional interactions, such as hydrogen bonds or ionic bonds. It seems however, that the hydrophobic force between the hydrophobic patches of two hydrophobin monomers is rather weak. This would be plausible considering the natural function of the hydrophobins. Experimental methods used to study dynamic solution behavior rely on fluid flow, which exerts cutting forces in the liquid, possibly strong enough to break all but the smallest base units. For hydrophobin this might be the dimer, or tetramer, and observation of larger assemblies fails. It is easily understood how the tetramer could form a continuing lamellar structure. The crystal structure of the hydrophobin oligomers shows, that the hydrophobic patches are not exactly aligned, but twisted (Figure 2a), possibly allowing subsequent units to link to the assembly.
The fact that neither the interfacial activity nor the ATPS process is largely disturbed by the connection of fusion partners, would support the fact, that the cross-sectional area of the fusion partner is more important than the whole size of the protein. The driving force for the hydrophobin to self-assemble at interfaces is not lost upon fusion to other proteins. In other words, the effective amphiphilicity of the hydrophobin is not disturbed. Due to the sulphur-bridged inner structure, the hydrophobins are rigid and robust, they do not lose structure. However, this does not mean that the chemical surface of the hydrophobin molecule is inert to ambient conditions. Factors that affect the hydrophobic effect also affect hydrophobin structure and function. This means, that certain changes, such as temperature or salt concentration can cause tighter packing of the hydrophobic parts, whereas hydrophilic parts become more hydrated. Thus, the hydrophobin structure can be effectively distorted.

Geometrical changes in the hydration shell of the amphiphilic hydrophobin protein could play a role in the two-phase separation process in respect to choice of detergent. Accordingly, increased hydrophilicity in the detergent EO chain has been noted to be beneficial for the two-phase separation of the more hydrophilic hydrophobin fusion proteins. These surfactants include more water and physical space around the head groups in the micellar phase. In addition, longer EO chains have more conformational freedom to comply with the extending, curved protein surface.

In vitro assays take place in dilute aqueous solutions containing vast volumes of free water. It may be that in such a situation, confinement of water molecules has more pronounced effects. Biomolecules in vivo operate in a chemical microenvironment which is largely shaped by electrostatic and hydrophobic interactions with surrounding chemical groups. Although the structure of water is locally disrupted at molecular interfaces, lost hydrogen bonds may be compensated by other interactions with vicinal molecules. Protein aggregation is a consequence of uncontrolled hydrophobic interactions taking over, but also this problem is solved in vivo via specific stabilizing molecular interactions.

Macromolecular crowding agents are sometimes used in vitro to mimic in vivo conditions. However, use of polymeric additives may result in unwanted precipitation. Compensating the loss of hydrogen bonds specifically could have the same result in a more controllable manner. It is known, that the cpp value of surfactants (Equation 3) is affected by associated small molecule alcohols, also affecting the geometry (solvation energy) of the micellar assemblies. Similarly, suitable additives or protein stabilizers could be considered to stabilize biomolecular assemblies in vitro.

Application step

The application step of hydrophobin fusion design introduces case-sensitive variables to be considered. As has been stated previously, HFBI is an excellent tag for ATPS based processes. Neither the overall hydrophilicity nor the immediate size of the fusion partners evidently prohibit phase partition of the hydrophobin. Development of applications relying on this detergent interaction is thus well grounded. Detergent composition is one of the most important variables in applications utilizing a
two-phase system. The optimal non-ionic detergent should provide aliphatic support for the hydrophobic patches, and at the same time offer sufficient ionic and hydrogen bonds to incorporate the hydrophilic complex. The ATPS systems for hydrophobin fusions could be further enhanced by introducing a pulling force from the detergent phase, for example in the form of mixed micelles or specific interactions to charged amino acid residues. It is also important to ensure that the chosen detergent is tolerated by the fusion partner.

The measured sensitivities of the G-FET sensor demonstrated the suitability of hydrophobin fusions for biofunctionalization in electric devices. The model analytes were selectively measured with high sensitivity. Although the HFBI layer was observed to act as a dielectric in previous experiments, charges could be sensed by the graphene, presumably via holes and cavities in the protein layer. The protein layer is hydrated to some extent, thus allowing mobility of ions to connect the solution environment and graphene surface. Molecular movement near the surface causes displacement of the solution ions and changes in the electrostatic interactions.

The effect of ionic screening by the electric double layer was smaller than was expected. This was assumed to be due to low water confinement and discontinuity of the Debye length (Equation 1) in the biomolecular structures, in accordance with a previous computational study (Stigliano et al. 2013). The phenomenon is closely related to the previously discussed entrapment of water in supramolecular assemblies of amphiphilic molecules (Chandler 2005). Adaptation of hydrophobin fusion technology to environments containing authentic biological samples should be studied further.

Exfoliation and solubilization of carbon nanotubes or graphene by hydrophobin is actually an example of a 3D system composed of a 2D interface. Architectures based on a continuous phase of 2D interfaces ensure the optimal binding interactions of hydrophobins to be employed in 3D systems. In freely associated 3D systems such as the HFBI-ProteinA antibody harvesting method, control over the system is more difficult to achieve. Most importantly, molecule stoichiometry needs to be considered and protein interconnectivity avoided.

The exceptionally large, exposed hydrophobic patch of the hydrophobin HFBI leads to a specific affinity for interfaces. Understanding the mechanisms of hydrophobin function is beneficial for development of hydrophobin fusion technology. However, the detailed mechanism relating the events of surface adsorption and solution behavior are unknown. The design and application of various hydrophobin fusion proteins implied, that certain key factors are associated with molecular behavior. Most importantly, it is the amphiphilic structure, which dictates the final mode of hydrophobin assembly. As a consequence of the interaction of the hydrophobic patches with matching interfaces, critical geometrical restrictions are exerted to the connected fusion partners.

In light of the described energetic and geometrical limitations, it seems plausible, that destabilization of solution multimers of the hydrophobin fusion proteins would lead to an increased concentration of the monomeric form of the fusion protein.
Thus, the fusion protein would actually be more available for adsorption at an available interface. In this view, wild-type hydrophobins and their fusion proteins do not compete equally for interfacial adsorption sites.

Hydrophobin fusion technology shows promise especially in 2D biofunctionalization of interfaces in high-technology applications. To further advance the robustness of the hydrophobin fusion technology, control over surface composition and coverage as well as binding affinity to different solid substrates needs to be mastered. Examination of the design process may help to identify variables, which are significant for the hydrophobin fusion engineering. The findings described in this thesis show, that successful application of hydrophobin fusion technology requires consideration of the final system architecture and molecular environment. Use of more flexible production organisms, smart bioconjugation chemistry, and thorough understanding of related molecular mechanisms and geometrical features at the interface may simplify the design process to create a more functional, iterative practice.
5. Conclusions

In conclusion, the work presented in this thesis demonstrates the design and use of protein functionalities for creation of biomolecular assemblies based on self-assembly of class II hydrophobin HFBI. The focus of the research was to evaluate the functionality of the fusion proteins in liquid environment and solid-liquid interfaces in relevant model applications. The results underline the importance of considering protein architecture and stoichiometry in the design process, while also bringing out holistic aspects in the final application stage.

For the hydrophobin to act in its natural function, the affinity of the hydrophobin monomer to interact with another via the hydrophobic patch must be weaker than its affinity for an interface. The solution equilibrium includes exchange of hydrophobin monomers in the oligomeric assemblies, producing solubilized monomers in the process. However, contributions of the hydrophobic effect, i.e., the enthalpic cost of exposing the hydrophobic patch to water, is the same, regardless of the volume of the hydrophilic end. The hydrophilic parts remain in the aqueous environment throughout the process. Thus, the immediate size of the hydrophilic fusion partner does not disturb the functionality of the hydrophobin. However, available configurations of the hydrophilic parts are restricted in the supramolecular assembly. This causes disturbance to the structure of vicinal water molecules, i.e., increases the entropic element of the solvation free energy of the molecular assembly. Accordingly, simultaneous existence of high affinity multimerization and exposure of the hydrophobic patch may result in an unstable, hardly controllable system, as in the case of the HFBI-ProtA antibody complex.

Geometrical issues to consider relate to the fit of the solid substrate and the hydrophobic patch. This effect becomes prominent in nanotechnological applications, but may also affect the outcome of hydrophobin-stabilized liquid based systems. Hydrophobin-derived fusion proteins most applicable at the solid-liquid interface. Introducing a 2D continuous phase in 3D systems may be beneficial for system stability, as well as lessening the multimerization degree of the fusion partner. In conclusion, reflection of interface geometries, solution dynamics and molecule assembly characteristics is necessary throughout the design process. Final application design is a cross-disciplinary effort, which requires understanding throughout the physicochemical fields.
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Proteins have become a central focus of research in the fields of biotechnology and material development. Nature has created detailed and precise function to these molecules, which can be harnessed to build new materials and applications. The art of protein engineering may be used to join and modify elements in new combinations.

This research focuses on the design and use of fusion proteins of hydrophin protein HFBI. Hydrophobins are small fungal proteins with interfacial function. A central theme throughout this research was to evaluate aspects such as protein component stoichiometry, material geometry and charge effects, as well as holistic factors influencing application design. The results presented in this thesis demonstrate the design and use of protein functionalities for creation of biomolecular assemblies based on the self-assembly of hydrophin HFBI.