Directed Interface Modifications by Genetically Engineered Surface Active Proteins

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'Light up the darkness'

by Bob Marley
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List of Abbreviation

aa Amino acid
AFM Atomic force microscopy
AFAM Atomic force acoustic microscopy
a.u. Arbitrary units
AOB Artificial oil body
BSA Bovine serum albumin
*C. fusiformis* *Cylindrotheca fusiformis*
CA Contact angle measurement
CAC Critical aggregation concentration
Ceg2 Class I hydrophobin of *N. crassa* encoded in clock controlled gene
CdS/ZnS Cadmium sulfide/zinc sulfide
CdSe Cadmium selenide
CMC Critical micelle concentration
CTAB Cetyltriammonium bromide
ddT2O Double distilled water
DLS Dynamic light scattering
E Fluorescence quenching efficiency
EAS Easy wettable
EGFP Enhanced green fluorescent protein
EOD Excitable oil droplet
FFT Fast FOURIER transformation
FRET Fluorescence resonance energy transfer
*G. stearothermophilus* *Geobacillus stearothermophilus*
GLuc Luciferase of *Gaussia princeps*
HA Epitope tag of the human influenza hemagglutinin protein
HFB Class II hydrophobin of *T. reesei*
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>KD</td>
<td>KYTE-DOOLITTLE hydrophobicity index</td>
</tr>
<tr>
<td>MCD</td>
<td>Methyl-β-cyclodextrin</td>
</tr>
<tr>
<td>N. crassa</td>
<td>Neurospora crassa</td>
</tr>
<tr>
<td>Ni-IDA</td>
<td>Iminodiacetic acid; modified agarose for nickel chelating</td>
</tr>
<tr>
<td>OD&lt;sub&gt;600&lt;/sub&gt;</td>
<td>Optical density at 600 nm</td>
</tr>
<tr>
<td>ODE</td>
<td>1-octadecene</td>
</tr>
<tr>
<td>PCL</td>
<td>Poly(ε-caprolactame)</td>
</tr>
<tr>
<td>PDMS</td>
<td>Polydimethylsiloxane</td>
</tr>
<tr>
<td>pH</td>
<td>Potential hydrogen (pondus hydrogenii)</td>
</tr>
<tr>
<td>pI</td>
<td>Isoelectric point</td>
</tr>
<tr>
<td>QDs</td>
<td>Quantum dots</td>
</tr>
<tr>
<td>R5P</td>
<td>Repetitive subunit of the silaffin protein from C. fusiformis</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>S. ureae</td>
<td>Sporosarcina ureae</td>
</tr>
<tr>
<td>S-layer</td>
<td>Surface layer</td>
</tr>
<tr>
<td>SASA</td>
<td>Solvent accessible surface area</td>
</tr>
<tr>
<td>SCWP</td>
<td>Secondary cell wall polymers</td>
</tr>
<tr>
<td>sd</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>T. reesei</td>
<td>Trichoderma reesei</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>tRFP</td>
<td>Turbo red fluorescent protein</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-borate-EDTA buffer</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TBS/T</td>
<td>Tris-borate-sodium/Tween 20</td>
</tr>
<tr>
<td>TE buffer</td>
<td>Tris-EDTA buffer</td>
</tr>
<tr>
<td>TOPO</td>
<td>Triocetylphosphine oxide</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>X-ray</td>
<td>RÖNTGEN radiation</td>
</tr>
<tr>
<td>XSR5P</td>
<td>N- and C-terminal truncated version of the R5P</td>
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Abstract

This work was performed in the framework of an interdisciplinary graduate program that focuses on the establishment and extension of innovative compounds for the packaging of electronic systems. Such chemically or biotechnologically tailored compounds can be used for the direct patterning of optically, magnetically or biologically functional structures in nano- and biotechnical products. In order to organize matter at the nanometer scale, imprinting lithography techniques or self-organization processes are appropriate. Fine-tuning of numerous engineering processes requires continuous and high precision monitoring as well as control of diverse parameters. These demands are only partially met by physical or chemical components since they use surrogate parameters, measure off-line, or provide insufficient performances. Biological compounds, in particular protein-based feedback systems, fulfill certain system requirements to a considerable degree.

Hydrophobins and S-layer proteins are surface active proteins, produced by filamentous fungi or bacteria. In nature, these (self-)assembly proteins form highly ordered and robust structures. In addition, their tolerance for different sequence manipulations and chemical modifications allows extensive functionalization of these nanometer-sized proteins. Hence, these surface active proteins can also be fused with other protein domains to create chimera, which retain function of both original proteins. In conclusion, both hydrophobins and S-layer proteins represent a versatile tool in numerous fields of applied biotechnology, medicine or diagnostics. But until now, efficient in vitro operation in molecular designed protein coatings is strongly restricted due to their complex assembly mechanism.

In the first phase of this work, it was demonstrated, that representatives of class I and class II hydrophobins tend to form multilayered structures on solid surfaces. It was found that only two protein orientations seems to be preferentially formed. In the process of assembly, the orientation of the first hydrophobin layer strictly depends on the substrate wettability. Consequently, each of the following hydrophobin layers is inverse oriented to the layer before. This alternating assembly mechanism has to be taken into account, when working with
functionalized hydrophobins, because a hydrophobin-fused functional protein domain is exclusively located on one side of the protein. Due to the densely packed structure of surface active proteins, a fused functional domain, embedded between two hydrophobins is barely available for external reagents. Basically, the simultaneous existence of a broad spectrum of ordered and disordered assembly structures, demonstrated the need of an uniform protein film assembly for applications in fine-diagnostics or biomedicine.

With regard to molecular designed protein coatings, this work further aimed at establishing conditions to develop a method for a ‘layer-by-layer’ assembly of protein chimeras. Based on their amphiphilic character, self-assembly behavior of surface active proteins can be influenced by conventional ionic surfactants. In order to study the effect of surfactants on the composition and morphology of adsorbed protein films, contact angle measurements, nulling ellipsometry, SEM, AFM and AFAM were performed. It was found that the layer thickness of assembled protein films is strictly dependent on the amount of added surfactant. At certain threshold surfactant concentrations, hydrophobins and S-layer proteins assemble in uniform layers, which are as thick as expected for a protein monolayer or a bilayer. Assembled protein films are covered by a smooth surfactant layer, which prevents further protein assembly. AFAM measurements reveal the formation of well defined lattice structures under the coverage of surfactants. Even the removal of the surfactant layer is possible without interfering with protein specific secondary structures. Solvent accessibility and functionality of protein-fused domains was successfully demonstrated. As compared to conventional assembly techniques, this novel protein deposition method offers a possibility for a ‘directed’ protein coating on solid surfaces. In addition, it guarantees broadly ranged homogeneous assembly of protein chimeras on non-planar or even porous surfaces independent of their position.

Finally, a prototype for an interfacial FRET was developed in a close collaboration with the Institute of Physical Chemistry (TUD). This innovative FRET between semiconducting nanoparticles and illuminating protein chimeras takes place across an oil/water interface. Hydrophobins were used to stabilize artificial oil droplets in aqueous solution. These small proteins possess the ability to attach fused functional domains very close to an oil/water interface. When, in addition to this, an optically active nanostructure directly docks to the hydrophobin, the distance of a protein-fused domain and the nanostructure are in the range of the FÖRSTER radius. It was successfully demonstrated that quantum dots and fluorescent proteins fulfill the spectroscopic requirements of such a donor/acceptor pair. The FRET performance of these excitable oil droplets was examined as a ‘proof of concept’. Due to its modular design, this signal amplification setup could be exploited in numerous fields of technical application ranging from quantification of micronutrient to photothermal cancer therapy.
I. Introduction

Self-organization is a synergistically cooperative process whereby several building blocks individually interact by forming complex and highly ordered structures. According to Matutana & Varela (1980) self-organizing systems are characterized by four properties: (1) complexity, (2) autonomy, (3) redundancy and (4) self-reference (Varela et al., 1974; Probst, 1987).

However, the directed formation of ordered structures by self-assembly of several, independent building blocks is hindered by entropy, a measure of disorder or randomness. The term entropy was coined in 1865 by Rudolf Clausius and describes the favored tendency of a process (Clausius, 1865). Unfortunately, by the lack of external compulsions, a highly ordered system never reflects the preferred situation. For this reason, the shards of broken pottery will never jump up the table to form an intact piece.

In biology, self-organization through molecular recognition is normal, and perhaps most prominently seen in DNA duplex formation or folding of proteins (Watson & Crick, 1953; Anfinsen, 1972). Complex intermolecular self-assembly of biomacromolecules was demonstrated by both DNA origami and the formation of protein supercomplexes, for example in the respiratory chain or in the photosystems of photosynthesis (Rothemund, 2006; Schägger & Pfeiffer, 2000; Minagawa, 2011). A further example is the formation of lipid bilayer membranes by phospholipids in almost all living organisms and many viruses. This impermeable barrier hinders uncontrolled ion and protein diffusion into extracellular space (Bangham & Horne, 1964).

Seen in historical terms, copying nature is a real ‘old shoe’. It is known from Greek mythology that even Icarus tried to reach the sky by imitating eagle wings. Unfortunately, the wax of his wings melted when he came too close to the sun. He crashed and died.

Since 1960, the decoding of living nature and its innovative implementation in daily life is called bionics (Lipetz, 1961). The term was deduced from the Latin noun ‘bios’ (unit of life)
and the suffix ‘nic’ (with nature of...). Of course examples are very varied. Especially an enormous increase in energy efficiency can be achieved by a direct transfer of surface structures from ornithology or ichthyology in the fields of aerodynamics and hydrodynamics. The well known lotus effect was deduced from botanics. It serves as nonchemical passivation of surfaces by the formation of regular raised structures (VON BAYER, 2000).

Novel sensor surfaces are structured similarly and consist of repetitive subunits. The complexity of these systems is found in both the homogeneity of the structures applied to an interface as well as the guaranteed accessibility of active centers. Because chemical reactions are insufficiently selective and sensitive, and enzymatic reactions are difficult to imitate, proteins and polypeptides seem to be an even more attractive application alternative. Unfortunately, biomolecular structures are difficult to synthesize and more labile in an extracellular environment, which puts into question the economic and ecological benefit. Because of their ability to form self-assembled structures, fungal hydrophobins and surface layer (S-layer) proteins from bacteria could show a way out of this dilemma. These thermally and chemically stable proteins have a high surface activity, this is why they assemble on surfaces.

The amphiphilic character of hydrophobins enables fungal hyphae to break through the water-air interface, which allows effective spreading of fungi (WÖSTEN, 2001). Besides the protective effect, S-layer proteins also serve as membrane filters, virulence factors and anchoring matrices for extracellular proteins (EGELSEER et al., 1995; WEIGERT & SÁRA, 1996; SEKOT et al., 2011).

The possibility to functionalize these proteins predestinates them for modules of a biosensor. The tendency of these surface active proteins to self-assemble in vitro complicates the directed interface mobilization. For this reason, working with monodisperse protein solutions is essential, but is hindered by aggregation processes. A previous precipitation of larger protein aggregates is the base for all common deposition methods such as Langmuir-Blodgett or Langmuir-Schäfer method and layer-by-layer techniques. This is economically inefficient and partially leads to hindrance in the accessibility of active and functionalized protein domains during the immobilization process.

In this thesis, the assembly of genetically modified representatives of hydrophobins and S-layer proteins has been studied on solid substrates and at water/oil interfaces. The self-assembled films have been characterized by fluorescence analysis, contact angle measurement, ellipsometry, scanning electron microscopy and atomic force microscopy. Especially
1.1.1 S-layer proteins - Bacterial surface layer proteins

High resolution analytical methods were used to determine the homogeneity of surface assembled protein layers. Macroscopic measurement techniques served to evaluate and to quantify the direction of fused protein domains. By the combination of different analytical methods, it was able to deduce a relation between the thickness of observed protein layer and its tag accessibility. This acquired knowledge of protein layer formation helped to develop a novel procedure of directed protein immobilization on solid substrates, while guaranteeing tag accessibility.

1.1 Self-assembly proteins

In principle, a wide variety of protein deposition positions can be assumed, which would result in a great diversity of layer formation. Commonly, two preferred protein orientations are obtained after deposition. A reason is that most surface active proteins consist of a distinct hydrophilic and a hydrophobic protein patch. Depending on the surface properties, only one protein-surface interaction is preferred. Furthermore, in theory, protein-protein interaction supports a directed formation of complete and homogeneous protein layers (self-reference). In this case, the surface characteristics should be equal at every measuring point. Nevertheless, differences in surface properties are detectable, hilly landscapes are observed via high-resolution techniques after in-vitro assembly. The data implies, that not strictly one side is exposed to the medium.

The assembly behavior of surface active protein species is determined by the amino acid sequence of the polypeptide chains and consequently their tertiary structures. By the formation of self-assembled protein structures in solution, the deposition of such protein agglomerates leads to a defective ordering at the interphase. That is why protein solutions containing almost exclusively monomers are preferred. Unfortunately, the protein specific critical aggregation concentration (CAC) depends on several environmental parameters such as temperature, pH value, ionic composition and/or ionic strength (Pum et al., 1999). The orientation of the recrystallized protein structures is also influenced by the physicochemical properties of the substrate. In most cases, moderately hydrophilic/polar or hydrophobic/nonpolar interfaces are not sufficient to impose homogeneous layer formation on proteins. In order to support a defined protein orientation, specific substrates or modification of the
substrate surface are necessary to direct the relative monomer orientation. For example S-layer proteins specifically anchor to hydrophilic secondary cell wall polymers (SCWP) or poly-L-lysine coated substrates (SÁRA et al., 1998). Similarly, hydrophobins prefer immobilization on distinctly hydrophobic surfaces, for example Teflon®, Nafion®, Kevlar® and PDMS, or on highly hydrophilic surfaces, like glass or mica (LUMSDON et al., 2005; QIN et al. 2007).

1.1.1 S-layer proteins - Bacterial surface layer proteins

Ultrastructural analyses in combination with chemical and genetic studies have revealed that prokaryotic organisms have developed a broad spectrum of cell-envelope structures for adaption to specific ecological and environmental conditions (SLEYTR & BEVERIDGE, 1999). One of the nearly universal surface structures on archaea and bacteria are monomolecular crystalline arrays of proteinaceous surface layers (SLEYTR, 1978; SLEYTR et al., 1993). They cover the cell surface during all stages of cell growth and division. S-layers are isoporous lattices, which are formed by self-assembly of single protein or glycoprotein subunits. The S-layer lattices can have oblique (p1, p2), square (p4), or hexagonal (p3, p6) symmetries (Figure 1.1). Hexagonal symmetry is predominant among archaea (SÁRA & SLEYTR, 2000). Few homologies in the primary structure of S-layer proteins implies multiple evolutionary parallel developmental path of this abundant cell surface components (SÁRA & SLEYTR, 2000).

![Figure 1.1: Symmetries of S-layer lattices.](image)

Figure 1.1: Symmetries of S-layer lattices. Schematic drawings of different S-layer lattice types are presented on the left side. Three-dimensional reconstruction obtained by atomic force microscopy imaging of an squared S-layer lattice, consisting of SbpA from *Bacillus sphaericus* CCM 2177, right side.
Despite the pronounced diversity in their primary structure, S-layer proteins of different bacteria show uniform physicochemical characteristics. It was shown that similarities in the surface net charge and in the hydrophilicity of different protein compartments are responsible for the proper orientation and incorporation of the S-layer subunits. Amino acid analysis revealed that S-layer proteins are also rather similar in their overall composition (SÁRA & SLÉYTR, 1996; BEVERIDGE, 1994; SLÉYTR & MESSNER, 1983). Typically, S-layer proteins possess a high proportion of acidic and 40-60% hydrophobic amino acids. The presence of cysteine in thermophilic and hyperthermophilic S-layer proteins of archaea increases protein stability by forming disulfide bonds and allows posttranslational modifications (CLAUS et al., 2005). In general, the outer surface of S-layers is more hydrophobic and charge neutral, whereas the inner surface is negatively net charged. Contrary to the S-layer side faced toward the cell surface, the S-layer side faced toward the environment reveals a smoother and more regular structure (SLÉYTR et al., 2001).

In contrast to many archaeal S-layer proteins, those of bacteria are noncovalently linked to each other and to the supporting cell-wall components. By sequence comparison, S-layer-homology (SLH) motifs have been identified at the N-terminal part of many S-layer proteins (LUPAS et al., 1994). It is evident that SLH-like motifs in S-layer proteins serve as anchoring structures to SCWP (KUEN et al., 1996; SÁRA et al., 1998; SÁRA, 2001).

1.1.1.1 SbsC - an oblique S-layer from *Geobacillus stearothermophilus*

From *Geobacillus* (*G.*) *stearothermophilus*, a wide variety of S-layers could be identified, all of which are quite different in morphology and crystalline structure to each other. For example, the genome of the wild type strain *G. stearothermophilus* PV72 encodes for two S-layer proteins, which differ considerably in their primary protein structure (KUEN et al., 1996). Furthermore, SbsA forms defined hexagonally ordered lattice structures, whereas SbsB assembles into an oblique lattice type symmetry (KUEN et al., 1996; SÁRA et al., 1996). Another S-layer protein was isolated from the *Bacillus* strain ATCC 12980. The gene *sbsC* encodes for a protein of 1099 amino acids with a molecular weight of 115.41 kDa (JAROSCH et al., 2000). Unlike the S-layer protein encoding genes *sbsA* and *sbsB*, the third identified S-layer gene *sbsC* from the strain *G. stearothermophilus* ATCC 12980 could be successfully expressed in *E. coli* without encoding a signal sequence or transcription terminator.
As determined by computer image reconstruction, the self-assembly products of the heterologously expressed *sbsC* exhibited the formation of a regular two-dimensional lattice structure with an oblique p2 unit cell (\(a = 11.6 \text{ nm}, b = 7.2 \text{ nm} \text{ and } \gamma = 81^\circ\)) (JAROSCH et al., 2001). Comparison of the sequences of SbsC and SbsA has revealed that their N-terminal regions (amino acids 31-270) are 85% identical (JAROSCH et al., 2000). Affinity studies have demonstrated that the N-terminal regions merely recognize an identical type of SCWP, mediating the attachment of S-layers to the underlying peptidoglycan meshwork (EGELSEER et al., 1998; JAROSCH et al., 2000). A site-specific deletion of this region does not interfere with the self-assembly process. By the generation of various C-terminally truncated SbsC versions, it could be demonstrated that the last 179 amino acids are not required for the formation of the lattice structure. Based on the study results, the protein amino acid sequence can be divided into three different functional domains, but only the central protein domain contains all information for self-assembling in a regular lattice structure.

Neither partial deletion nor fusion of the C-terminally truncated SbsC with other functional protein domains interfere with the self-assembly process and the formation of an oblique lattice structure. Activity studies of a recombinant SbsC fusion protein comprising a C-terminal birch pollen allergen (Bet v1) demonstrate maintenance of the ability to self-assemble into oblique lattice structures (BREITWIESER et al., 2002). Immunoblotting analyses confirmed the accessibility of antibodies against the allergen moiety to self-assembled products on the outer surface of the S-layer lattice (BREITWIESER et al., 2002; GERSTMAYR et al., 2007). Furthermore, in vivo localization analysis of the mature S-layer proteins was realized by a C-terminal fusion with a green fluorescent protein (eGFP). Expression of the fusion constructs in *Saccharomyces cerevisiae* and HeLa-cells leads to the formation of tubular assemblies in the cytosol and in vitro (BLECHA et al., 2005; KORKMAZ et al., 2010; KORKMAZ et al., 2011).

### 1.1.1.2 S13240 from *Geobacillus stearothermophilus*

The open reading frame *s13240* consists of 3210 bp, which encodes for a 1069 amino acid embracing S-layer protein of another strain from *G. stearothermophilus*. The S-layer protein with a theoretical molecular mass of around 113 kDa was first described by BLECHA (2005) from the strain DSM 13240. A protein sequence based alignment between the primary structure of SbsC and S13240 shows 95.6% identity in the N-terminal region (amino acid 1-
In contrast, no significant sequence homologies are recognizable in the C-terminal region.

The morphology of the self-assembly products formed by S13240 subunits depend significantly on the pH value and medium composition. Dialyzing against alkaline solutions leads to the formation of typical S-layer structures. However, only tubular structures with a diameter of around 350 nm could be observed by treatment with acidic solutions or 50% ethanol. TEM images of S13240 assemblies imply formation of an oblique lattice symmetry with a periodicity of around 14 nm (BLECHA, 2005).

Similar to previous studies of sbsA and sbsB, a stable cloning of the s13240 gene in E. coli was only possible with its authentic signal sequence and its own transcriptional terminator. Heterologous gene expression in E. coli leads to a protein accumulation in the insoluble protein fraction. The signal peptide appears to significantly influence neither the S-layer formation nor the lattice symmetry of the assemblies.

1.1.1.3 SslA - a squared S-layer from Sporosarcina ureae

The cell wall structure of Sporosarcina (S.) ureae strain ATCC 13881 was first described as periodic ordered structure in 1969 by HOLT and LEDEBETTER. Ten years later, electron microscopic images of the outer cell wall layers of S. ureae showed formation of periodic square type symmetry with a lattice constant of 12 nm (BEVERIDGE, 1979). The abundant surface protein was isolated and characterized on the biomolecular level. It was named SslA. The 3D reconstruction of the S-layer monomer via high resolution electron microscopy was based on an apparent molecular weight of 115 kDa (ENGELHARDT et al., 1986). Because recent studies have shown that the reassembled S-layer lattices of S. ureae represents an excellent biotemplate for metal nanocluster formation, the SslA encoding gene (sslA) was isolated and sequenced (MERTIG et al., 1999; RYZHKOV et al., 2007a). Detailed research of the 3294 bp comprehensive reading frame revealed the primary sequence of a precusor protein with an exact molecular weight of 116.25 kDa. The secreting signal of the protein includes the first 31 amino acids, which are split off after protein secretion to the cell surface. Based on sequence analysis between SslA, SbsC (G. stearothermophilus) and SbpA (B. sphaericus), truncated SslA-variations were generated and heterologously expressed in E. coli. After protein isolation, they still showed in vitro reorganization activity (RYZHKOV et al., 2007b).
1.1.2 Hydrophobins - Fungal surface active proteins

The presence of evident tubular like structures on fungal surfaces was first observed in 1967 on the conidia of *Penicillium megasporum* many years before hydrophobins were first described (Remsen et al., 1967). In 1990, a family of homologous genes was identified in *Schizophyllum commune* (Schuren & Wessels, 1990). Because the genes encoded proteins are characterized by quite hydrophobic amino acid patterns, they were called hydrophobins (Wessels et al., 1991). Stringer et al. were the first to verify that these small proteins are the principal monomeric building blocks for the fibrillar rodlet structures in *Aspergillus* (Stringer et al., 1991). Hydrophobins are proteins that occur uniquely in mycelial fungi. Until now, these proteins have been isolated from Ascomycetes and Basidomycetes, which are representatives of the higher fungi (Eumycetes) (Wessels, 1997). As we now know, hydrophobins are secreted to fulfill a broad spectrum of functions in fungal development, by lowering the high interfacial tension of water (Wösten, 2001).

The hydrophobin open reading frame encodes the mature hydrophobin and a short N-terminal signal peptide sequence. As in other eukaryotes extracellular secretion of proteins in filamentous fungi includes a multi-process secretory pathway. (i) Translation of the protein sequence first occurs and is translocated into the endoplasmatic reticulum (ER). (ii) Quality control happens in the ER, which involves the combined action of chaperones and foldases (e.g. protein disulfide isomerase). After (iii) transport to the Golgi equivalent complex where glycosylation and processing occur, (iv) hydrophobins are transported to the plasma membrane. In both of the transport steps, the hydrophobin is packaged inside membrane vesicles coated by specific proteins (Saloheimo & Pakula, 2012). For hydrophobin secretion, vesicles dock and fuse with the plasma membrane and release their content to the outer membrane space (Spang, 2008). Finally, secreted hydrophobins rapidly assemble with the mycelial cell wall (McCabe & Van Alfen, 1999).

Hydrophobins fulfill a broad spectrum of functions in fungal growth and development. For instance, they are involved in the formation of hydrophobic aerial structures (e.g. aerial hyphae and fruit bodies) (Wessels, 1996, 1997; Wösten & Wessels, 1997; Wösten et al., 2000). Furthermore, unbound hydrophobins reduce the water surface tension, which mediates attachment of fungal hyphae to hydrophobic interfaces (Wösten et al., 2000). Disruption of hydrophobin genes via site-directed mutagenesis reduces the formation of aerial hyphae and changes the wettability (Van Wetter et al., 1996; Askolin et al., 2005). Finally, it was
shown, that hydrophobins directly influence cell wall composition by direct matrix integration (WÖSTEN, 2001).

Hydrophobins not only allow fungi to escape their aqueous environment or mediate attachment of hyphae to hydrophobic surfaces but also function as protective structures against adverse environmental conditions (WÖSTEN et al., 1994). Furthermore, hydrophobins appear to be involved in fungal pathogenicity, whereby they act as toxins and/or attach fungal structures to the host surface (WÖSTEN, 2001).

1.1.2.1 Structure, properties and classification

With sizes of around 100 amino acids, hydrophobins are relatively small, surface active proteins, which are produced and secreted by a variety of filamentous fungi. The primary amino acid sequences between different hydrophobins show few similarities. However, they are characterized by a typical hydropathy pattern and a conserved pattern of eight cysteine residues, which pair to create four intramolecular disulfide bridges, in an apparently conserved manner (SCHUREN & WESSELS, 1990; DE VRIES et al., 1993). As an effect, hydrophobins are extremely resistant against harsh chemical and enzymatic conditions (WÖSTEN, 2001).

Because of their amphiphilic character, hydrophobins are capable to self-assemble at interfaces, whereas they convert hydrophobic surfaces into hydrophilic and vice versa. Based on their hydropathy patterns and solubility characteristics, hydrophobins were divided into two classes (WESSELS, 1994). Both hydrophobin classes can also be distinguished on the basis of their characteristic spacing between the cysteine residues (WÖSTEN & WESSELS, 1997). Analysis of their properties has shown that the division is useful since it reflects some clear functional differences. Both classes form different types of supramolecular assemblies. The class II assemblages can be dissociate more readily in e.g. 60% ethanol or 2% SDS (WESSELS, 1997; WÖSTEN, 2001). In contrast, class I hydrophobins form stable nanometer sized rodlet assemblages dissociable only in reagents like TFA and formic acid (WESSELS et al., 1991; DE VRIES et al., 1993). Based on reactions with the Congo Red stain some insoluble assemblages of class I members show similarities to amyloid fibrils (MACKAY et al., 2001).

Class II hydrophobins represent a uniform group in the phylogenetic tree. They have only been observed in Ascomycets, whereas class I hydrophobins are present in both Basidomycetes and Ascomycetes (LINDER et al., 2005). That is why WHITEFORD and SPANU speculated
that class II hydrophobins would have evolved independently of the class I hydrophobins (Whiteford & Spanu, 2002).

1.1.2.2 Ccg2 - class I hydrophobin from Neurospora crassa

In 1976 the eas (easily wettable) gene locus has been genetically mapped in Neurospora (N.) crassa, when site-directed gene mutation changed the wettability of its asexual spores. In contrast to the wild-type conidia (asexual spores), which are hydrophobic, the absence of parallel bundles of roldets leads to an increase in their surface wettability and a less efficient dispersal by air (Selitrennikoff, 1976; Beever & Dempsey, 1978).

The expression of the eas gene is controlled by a promoter region known as the activating clock element, and is also induced by light (Bell-Pederson et al., 1992). This transcriptionally activation by the circadian clock and blue light led to the identification of the same locus also referred to as ceg-2 (clock-controlled gene) and bli-7 (blue light inducible) (Bell-Pederson et al., 1992; Lauter et al., 1992).

Sequence analysis and biophysical characterization of the gene product revealed that EAS is a member of class I hyrdrophobins (Mackey et al., 2001).

The three-dimensional structure of EAS illustrates that the monomer forms a β-barrel structure with two disordered loops (Figure 1.2). It could be shown that the large unstructured loop is extremely hydrophobic, but deletions within this region have no effect on the folding or structure of the EAS monomer (Mackay et al., 2001). Recently, site-directed mutagenesis and peptide inhibition assay revealed that a short peptide sequence (F72-N76) located between cysteines 7 and 8 is the driving force for intermolecular association and roldlet formation (MacIndoe et al., 2012). It is assumed that during roldlet assembly a cross-β amyloid structure arise through a conformational change in this region. If this amyloidogenic region is transplanted into a class II hydrophobin, the monomers are able to form amloid-like roldlet structures (MacIndoe et al., 2012).

The model for the EAS hydrophobin roldlet structure shows that the monomers are arranged antiparallel to minimize steric interference. The side chains of the short peptide sequence are aligned and stack very well across adjacent monomers allowing the formation of interstrand hydrogen bonds (MacIndoe et al., 2012). The calculated thickness of such an EAS fibril is in
(accordance with the AFM determined layer thickness of around 2.5 nm (MACINDOE et al., 2012; GRUNER et al., 2012).

**Figure 1.2: Solution structure of Ccg2 before assembly into tubular-like structures.** Ribbon diagram of class I hydrophobin Ccg2 from *N. crassa* reveals two disordered loops (left). The small disordered loop is essential for rodlet formation, which was first observed on conidia using electron microscopy (right).

### 1.1.2.3 HFBI - class II hydrophobin from *Trichoderma reesei*

*Trichoderma* (*T.*) *reesei* is a cellulolytic filamentous fungus, which degrades plant material in its natural environment. The efficient secretion of cellulose- and hemicellulose-degrading enzymes, and the cheap and easy cultivation of *T. reesei* makes it a useful organism for a variety of industrial applications. The genome sequence of *T. reesei* contains six genes (*hfb1-6*) encoding class II hydrophobins (NAKARI, et al., 1993; NAKARI-SETÄLÄ et al., 1996, 1997; PENTTILÄ et al., 2000; RINTALA, 2001; NEUHOF et al., 2007). Although there are several class II hydrophobin genes present, their expression pattern strictly depends on different physiological conditions such as light, carbon starvation and mechanical stress (MIKUS et al., 2009).

Until now, three major class II hydrophobins (HFBI, HFBII and HFBIII) have been characterized at molecular level. HFBI and HFBII were isolated from cell walls of submerged hyphae and aerial spores, respectively, as well as from the culture medium (NAKARI-SETÄLÄ et al., 1996, 1997). The amino acid similarity between HFBI and HFBII is 69% (NAKARI-SETÄLÄ et al., 1997). As a result, according to protein crystallography, the folded monomers (Fig. 1.3) (HAKANPÄÄ et al., 2004, 2006), as well as their behavior in solution and on solid substrates are very similar. The hydrophobin HFBIII was purified from the mycelium of an
Over-expressing *T. reesei* strain (Linder *et al.*, 2005; Rintala, 2001). At the air/water interface, all three hydrophobins self-assemble into hexagonally ordered crystalline films (Kisko *et al.*, 2007, 2009).

In solution, HFBI forms oligomers in a concentration dependent manner. At protein concentrations less than 50 ng/µL, HFBI is monomeric, whereas the amount of dimers and tetramers increases with higher concentrations. A maximum level of multimerization is attained at approximately 150 ng/µL, where the tetrameric state is found to be predominant (Szilvay *et al.*, 2006).

[Figure 1.3: X-ray crystal structure of the class II hydrophobin HFBI from *T. reesei*. The model shows the conserved amphiphilic ternary structure of HFBI. Hydrophobic amino acid residues form a planar patch (blue), however, polar amino acids represent the main protein corpus (red). After monomer assembly at a hydrophobic surface, the α-helix, which is formed by charged residues is facing the environment (Szilvay *et al.* 2007).]

The authentic *hfb*1-allele contains a 428 bp open reading frame, which is interrupted by two introns (70 bp and 64 bp). It encodes a moderately hydrophobic protein of 97 amino acids, which contains a typical signal sequence for secretion. When the signal peptide is cleaved off, the mature hydrophobin has a length of 75 amino acids, with a molecular mass of around 7.5 kDa (Nakari-Setälä *et al.*, 1995). Recent studies have shown that self-assembly or surface activity of the hydrophobin is not affected by N- or C-terminal modifications. For that reason, HFBI is suitable as an assembling domain for the construction of versatile N- or C-terminal linked, bivalent fusion proteins (Asakawa *et al.*, 2009; Joensuu *et al.*, 2010; Laaksonen *et al.*, 2010; Lahtinen *et al.*, 2008; Linder *et al.*, 2002, 2004).
1.1.3. Application potential of S-layer proteins and hydrophobins

The application potential of surface active proteins generally depends on their bioavailability and the possibility of a simple protein isolation method. Heterologous gene expression is a promising option to generate high amounts of proteins. In contrast to many archaeal S-layer proteins, those of bacteria as well as hydrophobins are non-covalently linked to each other. Moreover, the high resistance of these S-layer proteins and hydrophobins against disrupting solvents or chaotropic agents enables simple protein purification.

Furthermore, the attractiveness of isolated surface active proteins for a broad spectrum of application lies in their intrinsic ability to assume coherent supramolecular lattice structures in suspension, on suitable solid surfaces (e.g. metals and polymers), or at interfaces (e.g. lipid films and liposomes) (SÁRA & SLEYTR, 2000; EGESEER et al., 2008; LUMSDON et al., 2005; VALO et al., 2010). Nevertheless, for many applications in nanotechnology of surface active proteins, external and spatial control of the reassembly is mandatory (PUM et al., 2006). Unfortunately, S-layer proteins with excellent self-assembly properties, cannot be kept in a water-soluble state, and thus, they are not suitable to recrystallize on artificial surfaces (EGESEER et al., 2008).

Many technological applications depend on the ability of isolated surface active proteins to assemble into monomolecular, crystalline lattice structures with a defined arrangement of functional groups and pores in the nanometer dimension. That is why S-layer proteins and hydrophobins can be used as building blocks and templates for a functional mediation of nanostructures at the meso- and macroscopic scale for both life and non-life science applications.

Because S-layers represent highly isoporous protein meshworks with pores of identical size in the 2-6 nm range and uniform morphology, they are suitable for the production of ultrafiltration membranes (SÁRA & SLEYTR, 1987). The possibility to modify carboxyl groups at the surface of S-layers enables synthesis of different charged ultrafiltration membranes with different surface hydrophilicity. Furthermore, defined arrangement of functional groups in surface active proteins allows posttranslational immobilization of macromolecules (SLEYTR et al., 2001; PALOMO et al., 2003, WANG et al., 2010a; ZHANG et al., 2011a). Due to their high metal ion affinity, S-layers support controlled precipitation of minerals (SCHULTZE-LAM et al., 1992; THOMPSON et al., 1990). It has been demonstrated that S-layer lattices may be used as biological templates for binding metallic or semiconducting nanoparticles into perfectly ordered arrays as required in molecular electronics, biocatalysis and nonlinear optics (PUM &
Hydrophobins were also successfully used for deposition of titanium dioxide thin films, which increase biocompatibility of implants, and is necessary for other biomedical applications (Santhiya et al., 2010). Varying the surface hydrophobicity by coating with hydrophobins can also increase biocompatibility and improve cell adhesion (Hou et al., 2009; Janssen et al., 2002). So far, hydrophobins do not cause allergenic reactions, which enable applications as emulsifier in food industry. Stabilization of oil droplets and nanoparticles conceded usage in drug-delivery (Valo et al., 2010; Wessels et al., 1997).

In previous studies, S-layer proteins were recrystallized on positively charged liposomes (Küpcü et al., 1995). Such S-layer-coated liposomes represent simple model systems resembling the architecture of artificial virus envelopes. For that reason, these thermal and mechanical stable liposomes could reveal a broad application potential, particularly in drug-delivery, gene therapy, or sensor technology (Mader et al., 1999, 2000; Sleytr et al., 1999).

Synthesis of genetically engineered fusion proteins based on S-layer proteins (SbpA, SbsA, SbsB, and SbsC) or hydrophobins (HFBI, HFBII) offer further perspectives in medical, bioanalytic or biocatalytic applications. By maintaining the capability of self-assembling, such functionalized chimeric surface active proteins can be used as versatile templates for arranging a variety of functional domains (e.g. enzymes, antibodies, and immunogenes) on the outermost surface of a geometrically well-defined lattice (Breitwieser et al., 2002; Pleschberger et al., 2003; Riedmann et al., 2003; Schäffer et al., 2007; Völlenkle et al., 2004; Linder et al., 2002). Fusion of hydrophobins to proteins of interest allows their purification by aqueous two-phase separation (Linder et al., 2004). Linder et al. (2004) demonstrated that recombinant proteins with a HFBI-tag can be purified with high selectivity and good yield using non-ionic surfactants.
1.2 Illuminating proteins

An illuminating protein is a biological macromolecule, which can be transferred into an excited transition state by interaction with light particles (photons) or electrons. As a result of radiative relaxation into its electronic ground state, the absorbed energy can be released into the environment in form of less energetic photons (fluorescence) or electrons.

In 1817, Pelletier and Caventou first extracted a green illuminating pigment from plants (Pelletier & Caventou, 1817). It turned out that this photoactive pigment serves as a transmitter for light-excited electrons in photochromic systems to synthesize ATP and NADPH. Both molecules can be used for converting carbon dioxide into sugar. The source of electrons varies depending on the organism, but of particular importance for evolution is the splitting of water, which releases oxygen (Vermaas, 2002). Performed by plants, algae and cyanobacteria, the so-called oxygenic photosynthesis is vital for all aerobic life on earth.

Autofluorescence is a natural phenomenon found in certain beetles, bacteria, and marine species. Shimomura et al. first discovered a blue light emitting protein (aequorin) from the hydrozoan jellyfish Aequorea aequorea in 1962 (Shimomura et al., 1962). Evidently, as it turned out, the protein itself just catalyzes a special class of chemiluminescence reactions found in living organisms. In general, the so-called bioluminescence is a form of light typically produced by the oxidation of a photon emitting-compound (luciferin) in conjunction with a catalyzing enzyme (luciferase or photoprotein). In addition, luciferases are able to catalyze the oxidation of reduced flavin mononucleotides and long-chain aliphatic aldehydes in the presence of oxygen to yield blue light. Organisms utilize bioluminescence for a variety of vital functions, which can be divided into four categories: defense, offence, communication, and dispersal (Wilson & Hastings, 1998). The fact that light-emitting systems ranging from bacteria to fish show little homology to each other suggests that they have evolved independently several times, and are important for organisms (Wilson & Hastings, 1998). Depending on their required cofactors, luciferases can be applied for real-time monitoring of intracellular calcium, magnesium or ATP levels (Saran et al., 1995; Nguyen et al., 1988). Furthermore, luciferases are successfully used as genetic reporters for gene expression studies (Tanahashi et al., 1990; Hill et al., 2002).

If there is sufficient overlap between the emission spectrum of the primary emitter and the absorption spectrum of a second chromophore, radiationless energy transfer can take place at very close distances via fluorescence resonance energy transfer (FRET, see below). One
prominent relationship between bioluminescence and fluorescence is that brightly autofluorescent proteins may be colocalized with their bioluminescent counterpart (HADDOCK et al., 2010). Once again, the first example was confirmed as part of further bioluminescence studies of the jellyfish *Aequorea aequorea*. SHIMOMURA et al. isolated a green fluorescent protein (GFP) (SHIMOMURA et al., 1962). This autofluorescent protein acts as energy transfer acceptor for the blue light emitted by aequorin and transduces the energy into a higher wavelength. Because of the tight in vivo association between aequorin and GFP, the bioluminescence from the living organism is green (JOHNSON et al., 1962). The most revolutionary application potential of autofluorescent proteins is in biological research, where they have become powerful tools as markers for studying subcellular organelles or localization of recombinant chimeras in living cells (RIZZUTO et al., 1995; BALLESTREM et al., 1998). By exploiting the FRET effect, fluorescent proteins can also be used to obtain information on the proximity of two proteins within a 10-nm distance.

Figure 1.4: The turbo red fluorescent protein (tRFP). TRFP is a red-shifted fluorescent protein, derived from the *Entacmaea quadricolor*-based GFP using random mutagenesis. The protein, composed of 231 amino acids, adopts the characteristic β-barrel architecture (A) and a central α-helix containing the chromophore (B). The tRFP is a dimeric fluorophore with excitation/emission peaks of 553/574 nm. It is characterized by high pH-stability and a high fluorescent quantum yield of 0.67 (MERZLYAK et al., 2007).
1.3 Quantum dots

Quantum dots (QDs) are optically active nanostructures, which stray far from the laws of ‘school-level’ physics. Due to their small size, they are characterized by distinct extraordinary optoelectronic, mechanical and thermal properties. This effect is known as quantum confinement. Indeed, controlling size and shape of QDs allows for selective design of these properties. Nowadays, QDs can be tuned to emit light of any color and in a precisely defined wavelength. In comparison to conventional organic fluorophores, QDs have many advantages such as high photostability and brightness, as well as resistance to photobleaching (Bera et al., 2010). Because of this robustness, QDs can be applied in various aspects of our everyday life. They are mainly applied in electro-optic devices, such as lasers, QD-based light emitting diodes (QLEDs) and photovoltaics (Klimov et al., 2000; Coe-Sullivan, 2009; Cui et al., 2006; Dayal et al., 2010). Recent studies promise great potential for the application in new ultra-efficient solar cells (Shao & Balandin, 2007).

Despite the advantages, conventional QDs are characterized by low water and oxygen stability. However, as an example, bioimaging applications require high-quality water-soluble QDs (Yu et al., 2006). Furthermore, most QDs contain cytotoxic ions (e.g., cadmium, selenium and tellurium) (Bera et al., 2010). To increase biocompatibility of QDs, an appropriate water-resistant coating, such as functionalized organic ligands or amorphous silica layers, are necessary (Yu et al., 2006; Yang et al., 2006; Mazumder et al., 2009). Additionally, for better passivation and to compensate for surface defects, QDs can be surrounded by a thin shell of inorganic material (Peng et al., 1997; Bera et al., 2010). However, because of their large surface to volume ratio surface modifications have significant effects on optical absorption, quantum efficiency, luminescence intensity, spectrum and aging effects (Bera et al., 2010). For that reason, shell materials should have a lattice parameter within 12% of the core to encourage epitaxy and minimize strain (Bera et al., 2010).

Since their first appearance in biological contexts (Bruchez Jr. et al., 1998; Chan & Nie, 1998), QDs have found widespread use in a myriad of biosensing applications including immunoassays, nucleic acid detection, resonance energy transfer studies, clinical/diagnostic assays, and cellular labeling (Giepmans et al., 2006; Mazumder et al., 2009).
## 1.4 Energy transfer processes

Electronic energy can be transferred from one excited ‘donor’ molecule to another unexcited ‘acceptor’ molecule in several ways. At the end of these processes the donor is in its ground state, and the acceptor is in the excited state. One way is ‘trivial’; in a radiative process, a primary excited fluorophore re-emits light, which is simply absorbed by a second accessory chromophore (Wilson & Hastings, 1998). In contrast, radiationless energy transfer can happen by one of two processes. When the distance between energy donor and acceptor is in the range of 6-20 Å, an electron can jump (tunnel) between both chromophores. This so-called Dexter energy transfer is known as through-bond energy transfer based on a double electron exchange mechanism between donor and acceptor (Dexter, 1953).

Alternatively, if there is sufficient spectral overlap between the fluorescence spectrum of the primary emitter and the absorption spectrum of a second chromophore, a long-range coupling of the donor and acceptor dipoles can take place. The transfer efficiency is an inverse function of $r^6$, where $r$ is the distance between donor and acceptor, and strongly depends on their mutual orientation ( Förster et al., 1948). FRET, also called through-space energy transfer is favored when donor and acceptor are held in rigidly good alignment of 10-100 Å. That is why the established applicability of FRET for nanotechnology is primarily in the use of fluorophores (atoms, molecules, semiconducting particles, proteins, etc.) as an ‘optical nanometer measure’.

The light emitted from a bioluminescent protein can also be exploited for FRET to an appropriate acceptor. This process, known as bioluminescence resonance energy transfer (BRET), is similarly efficient for donor-acceptor separation distances from 10-100 Å (Sapsford et al., 2006). The principal advantage offered by BRET is that no excitation light source is required to excite the donor. Due to this fact, interferences can be reduced such as from light scattering, high background noise, and direct acceptor excitation. Additionally, the possibility that donor and acceptor can be co-expressed as fusion-proteins and the excitation follows a localized event allowing target specific excitation, which is especially important for applications in vivo (Boute et al., 2006)
1.5 Aim of work

The complex mechanism of \textit{in vivo} self-organization is still not completely understood. But, its outstanding role in diverse natural phenomena provides evidence for their enormous capability. For example, spontaneous folding of biomacromolecules and the formation of membranes are fundamental requirements for the existence of life. More difficult is the arrangement of multienzyme complexes, which are essential for key reactions that produce energy or biomass. Finally, communication or motility of organisms also requires cooperation of various building blocks. With respect to an improved performance in technical applications it could be helpful to transfer these precise systems into technical applications.

One quite recently discovered group of self-organizing structures are surface active proteins, which seem to be widespread in nature. These proteins fulfill a variety of tasks in growth, development, communication or protection of organisms. For this reason, the work was aimed to characterize the surface assembly behavior of fungal hydrophobins and bacterial S-layer proteins, two subfamilies of surface active proteins. Representatives of class I and class II hydrophobins (Ccg2, HFBI) should be heterologously produced in \( E. \text{ coli} \), isolated and subsequently assembled on solid surfaces. Assembled protein films shall be investigated using contact angle measurement, nulling ellipsometry, scanning electron microscopy and atomic force microscopy. Based on these results, an effective and reproducible method for an ordered assembly of uniform protein layers should be developed. Considering technical applications, usage of fusion proteins is favored. Therefore, several protein chimeras were generated, which consist of a surface active protein domain as well as of a functional domain. Beside hydrophobins, bacterial S-layer proteins (S13240, SbsC, SslA) were also used as a surface active protein domain. Functional domains were antigens (HA-tag, His-tag), an enzymatic domain (luciferase) and fluorescent proteins (eGFP, tRFP). Accessibility of fused peptide domains was verified by fluorescence microscopy and microplate reader-based spectrophotometry.

In chemistry, organic solvents were used to stabilize QDs, which exhibit great application potential due to their optoelectronic properties. Unfortunately, these properties suffer, if QDs were transferred into aqueous solutions. Hydrophobins are distinct small proteins that offer the opportunity to attach fused protein domains close to an oil/water interface. In this context, hydrophobins might act as a mediator to connect QDs, still embedded in the oil phase with hydrophobin-fused domains in aqueous solution. As a ‘proof of concept’, it should be examined, whether illuminating protein domains are able to interact with oil-embedded QDs (CdSe, CdS/ZnS) via an energy transfer.
II. Materials and Methods

2.1 Laboratory equipment

Table 2.1: Special laboratory equipment used in this work.

<table>
<thead>
<tr>
<th>Description</th>
<th>Name</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electroporation system</td>
<td>Gene Pulser II</td>
<td>BIO-RAD LABORATORIES GMBH (Munich, Germany)</td>
</tr>
<tr>
<td>Electrotransfer system</td>
<td>PerfectBlue Semi-Dry Electro Blotter Sedec M</td>
<td>PEQLAB BIOTECHNOLOGIE GMBH (Erlangen, Germany)</td>
</tr>
<tr>
<td>Fluorescence microscope</td>
<td>Axio Observer.Z1 Inverted microscope</td>
<td>CARL ZEISS MICROIMAGING GMBH (Jena, Germany)</td>
</tr>
<tr>
<td>Fluorescence microscope</td>
<td>BZ-8100E</td>
<td>KEYENCE DEUTSCHLAND GMBH (Neu-Isenburg, Germany)</td>
</tr>
<tr>
<td>High pressure homogenizer</td>
<td>French® Pressure Cell Press</td>
<td>SIM AMICO SPECTRONIC INSTRUMENTS INC. (Rochester, USA)</td>
</tr>
<tr>
<td>Horizontal electrophoresis system</td>
<td>PerfectBlue Gel System Mini M/Midi S</td>
<td>PEQLAB BIOTECHNOLOGIE GMBH (Erlangen, Germany)</td>
</tr>
<tr>
<td>Microplate reader</td>
<td>Infinite M200</td>
<td>TECAN GROUP LTD. (Männedorf, Switzerland)</td>
</tr>
<tr>
<td>Laboratory pH-meter</td>
<td>pH-Meter 766 Calimatic</td>
<td>KNICK (Berlin, Germany)</td>
</tr>
<tr>
<td>Ultrasonic homogenizer</td>
<td>Bandelin Sonopuls UW 2070</td>
<td>BANDELIN ELECTRONIC GMBH (Berlin, Germany)</td>
</tr>
<tr>
<td>UV Transilluminator</td>
<td>AlphaImager HP Imaging System</td>
<td>BIOZYM SCIENTIFIC GMBH (Hessisch Oldendorf, Germany)</td>
</tr>
<tr>
<td>UV/VIS Spectrophotometer</td>
<td>Nanodrop ND-1000</td>
<td>PEQLAB BIOTECHNOLOGIE GMBH (Erlangen, Germany)</td>
</tr>
<tr>
<td>UV/VIS Spectrophotometer</td>
<td>Ultrospec 3000</td>
<td>PHARMACIA BIOTECH (Munich, Germany)</td>
</tr>
<tr>
<td>Vertical electrophoresis system</td>
<td>PerfectBlue Dual Gel System Twin ExW S</td>
<td>PEQLAB BIOTECHNOLOGIE GMBH (Erlangen, Germany)</td>
</tr>
</tbody>
</table>
2.2. Laboratory materials

2.2.1 Antibodies

Table 2.2: Antibodies with applied dilutions for immunostaining. All antibodies were diluted in TBS-T containing 5% (w/v) non-fat dried milk powder.

<table>
<thead>
<tr>
<th>Antibody Specifity</th>
<th>Dilution</th>
<th>Purchaser</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary antibodies</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-tetra-His-antibody (from mouse IgG)</td>
<td>6xHis-tag</td>
<td>1:2,000</td>
</tr>
<tr>
<td>Mouse-anti-HA</td>
<td>HA-epitope</td>
<td>1:1,000</td>
</tr>
<tr>
<td>Anti-GFP (from mouse IgG)</td>
<td>EGFP, EGFPpest, EGFPuv</td>
<td>1:3,000</td>
</tr>
<tr>
<td>Anti-RFP (from rabbit IgG)</td>
<td>TurboRFP</td>
<td>1:10,000</td>
</tr>
<tr>
<td>Anti-HA, Alexa Fluor® 488 Conjugate</td>
<td>HA-epitope</td>
<td>1:300</td>
</tr>
<tr>
<td>Secondary antibodies</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ECL Mouse IgG, HRP-conjugated whole Ab (from sheep)</td>
<td>Mouse IgG</td>
<td>1:5,000</td>
</tr>
<tr>
<td>ECL Rabbit IgG, HRP-conjugated whole Ab (from donkey)</td>
<td>Rabbit IgG</td>
<td>1:5,000</td>
</tr>
</tbody>
</table>

2.2.2 Chemicals

Laboratory chemicals not listed below were obtained from MERCK KGAA (Darmstadt, Germany), SIGMA–ALDRICH CO. LLC. (Seelze, Germany), CARL ROTH GMBH + CO. KG (Karlsruhe, Germany) or APPLICHEM GMBH (Darmstadt, Germany) in analytical quality. Media and solutions were prepared with distilled water and, if required, either heat sterilized at 121°C for 20 min or filter-sterilized.

Table 2.3: Special and ultra-pure chemicals.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agar</td>
<td>FORMEDIUM (Norfolk, United Kingdom)</td>
</tr>
<tr>
<td>Agarose (low melting point)</td>
<td>BIOZYM SCIENTIFIC GMBH (Hessisch Oldendorf, Germany)</td>
</tr>
</tbody>
</table>
2.2.3 Consumables

Table 2.4: Special consumables used in this work.

<table>
<thead>
<tr>
<th>Description</th>
<th>Specification/Brand</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autoradiography films</td>
<td>Amersham Hyperfilm ECL</td>
<td>GE HEALTHCARE EUROPE GMBH (Munich, Germany)</td>
</tr>
<tr>
<td>Blotting filter paper</td>
<td>Rotilabo, 0.36 mm</td>
<td>CARL ROTH GMBH + CO. KG (Karlsruhe, Germany)</td>
</tr>
<tr>
<td>Dialysis membrane</td>
<td>Millipore V’-series membrane, 0.025 µm, Ø 25 mm</td>
<td>MILLIPORE (Schwalbach/Ts, Germany)</td>
</tr>
<tr>
<td>Electroporation cuvettes</td>
<td>Gap distance 2 mm</td>
<td>BIO-RAD LABORATORIES GMBH (Munich, Germany)</td>
</tr>
<tr>
<td>Injection needles</td>
<td>Sterican Gr. 14, 23G 1/4”, Ø 0.6x30 mm</td>
<td>B. BRAUN MELSUNGEN AG (Melsungen, Germany)</td>
</tr>
<tr>
<td>Substrate</td>
<td>Parafilm® M</td>
<td>HEATHROW SCIENTIFIC LLC (VERMON HILLS, USA)</td>
</tr>
<tr>
<td>PVDF membranes</td>
<td>Immobilon-P 0.45 µm</td>
<td>MILLIPORE (Schwalbach/Ts, Germany)</td>
</tr>
<tr>
<td>96-well microplates, black</td>
<td>BRAND plates pureGrade S #781668</td>
<td>BRAND GMBH + KO. KG (Wertheim, Germany)</td>
</tr>
<tr>
<td>96-well microplates, transparent</td>
<td>#473-810</td>
<td>DR. ILONA SCHUBERT LABORHANDEL (Leipzig, Germany)</td>
</tr>
</tbody>
</table>

2.2.4 Enzymes and size standards

Table 2.5: Enzymes for DNA restriction, amplification or modification, and size standards for DNA and protein electrophoresis. All enzymes were used with purchased buffers and additives due to the supplier’s instruction.

<table>
<thead>
<tr>
<th>Enzyme/size standard</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzymes for Cloning</td>
<td></td>
</tr>
<tr>
<td>Antarctic Phosphatase</td>
<td>NEW ENGLAND BIOLABS GMBH (Frankfurt am</td>
</tr>
</tbody>
</table>
2.2.5 DNA oligonucleotides

DNA oligonucleotides were synthesized by BIOMERS.NET GMBH (Ulm, Germany) or EUROFINS MWG OPERON (Ebersberg, Germany). Synthesized oligonucleotides were dissolved in Tris/HCl buffer (50 mM, pH 7.6) to a stock concentration of 100 pmol µL⁻¹.

Table 2.6: Oligonucleotides for PCR amplification of hydrophobin based fusion constructs. Sequences complementary to the target DNA sequence are written in capital letters. Cleavage sites for restriction enzymes are written in italics.

<table>
<thead>
<tr>
<th>Description</th>
<th>Sequence (5’ → 3’)</th>
<th>Cleavage sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ccg2-L-GLuc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NdeI-Ccg2 for</td>
<td>tagcgacatatgATCGGCCCAACACCTGCTCC</td>
<td>NdeI</td>
</tr>
<tr>
<td>Ccg2-L rev</td>
<td>CGCTGCAAACCTCGTGTGCCggagccccggattgcc</td>
<td>-</td>
</tr>
<tr>
<td>L-GLuc for</td>
<td>ggagccccggattgccgagccccggatcaa</td>
<td>-</td>
</tr>
</tbody>
</table>

Additives for protein purification

<table>
<thead>
<tr>
<th>Additive</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNase II</td>
<td>CARL. ROTH GMBH + CO. KG (Karlsruhe, Germany)</td>
</tr>
<tr>
<td>RNase A</td>
<td>CARL. ROTH GMBH + CO. KG (Karlsruhe, Germany)</td>
</tr>
<tr>
<td>Complete Protease Inhibitor Cocktail Tablets</td>
<td>ROCHE DIAGNOSTICS GMBH (Mannheim, Germany)</td>
</tr>
</tbody>
</table>

Size standards

<table>
<thead>
<tr>
<th>Size Standard</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>GeneRuler™ 100 bp DNA Ladder</td>
<td>FERMENTAS GMBH (St. Leon-Rot, Germany)</td>
</tr>
<tr>
<td>GeneRuler™ 1 kb Plus DNA Ladder</td>
<td>FERMENTAS GMBH (St. Leon-Rot, Germany)</td>
</tr>
<tr>
<td>Lambda (λ) DNA/Pst Marker</td>
<td>FERMENTAS GMBH (St. Leon-Rot, Germany)</td>
</tr>
<tr>
<td>PageRuler™ Plus Prestained Protein Ladder</td>
<td>FERMENTAS GMBH (St. Leon-Rot, Germany)</td>
</tr>
</tbody>
</table>
### Material and Methods

<table>
<thead>
<tr>
<th>GLuc-S-XhoI rev</th>
<th>GGGGCCGTTGGTGAATTaaactcaggtttagctgat</th>
<th>XhoI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ccg2-L-tRFP</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NdeI-Ccg2 for</td>
<td>tagegacatatg ATCGGCCCCACACCCTGCTCC</td>
<td>NdeI</td>
</tr>
<tr>
<td>Ccg2-L rev</td>
<td>CGCTGCAACCTGGTTGCAagagggggggtcggg</td>
<td>-</td>
</tr>
<tr>
<td>L-tRFP for</td>
<td>gaggagggggtgctggaggggggcatca</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>AAGGGCACAGACAGACAGAAGGGGAG</td>
<td></td>
</tr>
<tr>
<td>tRFP-S-XhoI rev</td>
<td>CCTAGCAAAACTGGGCAACAGAataactcaggtttagctgat</td>
<td>XhoI</td>
</tr>
<tr>
<td><strong>HFBI-L-GLuc</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NdeI-HFBI for</td>
<td>tagegacatatg AGCAACGGCAACGGAATGGTTGC</td>
<td>NdeI</td>
</tr>
<tr>
<td>HFBI-L rev</td>
<td>AGACCGCCCGTCGTTGCTgagaggggtgctggg</td>
<td>-</td>
</tr>
<tr>
<td>L-GLuc for</td>
<td>gaggagggggtgctggaggggggcatca</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>AAGGGCACAGACAGACAGAAGGGGAG</td>
<td></td>
</tr>
<tr>
<td>GLuc-S-XhoI rev</td>
<td>GGGGCCGTTGGTGAATTaaactcaggtttagctgat</td>
<td>XhoI</td>
</tr>
<tr>
<td><strong>HFBI-eGFP</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NdeI-HFBI for</td>
<td>tagegacatatg AGCAACGGCAACGGAATGGTTGC</td>
<td>NdeI</td>
</tr>
<tr>
<td>eGFP-S-XhoI rev</td>
<td>GGACGAGCTGTCAAGAataactcaggtttagctgat</td>
<td>XhoI</td>
</tr>
<tr>
<td><strong>HFBI-L-(R5P)2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NdeI-HFBI for</td>
<td>tagegacatatg AGCAACGGCAACGGAATGGTTGC</td>
<td>NdeI</td>
</tr>
<tr>
<td>HFBI-L rev</td>
<td>AGACCGCCCGTCGTTGCTgagaggggtgctggg</td>
<td>-</td>
</tr>
<tr>
<td>Min for</td>
<td>GGAGGCCGTTGGTCTGAGGC</td>
<td>-</td>
</tr>
<tr>
<td>PR5P-S-XhoI rev</td>
<td>GCGCCGTTTCTCATCGCTCtaactcagagcageta</td>
<td>XhoI</td>
</tr>
<tr>
<td><strong>HFBI-L-(XSR5P)2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NdeI-HFBI for</td>
<td>tagegacatatg AGCAACGGCAACGGAATGGTTGC</td>
<td>NdeI</td>
</tr>
<tr>
<td>HFBI-L rev</td>
<td>AGACCGCCCGTCGTTGCTgagaggggtgctggg</td>
<td>-</td>
</tr>
<tr>
<td>Min for</td>
<td>GGAGGCCGTTGGTCTGAGGC</td>
<td>-</td>
</tr>
<tr>
<td>PXSR5P-S-XhoI rev</td>
<td>GGCTCGGGGTCCTCATCGCTCtaactcagagcageta</td>
<td>XhoI</td>
</tr>
<tr>
<td><strong>HFBI-L-tRFP</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NdeI-HFBI for</td>
<td>tagegacatatg AGCAACGGCAACGGAATGGTTGC</td>
<td>NdeI</td>
</tr>
<tr>
<td>HFBI-L rev</td>
<td>AGACCGCCCGTCGTTGCTgagaggggtgctggg</td>
<td>-</td>
</tr>
<tr>
<td>L-tRFP for</td>
<td>gaggagggggtgctggaggggggcatca</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>AAGGGCACAGACAGACAGAAGGGGAG</td>
<td></td>
</tr>
<tr>
<td>tRFP-S-XhoI rev</td>
<td>CCTAGCAAAACTGGGCAACAGAataactcaggtttagctgat</td>
<td>XhoI</td>
</tr>
<tr>
<td><strong>HFBI-L-ZiF</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NdeI-HFBI for</td>
<td>tagegacatatg AGCAACGGCAACGGAATGGTTGC</td>
<td>NdeI</td>
</tr>
</tbody>
</table>
Chapter 2 Material and Methods

HFBI-L rev  AGACGCCGCTCGGTGCTggaggcggtgggtctgg -
ZiF-S-Xhol rev  GCCGCCGTACTACTACTATTATTATTAC  Xhol
Taactegagcacc

<table>
<thead>
<tr>
<th>tRFP-L-HFBI-ArtOLEO</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>NheI-tRFP</em> for</td>
</tr>
<tr>
<td>tRFP-T rev</td>
</tr>
<tr>
<td>L-HFBI for</td>
</tr>
<tr>
<td>HFBI-L rev</td>
</tr>
<tr>
<td>L-ArtOLE for</td>
</tr>
<tr>
<td>ArtOLEO-Xhol rev</td>
</tr>
</tbody>
</table>

Table 2.7: Oligonucleotides for PCR amplification of S-layer protein based fusion constructs. Sequences complementary to the target DNA sequence are written in capital letters. Cleavage sites for restriction enzymes are written in italics.

<table>
<thead>
<tr>
<th>Description</th>
<th>Sequence (5’→3’)</th>
<th>Cleavage sites</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SbsC-L-(R5P)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rSbsC258<em>Ndel</em> for</td>
<td>atatatcatgGCAGCATTGACGCGGAAGG</td>
<td><em>Ndel</em></td>
</tr>
<tr>
<td>kSbsC-L rev</td>
<td>GGTCACAAATT ATATTATGCTGTAggaggcggtggtctg</td>
<td>-</td>
</tr>
<tr>
<td>Min for</td>
<td>GGAGGCGGCTGGGTCCTGGAGGC</td>
<td>-</td>
</tr>
<tr>
<td>PR5P-S-Xhol rev</td>
<td>GCGCCGTTTCCTCATCGCTaacagcagceta</td>
<td>Xhol</td>
</tr>
</tbody>
</table>

| **SbsC-L-(XSR5P)**  |
| rSbsC258*Ndel* for  | atatatcatgGCAGCATTGACGCGGAAGG  | *Ndel* |
| kSbsC-L rev  | GGTCACAAATT ATATTATGCTGTAggaggcggtggtctg  | - |
| Min for  | GGAGGCGGCTGGGTCCTGGAGGC  | - |
| PXSR5P-S-Xhol rev  | GGCTCGGGGTCCTCATCGCTaacagcagceta  | Xhol |

| **SbsC-L-ZiF**  |
| rSbsC258*Ndel* for  | atatatcatgGCAGCATTGACGCGGAAGG  | *Ndel* |
| kSbsC-L rev  | GGTCACAAATT ATATTATGCTGTAggaggcggtggtctg  | - |
| ZiF-S-Xhol rev  | GCCGCCGTACTACTACTATTATTATTAC  | Xhol |
| Taactegagcacc  | - |
Table 2.8: Oligonucleotides for PCR amplification of further constructs used as controls. Sequences complementary to the target DNA sequence are written in capital letters. Cleavage sites for restriction enzymes are written in italics.

<table>
<thead>
<tr>
<th>Description</th>
<th>Sequence (5´→3´)</th>
<th>Cleavage sites</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>tRFP-ArtOLEO in pET28b(+)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nhel-tRFP for</td>
<td>tRFGAGAGCTGATCAAGGAGAACATGC</td>
<td>Nhel</td>
</tr>
<tr>
<td>tRFP-T rev</td>
<td>CCCTAGCAAAACTGGGGCACAGA</td>
<td>-</td>
</tr>
<tr>
<td>L-ArtOLE for</td>
<td>Ggaggcggtgggtggaggcgggaca</td>
<td>-</td>
</tr>
<tr>
<td>ArtOLEO-Xhol rev</td>
<td>GCTGTTGCTGCTTGGTGGGCTGAGGATGC</td>
<td>Xhol</td>
</tr>
<tr>
<td><strong>GLuc in pET28b(+)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ndel-GLuc for</td>
<td>tRFGAGAGCTGATCAAGGAGAACATGC</td>
<td>Ndel</td>
</tr>
<tr>
<td>GLuc-S-Xhol rev</td>
<td>GGGGGCCGGTGTTGACATGCTGAGGATGC</td>
<td>Xhol</td>
</tr>
<tr>
<td><strong>HA-tRFP in pET23b(+)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ndel-HA-tRFP</td>
<td>tRFGAGAGCTGATCAAGGAGAACATGC</td>
<td>Ndel</td>
</tr>
<tr>
<td>tRFP-S-Xhol rev</td>
<td>GCTGTTGCTGCTTGGTGGGCTGAGGATGC</td>
<td>Xhol</td>
</tr>
</tbody>
</table>

2.2.6 Plasmids and vectors

Vector pUC18 (YANISCH-PERRON et al., 1985) was used as cloning vehicle to control the sequence of PCR products. For gene expression, verified DNA sequences need to be cloned into specific expression vectors (pET23b(+), pET28b(+)). Further plasmids given in Table 2.9 were used for gene expression or served as DNA templates for PCR amplification of target reading frames.

Table 2.9: Basic vectors and plasmids. Important features of bacterial vectors assigned for controlled expression, or for PCR-amplification of target sequences are given.

<table>
<thead>
<tr>
<th>Plasmid name</th>
<th>Relevant characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cloning vectors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pET23b(+)</td>
<td>pBR322 origin, T7-Tag, His-Tag, amp</td>
<td>NOVAGEN (Darmstadt, Germany)</td>
</tr>
<tr>
<td>pET28b(+)</td>
<td>pBR322 origin, lacI, T7-Tag, His-Tag,</td>
<td>NOVAGEN (Darmstadt, Germany)</td>
</tr>
<tr>
<td>Plasmid Name</td>
<td>Description</td>
<td>Origin</td>
</tr>
<tr>
<td>------------------------</td>
<td>------------------------------------------------------------------------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td>pUC18</td>
<td><em>kat</em>&lt;sup&gt;+&lt;/sup&gt;, <em>amp</em>&lt;sup&gt;+&lt;/sup&gt;, <em>ColE1</em> origin, <em>lacZ</em>&lt;sup&gt;+&lt;/sup&gt;</td>
<td>FERMENTAS GMBH (St. Leon-Rot, Germany)</td>
</tr>
<tr>
<td>pET17b-HFBIHA</td>
<td>HFBI&lt;sub&gt;(aa23-97)&lt;/sub&gt;, pBR322 origin, T7-Tag, <em>amp</em>&lt;sup&gt;+&lt;/sup&gt;</td>
<td>KURTZ (IfG, 2006)</td>
</tr>
<tr>
<td>pET23b-S13240&lt;sub&gt;(aa1-1069)&lt;/sub&gt;HA</td>
<td>S13240&lt;sub&gt;(aa1-1069)&lt;/sub&gt;, 3xHA-tag, Factor Xa, pBR322 origin, T7-Tag, His-Tag, <em>amp</em>&lt;sup&gt;+&lt;/sup&gt;</td>
<td>GRUNER (IfG, 2011)</td>
</tr>
<tr>
<td>pET28b-Ccg2&lt;sub&gt;(aa30-108)&lt;/sub&gt;HA</td>
<td>Ccg2&lt;sub&gt;(aa30-108)&lt;/sub&gt;, 3xHA-tag, Thrombin, Factor Xa, pBR322 origin, <em>lacI</em>, T7-Tag, His-Tag, kan&lt;sup&gt;+&lt;/sup&gt;</td>
<td>GRUNER (IfG, 2011)</td>
</tr>
<tr>
<td>pET28b-SbsC&lt;sub&gt;(aa258-920)&lt;/sub&gt;HA</td>
<td>SbsC&lt;sub&gt;(aa258-920)&lt;/sub&gt;, 3xHA-tag, Factor Xa, pBR322 origin, <em>lacI</em>, T7-Tag, His-Tag, <em>kat</em>&lt;sup&gt;+&lt;/sup&gt;</td>
<td>GRUNER (IfG, 2011)</td>
</tr>
<tr>
<td>pET28b-SsLA&lt;sub&gt;(aa32-1099)&lt;/sub&gt;HA</td>
<td>SsLA&lt;sub&gt;(aa32-1099)&lt;/sub&gt;, 3xHA-tag, Thrombin, Factor Xa, pBR322 origin, <em>lacI</em>, T7-Tag, His-Tag, <em>kat</em>&lt;sup&gt;+&lt;/sup&gt;</td>
<td>GRUNER (IfG, 2011)</td>
</tr>
<tr>
<td>pKLAC1-GLuc</td>
<td>GLuc, pMB1 origin, *P&lt;sub&gt;LAC4-PBI&lt;/sub&gt;, *P&lt;sub&gt;ADH2&lt;/sub&gt;, α-MF, <em>amds</em>, <em>amp</em>&lt;sup&gt;+&lt;/sup&gt;</td>
<td>NEW ENGLAND BIOLABS GMBH (Frankfurt am Main, Germany)</td>
</tr>
<tr>
<td>pUC57-ArtOLEO</td>
<td>ArtOLEO, pMB1 origin, <em>lacZ</em>, <em>kat</em>&lt;sup&gt;+&lt;/sup&gt;</td>
<td>ATG:BIOSYNTHETICS GMBH (Merzhausen, Germany)</td>
</tr>
</tbody>
</table>
2.3 Microorganisms

2.3.1 *Escherichia coli* strains

*Escherichia coli* (*E. coli*) strain TOP10F’ (*INVITROGEN GmbH; Darmstadt, Germany*) was employed for standard cloning procedures and propagation of plasmids (Tab. 2.10). Other listed *E. coli* strains were used for heterologous gene expression.

Table 2.10: Genotype of *E. coli* strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> BL21 (DE3) pLysS</td>
<td>F', dcm, ompT, hsdS*(r*'* m<em>K2</em>') *, gal, λ(DE3), (pLys cam®)</td>
<td>NOVAGEN (Darmstadt, Germany)</td>
</tr>
<tr>
<td><em>E. coli</em> SHuffle™ T7 lysY</td>
<td>MiniF lysY(cam®)/ Δ(ara-leu)7697 araD139 fluA2 lacZ::T7 gene1 Δ(phoA)PvuI phoR ahpC® galE (or U) galK λatt::pNEB3-r1-cDsbc (Spec®, lacI®) ΔtrxB rpsL150(Str®) Δgor Δ(malF3)</td>
<td>NEW ENGLAND BIOLABS GMBH (Frankfurt am Main, Germany)</td>
</tr>
<tr>
<td><em>E. coli</em> SHuffle™ T7 Express lysY</td>
<td>MiniF lysY(cam®)/ fluA2 lacZ::T7 gene1 [lon] ompT ahpC gal λatt::pNEB3-r1-cDsbc (Spec®, lacI®) ΔtrxB sulA11 R(mcr-73::miniTn10-—Tet®)2 [dcm] R(zgb-210::Tn10—Tet®) endA1 Δgor Δ(mcrCmrr) 114::IS10</td>
<td>NEW ENGLAND BIOLABS GMBH (Frankfurt am Main, Germany)</td>
</tr>
<tr>
<td><em>E. coli</em> TOP10F’</td>
<td>F’ mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu)7697 galU galK rpsL (Str®) endA1 nupG</td>
<td>INVITROGEN GMBH (Darmstadt, Germany)</td>
</tr>
</tbody>
</table>

2.3.2 Cultivation of *Escherichia coli*

Routine growth of *E. coli* cells was carried out in Luria-Bertani (LB) medium (MILLER, 1972) with required antibiotics at 37°C and constant shaking (180 rpm). After transformation of *E. coli* with plasmid DNA, cells were cultivated first in SOC medium (SAMBROOK et al., 1989) at 37°C without shaking for 1 h. Subsequently, cells were cultivated in liquid or onto solid LB-Miller medium containing respective antibiotics at 30°C. For gene expression a MOPS buffered LB-Miller medium (100 mM, pH 7.4) was used.

**LB-Miller medium**

(MILLER, 1972)  
10 g L⁻¹ Tryptone  
5 g L⁻¹ Yeast extract
Chapter 2 Material and Methods

10 g L\(^{-1}\) NaCl
15 g L\(^{-1}\) Agar (solid medium only)

SOC medium
(SAMBROOK et al., 1989)

20 g L\(^{-1}\) Tryptone
5 g L\(^{-1}\) Yeast extract
10 mmol L\(^{-1}\) NaCl
2.5 mmol L\(^{-1}\) KCl
10 mmol L\(^{-1}\) MgCl\(_2\)
10 mmol L\(^{-1}\) MgSO\(_4\)
20 mmol L\(^{-1}\) Glucose

2.3.3 Additives

Concentrated stock solutions of antibiotics and other media additives were prepared and sterilized by filtration (pore width 0.22 \(\mu m\)). Aliquots of stock solutions were stored at -20°C. Concentrations for stock solutions and concentration folds are listed in Table 2.11.

<table>
<thead>
<tr>
<th>Additive</th>
<th>Stock concentration</th>
<th>Concentration fold</th>
<th>Solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>100 mg mL(^{-1})</td>
<td>1000 x</td>
<td>ddH(_2)O</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>17 mg mL(^{-1})</td>
<td>1000 x</td>
<td>96% ethanol</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>30 mg mL(^{-1})</td>
<td>1000 x</td>
<td>ddH(_2)O</td>
</tr>
<tr>
<td>IPTG</td>
<td>1 mol L(^{-1})</td>
<td>1000 x</td>
<td>ddH(_2)O</td>
</tr>
</tbody>
</table>

2.3.4 Storage of *Escherichia coli*

*E. coli* cells were stored on solid LB-Miller medium at 4°C for four weeks in maximum before being replica-plated onto fresh medium. For long-term storage, glycerol stocks were prepared. Cells from 1.5 mL stationary culture were harvested by centrifugation (4,000 x \(g\), RT, 5 min). The supernatant was removed and the cell pellet was resuspended in fresh medium with required antibiotics and 30% (v/v) glycerol. This mixture was stored at -80°C.
2.4 Molecular biology techniques

2.4.1 Isolation of plasmid DNA

Standard extraction of bacterial plasmid DNA from *E. coli* was performed using a modified method of Birnboim and Doly (1979) (Gruner, 2009). For sequencing, small amounts of ultra-pure plasmid DNA were isolated with kit systems from Macherey-Nagel (‘NucleoSpin® Plasmid Kit’) or Invitek (‘Invisorb Spin Plasmid Mini Two’). Plasmid-DNA isolation was performed following the manufacturer’s instructions.

2.4.2 Electrophoretic separation of DNA

Analytical separation and purification of DNA fragments were performed using agarose gel electrophoresis. Depending on the size of DNA fragments, agarose concentrations in 1 x TBE buffered gels ranged between 1 and 2% (w/v). To visualize DNA under UV-light (365 nm), 0.1 \( \mu \text{g mL}^{-1} \) ethidiumbromid or 10 \( \mu \text{L} \) of 4,000 x RedSafe DNA staining solution were added.

Prior to use, the DNA was mixed with DNA loading dye. After sample loading, fragments were separated at a voltage of 100 V. Depending on DNA fragment size, three different ladders were used as size standards according to the manufacturer’s instructions. If required, fragments were excised using a sterile scalpel and purified (Section 2.4.3) for further processing.

\[
10 \times \text{TBE buffer} \quad \begin{align*}
890 \text{ mmol L}^{-1} \text{ Tris base} \\
890 \text{ mmol L}^{-1} \text{ Boric acid} \\
20 \text{ mmol L}^{-1} \text{ EDTA-Na}_2, \text{ pH 8.0}
\end{align*}
\]

\[
6 \times \text{DNA loading dye} \quad \begin{align*}
33\% \ (\text{w/v}) \text{ Glycerin} \\
0.2\% \ (\text{w/v}) \text{ Bromophenol blue} \\
100 \text{ mmol L}^{-1} \text{ EDTA-Na}_2, \text{ pH 8.0}
\end{align*}
\]

2.4.3 DNA purification

To purify DNA after PCR amplification and cleavage with restriction endonucleases, or to extract DNA fragments from agarose slices, the Invisorb® Fragment CleanUp-kit (Stratec Molecular GmbH; Berlin, Germany) was used according to the manufacturer’s instructions.
except for the elution step. Depending on the desired concentration, DNA was eluted with 20-50 µL double-distilled water.

### 2.4.4 Ethanol precipitation

The ethanol precipitation procedure of DNA allows removal of high salt concentrations from DNA or the concentration of diluted DNA solutions. For DNA precipitation six volumes of 98% ethanol, 0.1 volume of 3 M sodium acetate buffer (pH 5.2) and 0.04 volume of 20 mol L\(^{-1}\) glycogen were added to a sample, and incubated at -80°C for 1 h. After centrifugation (16,000 x \(g\), 4°C, 30 min), the pellet was washed twice with 500 µL of 70% ethanol. Remaining ethanol was evaporated in a Concentrator 5301 (EPPENDORF; Hamburg, Germany). In the end, DNA pellet was either dissolved in TE buffer or double-distilled water.

**TE buffer**  
10 mmol L\(^{-1}\) Tris/HCl, pH 8.0  
1 mmol L\(^{-1}\) EDTA

### 2.4.5 DNA amplification

For *in vitro* PCR amplification of DNA fragments targeted for cloning, DNA polymerases with proofreading activity were used in 50 µL reactions. Routine PCR was carried out with 1 unit DyNAzyme EXT DNA Polymerase. GC-rich templates were amplified with 1 unit Phusion High-Fidelity DNA Polymerase, the provided GC buffer and a maximum DMSO concentration of 5% (v/v) in the final reaction volume. Plasmids (Tab. 2.9) or synthesized oligonucleotides served as template-DNA in a final concentration per reaction of about 10 ng. The composition of a typical PCR mixture is given in Table 2.12. Amplification according to the supplier’s cycling instructions featured either 35 cycles with the same temperature during the annealing step or sequentially 10 cycles with a lower and 25 cycles with a higher annealing temperature (Tab. 2.13). The latter strategy was used for overlap extension PCR or in the case of primers with long overhangs.

Diagnostic check-PCR was performed in a 20 µL reaction volume with 1 unit *Taq* DNA Polymerase. Other ingredients and the cycling procedure were applied accordingly to instructions for DyNAzyme EXT DNA Polymerase.
Table 2.12: Composition of standard PCR mixtures. DNA polymerases were applied in an appropriate buffer and with further additives if required.

<table>
<thead>
<tr>
<th>Component</th>
<th>Final concentration/amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA polymerase buffer</td>
<td>1x</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>0.5-2 mmol L⁻¹</td>
</tr>
<tr>
<td>Template DNA</td>
<td>10-100 ng</td>
</tr>
<tr>
<td>Forward primer</td>
<td>0.5 μmol L⁻¹</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>0.5 μmol L⁻¹</td>
</tr>
<tr>
<td>dNTPs</td>
<td>0.2 mmol L⁻¹</td>
</tr>
<tr>
<td>DMSO (optional)</td>
<td>Maximum 5% (v/v)</td>
</tr>
<tr>
<td>DNA polymerase</td>
<td>1 unit</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>Add to 50 μL (20 μL)</td>
</tr>
</tbody>
</table>

Table 2.13: Cycling procedure for PCR amplification of DNA fragments. For each cycle step, temperature and duration are listed. Parameters for denaturation and extension steps depend on polymerase and template properties (see instructions of supplier). $T_{A1}$ and $T_{A2}$ are different annealing temperatures. Alternatively, 35 cycles with the same annealing temperatures were carried out.

<table>
<thead>
<tr>
<th>Cycle step</th>
<th>Temperature</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>94/98°C</td>
<td>2 min</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94/98°C</td>
<td>30 s</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>$T_{A1}$</td>
<td>15 s</td>
<td>10</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>0.5-1 min kb⁻¹</td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>94/98°C</td>
<td>30 s</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>$T_{A2}$</td>
<td>15 s</td>
<td>25</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>0.5-1 min kb⁻¹</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C</td>
<td>10 min</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10°C</td>
<td>Hold</td>
<td></td>
</tr>
</tbody>
</table>

2.4.6 DNA quantification

DNA concentration/purity for sequencing or PCR reactions was determined with a NanoDrop ND-1000 Spectrophotometer using the ‘Nucleic Acid’ application mode. Alternatively, for ligation reactions, the DNA concentration was estimated via agarose gel electrophoresis by comparing the signal intensity of DNA samples with that of size standards.
2.4.7 Enzymatic modification of DNA

2.4.7.1 DNA cleavage

Up to 1 µg of DNA was digested with 2 units of corresponding restriction endonuclase(s). For double digestion with two restriction enzymes, the buffer with the maximum activity for both enzymes was used. The reaction was incubated at recommended temperature (usually 37°C) for at least 3 h. After possible heat-inactivation (usually at 65°C for 20 min), DNA was purified for further processing.

2.4.7.2 Vector dephosphorylation

Restricted vector DNA was optionally dephosphorylated with Antarctic Phosphatase according to the supplier’s instructions prior to purification (Section 2.4.3) for further processing.

2.4.7.3 DNA ligation

Ligation of DNA fragments of cohesive ends and linearized vector-DNA was performed with a threefold molar excess of the insert in total amount of about 200 ng. The volume was adjusted to 10 µL with double-distilled water containing 1x concentrated ligase reaction buffer and 10 units of corresponding T4 DNA Ligase in the total volume. The reaction was incubated over night at 4°C. T4 DNA Ligase was heat-inactivated at 65°C for 20 min.

2.4.8 Transformation of *Escherichia coli*

2.4.8.1 Preparation of electrocompetent cells

An over-night culture was diluted 1:20 in 400 mL fresh LB-Miller medium. Cells were grown to an OD\textsubscript{600} of 0.4-0.5 and chilled on ice for 30 min. After cell harvesting via centrifugation (6,000 x g, 4°C, 10 min), pellet was washed twice with 200 mL ice-cold distilled water and once with 200 mL 10% (v/v) ice-cold glycerol. Finally, the pellet was carefully resuspended in 10% (v/v) glycerol to a final volume of 1-2 mL. Aliquots of 40 µL were stored until use at -78°C (for up to 6 months).
2.4.8.2 Drop dialysis

Before electroporation, a droplet of the DNA solution was placed onto a Millipore ‘V’-series membrane and dialyzed for 45 min against double-distilled water to remove excess salts.

2.4.8.3 Electroporation of *Escherichia coli*

*E. coli* cells were transformed with target DNA by electroporation (Dower *et al.*, 1988). Therefore, an aliquot of electrocompetent cells was mixed with 1-10 ng plasmid-DNA or 5 µL of a dialyzed ligation mixture. Electroporation was applied by a short electrical pulse (25 µF, 200 Ω, 2.5 kV). Then cells were immediately transferred to 1 mL sterile SOC medium and incubated at 37°C for 1 h. After centrifugation (4,000 x g, RT, 1 min), cells were resuspended in 250 µL fresh SOC medium and spread on LB-Miller medium with required antibiotics. Transformants were obtained after incubation at 37°C over night.

2.4.8.4 DNA sequencing

For standard sequencing at Eurofins MWG Operon (Ebersberg, Germany), ultra-pure plasmid DNA (Section 2.4.3) was mixed with sequencing primers following the service’s instruction.
2.5 Microbiological Techniques

2.5.1 Recombinant protein expression in *Escherichia coli*

For heterologous gene expression 20 mL LB-Miller medium supplemented with required antibiotics was inoculated with a single colony containing the expression plasmid. After over-night incubation at 30°C, and shaking at 180 rpm, pre-culture was diluted to an OD$_{600}$ approx 0.1 in 400 mL fresh LB-Miller medium containing antibiotics. When OD$_{600}$ reached 0.4-0.6 target gene expression was induced by adding 0.4 mM IPTG (Cho et al., 1985). Bacteria cells were harvested by centrifugation (4,000 $x$ $g$; 4°C; 10 min) 6 h later. Finally, the pellet was washed twice with 20 mL Tris buffer (pH 7.5). Pellets were stored at -20°C until further use.

<table>
<thead>
<tr>
<th>Tris buffer</th>
<th>50 mmol L$^{-1}$ Tris/HCl, pH 7.5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 x Complete Protease Inhibitor Cocktail Tablet</td>
</tr>
</tbody>
</table>

2.5.2 Determination of cell density

The cell density of liquid cultures was determined by measuring the optical density (OD) at a wavelength of 600 nm. If the OD$_{600}$-value exceeds the upper limit of 0.8, samples were diluted 1:10 in fresh medium. For *E. coli*, an OD$_{600}$ of 1.0 corresponds to approximately 1 x 10$^9$ cells mL$^{-1}$.

2.5.3 Cell disruption

For cell disruption, 100 mg wet weight of the bacterial cell pellet from the expression culture was resuspended in 2 mL Tris buffer (pH 7.5). The suspension was supplemented with 1 $\mu$g mL$^{-1}$ DNase II and 1 $\mu$g mL$^{-1}$ RNase A. For cell-lysis, the mixture was ultrasonicated (75%, 9 cycles, 1 min, 4°C) six times on ice and three times French pressed for cell lysis. After 20 min in a shaker (300 rpm; 37°C) insoluble ingredients were collected by centrifugation (20,000 $x$ $g$; 4°C; 10 min) and washed twice with 20 mL 50 mM Tris buffer (pH 7.5). Each washing step is followed by centrifugation (20,000 $x$ $g$; 4°C; 10 min). For protein extraction, 100 mg of the insoluble pellet was resuspended in 1 mL Lysis buffer and incubated at RT for 30 min. The supernatant was discarded after centrifugation (20,000 $x$ $g$, 4°C; 10 min). The extraction procedure was repeated twice at 37°C for 30 min and the supernatant was collected.
1 M Phosphate buffer (pH 8.0)  
18.2 mL 1 M KH$_2$PO$_4$  
81.8 mL 1 M Na$_2$HPO$_4$

Lysis buffer (pH 8.0)  
100 mmol L$^{-1}$ Phosphate buffer  
10 mmol L$^{-1}$ Tris/HCl  
8 mol L$^{-1}$ Urea

2.5.4 Protein purification by metal-chelate affinity chromatography

The selective purification of 6x Histidin-tagged fusion proteins was carried out by metal-chelate affinity chromatography according to HOCHULI et al. (1987). As described in the manufacturer’s protocol (NOVAGEN; Darmstadt, Germany), a column was filled with 15 mL of a resuspended Ni-IDA sepharose solution (His Bind® Resin, NOVAGEN) stored in 30% isopropanol and flushed with a filter. It was washed with ddH$_2$O to remove the remaining isopropanol. By adding 4 mL of a 1 M nickel sulphate solution, Ni$^{2+}$-ions were immobilized. Subsequently, the column was washed with ddH$_2$O to remove excess Ni$^{2+}$. To equilibrate purification conditions, column was rinsed with Lysis buffer until no more Ni$^{2+}$-ions were detectable as a Ni$^{2+}$-dimethylglyoxime complex (RAMMIKA et al., 2011). The column was loaded with 2 mL of the extraction solution. Afterwards the column was washed with Lysis buffer, followed by Wash buffer A and Wash buffer B. The elution of the target protein was initiated by adding 5 times 5 mL of the Elution buffer. The eluates were separately collected and stored at 4°C. For complete removing of all adsorbed protein, the column was treated with 1% SDS solution. For renewing the sepharose, column was washed with 100 mM EDTA-solution to remove the Ni$^{2+}$ ions followed by doubled distilled water and isopropanol.

Wash buffer A (pH 6.3)  
100 mmol L$^{-1}$ Phosphate buffer  
10 mmol L$^{-1}$ Tris/HCl  
8 mol L$^{-1}$ Urea  
20 mmol L$^{-1}$ Imidazole

Wash buffer B (pH 5.9)  
100 mmol L$^{-1}$ Phosphate buffer  
10 mmol L$^{-1}$ Tris/HCl  
8 mol L$^{-1}$ Urea  
40 mmol L$^{-1}$ Imidazole
2.5.5 Dialysis of protein solutions

2.5.5.1 Preparation of dialysis tubes

Dialysis tubes (about 10-20 cm) were boiled in a solution containing 0.1 M Na$_2$CO$_3$ and 10 mM EDTA (pH 8.0). Afterwards they were rinsed with double-distilled water and boiled again in 1 mM EDTA solution (pH 8.0). Washed tubes were stored in 1 mM EDTA (pH 8.0) at 4°C. Before usage, dialysis tubes were washed under running water.

2.5.5.2 Protein refolding by dialysis

Eluted fractions of purified proteins or precipitated cell fractions (2 mL) were immediately dialyzed against 2 L Refolding buffer over night. During this process, L-arginine suppresses aggregation because of an increased affinity for protein side chains that may be responsible for protein aggregation (TSUMOTO et al., 2004). The addition of the glutathione based redox system supports formation of correct disulfide bonds (HWANG et al., 1992).

Refolding buffer (pH 8.0)  
100 mmol L$^{-1}$ Tris/HCl  
200 mmol L$^{-1}$ L-arginine  
2 mmol L$^{-1}$ EDTA-Na$_2$  
1 mmol L$^{-1}$ Glutathione reduced  
0.2 mmol L$^{-1}$ Glutathione oxidized

2.5.6 Determination of protein concentration

Rough estimation of protein concentrations in cell-free solutions based on the determination method of WHITAKER & GRANUM (1980). Therefore, the absorbance at 235 and 280 nm was measured. Protein concentration was calculated by equation (2.1).

$$\text{Protein [mg mL}^{-1}] = \frac{E_{235nm} - E_{280nm}}{2.51}$$  \hspace{1cm} (2.1)
For accurate determinations of the protein concentration a Lowry et al. (1951) based DC-Protein Assay (BIO-RAD LABORATORIES GMBH, Munich, Germany) was carried out according to the manufacturer’s instructions. BSA was used to generate the reference curve.
2.6 Western Blot analysis

2.6.1 Methanol/chloroform precipitation

This protein precipitation method described by WESSEL et al. (1984) is suitable to remove detergents or salt from diluted protein solutions. A 150 µL protein solution was mixed with 4 volumes of methanol and 3 volumes of chloroform. For a better phase separation 3 volumes of double-distilled water were added, and the solution was mixed vigorously. After centrifugation (18,000 x g, RT, 5 min) the upper aqueous phase was removed, and the chloroform phase was supplemented with 3 volumes of methanol. The sample was mixed again, and the denatured protein was sedimented (18,000 x g, RT, 5 min). The liquid supernatant was discarded, and the protein residue is dried by short heating up to 60°C. Finally, the protein pellet was stored at -20°C or dissolved in Protein sample buffer.

6 x Protein sample buffer
(LAEMMLI, 1970)

- 300 mmol L⁻¹ Tris/HCl (pH 6.8)
- 26% (v/v) Glycerol
- 10% (w/v) SDS
- 0.04% (w/v) Bromophenol blue
- 5% (w/v) DTT

2.6.2 SDS-polyacrylamid gel electrophoresis

The SDS-polyacrylamid gel electrophoresis (PAGE) is applied for analysing the composition of a protein mixture under denaturing conditions. SDS addition allows protein separation by their mobility in the electric field, which is proportional to their molecular weight.

The electrophoresis was done in a discontinuous gel system according to a technique of LAEMMLI (1970). A vertical sandwich cassette was filled up to 4/5 with separating gel solution. After polymerization, the cassette’s top was filled with stacking gel solution, and a comb was inserted. Finally, the gel system was filled with Running buffer, and the comb was removed. Protein precipitates were dissolved in 1 x Protein sample buffer (see previous chapter). After boiling at 95°C for 5 min, samples and 3 µL PageRuler® Plus Prestained Protein Ladder were loaded onto the gel and separated by electrophoresis. Depending on size of used gel, protein separation takes place at a constant current of 15 A/gel respectively 45 A/gel at a maximum voltage of 120 V.
Table 2.14: Composition of SDS-polyacrylamide gels.

<table>
<thead>
<tr>
<th></th>
<th>Stacking gel</th>
<th>Separating gel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4%</td>
<td>8%</td>
</tr>
<tr>
<td>ddH₂O [mL]</td>
<td>3.05</td>
<td>7.025</td>
</tr>
<tr>
<td>30% (w/v) Acrylamid (Mix 34.5:5) [mL]</td>
<td>0.65</td>
<td>4</td>
</tr>
<tr>
<td>1 M Tris/HCl (pH 6.8) [mL]</td>
<td>1.25</td>
<td>-</td>
</tr>
<tr>
<td>1.5 M Tris/HCl (pH 8.8) [mL]</td>
<td>-</td>
<td>3.75</td>
</tr>
<tr>
<td>10% (w/v) SDS [µL]</td>
<td>50</td>
<td>150</td>
</tr>
<tr>
<td>10% (w/v) APS [µL]</td>
<td>25</td>
<td>75</td>
</tr>
<tr>
<td>TEMED [µL]</td>
<td>5</td>
<td>7.5</td>
</tr>
<tr>
<td><strong>Total [mL]</strong></td>
<td><strong>5</strong></td>
<td><strong>15</strong></td>
</tr>
</tbody>
</table>

Running buffer

- 25 mmol L⁻¹ Tris base
- 192 mmol L⁻¹ Glycine
- 0.1% (w/v) SDS

2.6.3 Coomassie staining and drying of protein gels

Following electrophoresis, SDS-polyacrylamid gels were treated with Coomassie staining solution for approx. 1 h under slight shaking. To visualize proteins, gel background was destained with Destaining solution to the desired contrast.

For fixation, gels are stored in 10% (v/v) glycerol and washed with water. After positioning on cellulose films, gels are dried in a gel dryer at 55°C under vacuum for 3 h.

**Coomassie staining solution**
- 40% (v/v) Methanol
- 10% (v/v) Acetic acid
- 0.1% (w/v) Coomassie® Brillant Blue R-250

**Destaining solution**
- 40% (v/v) Methanol
- 10% (v/v) Acetic acid
2.6.4 Electrotransfer of proteins

Separated proteins were transferred from a SDS-polyacrylamid gel onto a PVDF membrane (MILLIPORE; Schwalbach/Ts, Germany) by a semi-dry blotting procedure. Prior to use membrane was activated in 100% methanol and equilibrated in transfer buffer. The transfer stack was assembled according to the ‘Instruction Manual PerfectBlue Semi-Dry Electro Blotter’ (PEQLAB, v0211E). The electrotransfer was performed with a constant current of 1.5 mA cm$^{-2}$ and a maximum voltage of 20 V for 55 min.

2.6.5 Immunochemical detection of proteins

After electrotransfer, PDVF membrane was incubated in Blocking solution over night to avoid unspecific binding of antibodies. Target proteins were immunostained with specific antibodies (Tab. 2.2, Section 2.2) and detected with a chemiluminescent detection reagent following the instructions of the ‘ECL$^+$ Western Blotting Detection Reagents’ product booklet (GE HEALTHCARE LIFE SCIENCES, RPN2132PL AE 02-2011). Washing steps were performed in TBS-T for three times after incubation with the primary/secondary antibodies. The exposure time of an autoradiography film during chemiluminescent detection depended on the sample.

Before drying, uniform protein transfer on PVDF membranes was confirmed by reversible staining with Ponceau S.

<table>
<thead>
<tr>
<th>TBS-T</th>
<th>20 mmol L$^{-1}$ Tris/HCl (pH 7.6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>137 mmol L$^{-1}$ NaCl</td>
</tr>
<tr>
<td></td>
<td>0.1% (v/v) Tween 20</td>
</tr>
</tbody>
</table>

| Blocking solution               | 5% (w/v) non-fat dried milk powder in TBS-T |

<table>
<thead>
<tr>
<th>Ponceau S</th>
<th>3% (v/v) Trichloroacetic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.2% (w/v) Ponceau S</td>
</tr>
</tbody>
</table>
2.7 Sample preparation

2.7.1 Substrates for surface modifications

If not otherwise mentioned, glass slides, polished silicon wafers, paraffin waxed substrates or Teflon foils (HBM Germany; Darmstadt, Germany) were cut into 10 x 10 mm$^2$ squared platelets. Prior to handling, these substrates were cleaned with absolute ethanol and doubled-distilled water under ultrasonic treatment for at least 30 min. To minimize surface roughness and densify silanol groups (≡Si-OH), the surface of selected silicon wafers (111), covered by a native oxide layer (typical thickness 15-20 Å), was etched by an oxidizing ‘Piranha’ solution (30 vol% H$_2$O$_2$/H$_2$SO$_4$ 30:70) for 2 h at RT. After acid removal, wafers were rinsed with doubled-distilled water or Tris buffer (50 mM, pH 8.5) and dried in high pressure air flow.

2.7.2 Substrate coating

2.7.2.1 Sessile drop method

Substrate surfaces were loaded with a Tris-buffered (50 mmol L$^{-1}$, pH 8.5) protein solution in four different concentrations (10, 50, 100 and 200 ng µL$^{-1}$). After several contact times (15-60 min), surplus protein solutions were removed, and substrates were rinsed in a flow of double-distilled water for 1 min to wash away weakly adsorbed proteins before protein-coated substrates were stored in Dialysis buffer.

2.7.2.2 Drop-surface transfer method

To create a comparable S-monolayer of surface active proteins, the dialyzed protein solution was centrifuged (22,000 x g, 4°C, 10 min), and a drop of supernatant (concentration of 0.1 mg/mL) was placed on a hydrophobic solid. After incubation at ambient conditions for 10 min, the substrate was brought into contact with the drop’s surface to bind the protein film. By lifting away, the drop-surface was transferred to the substrate. To remove weakly bound proteins, substrate was washed with double-distilled water and stored in dialysis buffer for subsequent measurements.
2.7.2.3 Surfactant supported protein assembly

For the protein/surfactant adsorption on different substrates, freshly prepared surfactant solutions with concentrations varying around the critical micelle concentration (CMC) are added to a protein solution (≥ 50 mg/mL). After incubation for several minutes, when a mono-dispersed protein solution can be expected (Section 3.2.2), additives like bivalent cations or anions are added to support protein interactions. The pretreated 10 x 10 mm² rectangular substrate is brought into contact with 100 µL of filtered (0.22 µm) solution containing protein and surfactant, irrespective of the substrate orientation. After incubation at a temperature above the Krafft point of the surfactant for 30 min, protein solution is removed and sample is washed with ultra pure water (APPLiCHEM GMBH; Darmstadt, Germany) and stored in Dialysis buffer, filtered at 0.22 µm.

To generate multilayers of surface active proteins under ambient room temperature conditions, a protein solution with a final concentration of 200 ng/µL protein is prepared. Depending on the net surface charge of the used surface affine proteins, a 50 mM CaCl₂ stock solution were added stepwise to a final concentration of 5 mM. In HFBI solutions, pH is shifted stepwise using 50 mM Tris-buffer (pH 5.4). After incubation for 10 min, 20-100 µM SDS is added, and the pretreated wafer is coated with 100 µL of the modified protein solution. After one hour incubation at a temperature above the Krafft point of the surfactant, the solution is removed, the sample is washed with filtered Millipore water and stored in filtered Dialysis buffer.

2.7.3 Stripping of surfactant layer

For the removal of the surfactant-layer, the coated substrate was two times incubated in a stripping buffer solution containing 50 mM Methyl-β-cyclodextrin for 30 min in each case. The excess of the surfactant/sugar complex was removed in a double-distilled water bath or buffer. Subsequently, samples were stored in filtered Dialysis buffer or allowed to air dry.

2.7.4 Generation of artificial oil bodies

In preparation for constructing artificial oil bodies (AOBs), a 200 ng/µL protein solution in Tris buffer (50 mM, pH 8.5) was ultrasonified (2:2, 4 cycl., 75%, 4°C) for 20 s to break multiple protein structures and increase protein activity. Semiconducting quantum dots,
synthesized by the Institute of Physical Chemistry/Electrochemistry (TU Dresden, Germany), dissolved in an organic solvent were placed at the bottom of an Eppendorf tube. One third of the total protein solution was added, followed by ultrasonication (5 cycl., 75%) for 20 s. To stabilize AOBs, a further third of protein solution was added and well mixed. The sample was allowed to cool down for 5 min. Afterwards, the sonication was repeated one more time, before adding remaining protein solution.
2.8 Analytical methods

2.8.1 Fluorescence analysis

2.8.1.1 Microplate reader-based spectrophotometry

Fluorescence intensity of purified proteins and AOBs (Section 2.7.4) was quantitatively determined as arbitrary units (a.u.) by spectrophotometry using a TECAN Infinite M200 microplate reader. For statistical significance three 100 µL aliquots of each prepared sample were distributed on a black 96-well plate. Excitation/emission wavelength and the gain depends on the fluorophore to be detected but were kept constant during one experiment. Due to excitation/emission bandwidth, applied values were set at least 30 nm apart from each other. Frequently used settings are listed in Table 2.15.

<table>
<thead>
<tr>
<th>Parameter/setting</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light source</td>
<td>UV xenon flash lamp</td>
</tr>
<tr>
<td>Excitation wavelength/bandwidth</td>
<td>368-495/9 nm</td>
</tr>
<tr>
<td>Emission wavelength/bandwidth</td>
<td>400-700/20 nm</td>
</tr>
<tr>
<td>Gain</td>
<td>100-200</td>
</tr>
<tr>
<td>Number of reads per well</td>
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</tr>
<tr>
<td>Integration time</td>
<td>20 s</td>
</tr>
<tr>
<td>Lag time</td>
<td>2-5 µs</td>
</tr>
</tbody>
</table>

2.8.1.2 Fluorescence microscopy

For microscopy of *E. coli* cells, protein coated surfaces and AOBs, samples were mounted on a microscopy slide and covered with a cover slip. Bright field and fluorescence microscopy images were acquired with a Keyence BZ-8100E or Zeiss Axio Observer.Z1 inverted microscope using the objectives and filters listed in Table 2.16. For confocal analysis of AOBs, preparations were acquired on an ApoTome1 microscope (CARL ZEISS MICROIMAGING GMBH; Jena, Germany) equipped with a 100x 1.4 oil objective. Image overlays and z-stack merge were performed using the BZ Analyzer or Zeiss AxioVision software platform.

The accessibility of the HA-tag of immobilized fusion proteins was analyzed after incubation with fluorophore labelled antibodies (in 1x PBS; Tab. 2.2) for 30 min. Before imaging, sample was three times washed with 1x PBS by shaking (300 rpm).
1x PBS

2.7 mmol L⁻¹ KCl
137 mmol L⁻¹ NaCl
1.5 mmol L⁻¹ KH₂PO₄
8.1 mmol L⁻¹ Na₂HPO₄

Table 2.16: Objectives and filters for fluorescence microscopy.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Keyence BZ-8100E</strong></td>
<td></td>
</tr>
<tr>
<td>Objective</td>
<td>CFI Plan Apochromat 100x (NA1.4 WD0.13 oil immersion)</td>
</tr>
<tr>
<td>Filters</td>
<td>DAPI-BP (Excitation = 377/50 nm, Emission = 447/60 nm)</td>
</tr>
<tr>
<td></td>
<td>GFP-BP (Excitation = 472.5/30 nm, Emission = 520/35 nm)</td>
</tr>
<tr>
<td></td>
<td>TexasRed (Excitation = 562/40 nm, Emission = 624/40 nm)</td>
</tr>
<tr>
<td><strong>Zeiss Axio Observer.Z1</strong></td>
<td></td>
</tr>
<tr>
<td>Objective</td>
<td>Epiplan-Neofluar 100x (NA1.3 oil pol WD0.13)</td>
</tr>
<tr>
<td>Filters</td>
<td>FS49 DAPI (Excitation = 360/40; Emission = 460/50)</td>
</tr>
<tr>
<td></td>
<td>FS43 DsRed (Excitation = 545/25; Emission = 605/70)</td>
</tr>
<tr>
<td></td>
<td>FS38 GFP (Excitation = 475/40; Emission = 530/50)</td>
</tr>
<tr>
<td><strong>Zeiss ApoTome1</strong></td>
<td></td>
</tr>
<tr>
<td>Objective</td>
<td>Plan-Apochromat 100x 1.4 Oil</td>
</tr>
<tr>
<td>Filters</td>
<td>FS49 DAPI (Excitation = 360/40; Emission = 460/50)</td>
</tr>
<tr>
<td></td>
<td>DAPIExc-Cy3Em (Excitation = 350/50; Emission = 605/70)</td>
</tr>
<tr>
<td></td>
<td>FS43 Cy3 (Excitation = 545/25; Emission = 605/70)</td>
</tr>
</tbody>
</table>

2.8.2 Contact angle measurement

The effect of surface modifications on substrate wettability was estimated with contact angle measurements. To this end, the angle formed between the liquid/vapor interface of a water drop and a solid surface was determined with the sessile-drop method using a DSA10 system (KRÜSS GMBH; Hamburg, Germany) equipped with an automatic dispenser (dispensation rate 0.1 μL/s). DSA 1 software based on the YOUNG & LAPLACE equation allows calculation of the static contact angle. Drops of 2 μL double-distilled water were settled by contact with the surface. The standard deviation for contact angle measurements is 1-3°.
2.8.3 Ellipsometry

Nulling ellipsometry was performed to determine distributions in layer thicknesses on modified surfaces. The spectral variation of the ellipsometric parameters $\Psi$ (relative amplitude ratio) and $\Delta$ (relative phase shift) was measured at a fixed angle of incidence of 68°. To calculate layer thickness via multilayer box model consisting of silicon, silicon dioxide, protein and water, the refractive index of the protein layer was set to $n = 1.375$ (WERNER et al., 1999; REICHELT et al., 2009). The optical constants of silicon and silicon dioxide were taken from literature (SYNYTSKA et al., 2007). Determined values were averaged from six independent measurements recorded from different locations on a sample surface. All measurements were performed at $21 \pm 1^\circ{C}$ and constant humidity in an uniform air-conditioned room. The relative standard deviation is in the range between 1 - 5%.

2.8.4 Dynamic light scattering

The particle size in solution was determined by dynamic light scattering (DLS) using an ALV/DLS/SLS-5000 light scattering spectrometer (ALV GMBH; Langen, Germany). As the light source, a 22 mW He-Ne laser operating at 632.8 nm was used. To increase predictive significance, all measurements were made at angles of 30°, 90° and 120° and at a constant temperature of 25°C.

2.8.5 Scanning electron microscopy

Morphology characterization was carried out using a FE-SEM instrument (DSM 982 Gemini, ZEISS; Jena, Germany). To obtain high-quality images, the surface of the sample was coated with a thin layer of gold (~10 nm thickness) using a sputter coater.
2.8.6 Atomic force microscopy

Atomic force microscopy (AFM) is applicable to evaluate topology and morphology of nano-structured surfaces. A JPK Nanowizard® AFM (JPK INSTRUMENTS AG, Berlin, Germany) was used for imaging the protein films in liquid under ambient conditions at 21 ± 1°C. Films were analyzed in buffer (50 mM Tris, pH 8.5 or 100 mM sodium chloride) via tapping mode. The DNP-S10 silicon nitride cantilever (VEECO INSTRUMENTS GMBH; Mannheim, Germany) has a spring constant of 0.32 N·m⁻¹, a resonance frequency in aqueous solution of ~12 kHz, and a radius of curvature of ~10 nm (for objects below ~5 nm). Applied scanning parameters are integral gain at 0.1-0.5 V, proportional gain at 0-5 V, and scan rate along the slow axis at 0.5-1 Hz.

Atomic force acoustic microscopy (AFAM) imaging was performed at 22 ± 1°C using an Agilent 5600LS Scanning Probe Microscope (AGILENT TECHNOLOGIES GMBH; Böblingen, Germany) in contact mode. Rectangular silicon cantilevers (PPP-NCLR, Nanosensors GmbH & Co KG; Aidlingen, Germany) with a guaranteed tip radius of curvature < 10 nm, resonance frequencies between 120 and 190 kHz, and a nominal force constant of 48 N/m were used. Height and acoustic images have been simultaneously acquired.

2.9 Statistics and reproducibility

Unless otherwise stated, all experiments were carried out at least in triplicates to assure reproducibility. For fluorescence quantification experiments, mean values and standard deviation (sd) were calculated and depicted in plots produced with R. For qualitative studies, representative data are shown.
### 2.10 Software and databases

Table 2.17: Software and databases. URLs were last accessed on 31st July 2012.

<table>
<thead>
<tr>
<th>Name</th>
<th>Application</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AxioVision (v 4.8.2.0)</td>
<td>Microscope image overlays</td>
<td>CARL ZEISS MICROIMAGING GMBH (Jena, Germany)</td>
</tr>
<tr>
<td>BZ Analyzer (v 2.5)</td>
<td>Microscope image overlays, z-stack merge</td>
<td>KEYENCE DEUTSCHLAND GMBH (Neu-Isenburg, Germany)</td>
</tr>
<tr>
<td>DSA10 system software</td>
<td>Determination of contact angles</td>
<td>KRÜSS GMBH (Hamburg, Germany)</td>
</tr>
<tr>
<td>ExPASy-Compute pI/Mw tool</td>
<td>Estimation of the size (i.e. molecular mass) of proteins</td>
<td><a href="http://web.expasy.org/compute_pi/">http://web.expasy.org/compute_pi/</a></td>
</tr>
<tr>
<td>i-Control (v 1.1)</td>
<td>Microplate reader instrument control, data acquisition</td>
<td>TECAN GROUP LTD. (Männedorff, Switzerland)</td>
</tr>
<tr>
<td>ImageJ (v 1.43)</td>
<td>2D FFT, interactive 3D surface plot</td>
<td>Girish and Vijayalakshmi (2004)</td>
</tr>
<tr>
<td>Multiskop ellipsometry software (v 5.2)</td>
<td>Determination of layer specific parameters</td>
<td>OPTREL GbR (Kleinmachnow, Germany)</td>
</tr>
<tr>
<td>PubMed Central</td>
<td>Literature research</td>
<td><a href="http://www.ncbi.nlm.nih.gov/pubmed/">http://www.ncbi.nlm.nih.gov/pubmed/</a></td>
</tr>
<tr>
<td>Vector NTI Advance (v 11.0)</td>
<td>Determination of annealing temperature for PCR reactions</td>
<td>INVITROGEN COOPERATION (Carlsbad, USA)</td>
</tr>
</tbody>
</table>
III. Results

3.1 Characterization of assembled hydrophobin layers

In nanobiotechnology, the properties of surfaces are often the key to sensor applications. If analytes possess a low tolerance or affinity regarding the sensory substrate (surface), then the setup of mediators may be indicated. Hydrophobins enable biocompatible surface functionalization without significant restrictions of the physicochemical substrate properties. Because of the imperfect formation of hydrophobin films, a high variation in surface properties is observed. This section focuses on the relation between the film thickness of hydrophobin-coated solid surfaces and their wettability. It was found that the wettability of protein-coated surfaces strictly depends on the amount of adsorbed protein, as reflected in an oscillation of the contact angles of hydrophobin-coated silicon wafers. Fusion proteins of Ccg2 and HFBI, representatives of class I and II hydrophobins, document the influence of fused peptide tags on the wettability. Hydrophobin fusion proteins were assembled on various solid substrates using two different deposition methods (Section 2.7.2). It was found that the orientation of the first crystal nuclei plays a decisive role in the formation of the growing hydrophobin layers. Based on the combination of contact angle measurement (CA) and nulling ellipsometry, a simple method of deducing the film thickness of hydrophobin assemblies on solid surfaces was developed. Furthermore, the determination of the static contact angle allows predicting which part of the protein is exposed to possible analytes.

3.1.1 Effects of heterologous gene expression

Enhanced expression of heterologous genes was successfully applied in *E. coli* SHuffle® T7 Express strains. Empirical growth analysis as well as fluorescence microscopy imaging revealed that heterologous gene expression apparently did not affect growth or morphology of
3.1 Characterization of assembled hydrophobin-layers

E. coli cells. However, enhanced heterologous gene expression entails the risk of producing inactive protein chimeras. Moreover, fungal hydrophobins contain a conserved cysteine pattern, forming four intramolecular disulfide bonds, which reinforces the protein scaffold (De Vocht et al., 2000). A correct disulfide bond formation is required for monomer assembly (De Vocht et al., 2000; MacIndoe et al., 2012). The used SHuffle™ T7 Express cells contain DsbC, a disulfide bond isomerase, which additionally acts as a chaperone. Therefore, it enhances the amount of correctly folded protein in vivo both in the periplasm and in the cytoplasm. Nevertheless, after cell disruption, target proteins were primarily located in the insoluble cell fraction, which implies accumulation of active target proteins or the formation of inclusion bodies. For that reason, proteins were purified under denaturing conditions by nickel-chelate affinity chromatography using high amounts of chaotropic agents (see Figure A.2). For the removal of chaotropic agents, protein eluates were immediately dialyzed against appropriate buffers. Addition of L-arginine suppresses aggregation because of an increased affinity for protein side chains that may be responsible for protein aggregation (Tsumoto et al., 2004). The addition of the glutathion-based redox system supports formation of correct disulfide bonds (Hwang et al., 1992). In summary, the optimized procedure results in a final protein yield of approximately 7 mg g⁻¹ cell wet weight. Protein solutions obtained after dialyzes were used for the following surface coating procedures.

3.1.2 AFM imaging of assembled hydrophobin films

Hydrophobins represent a class of small proteins of about 100 amino acid residues. They are globular in shape with a diameter of about 2-3 nm (Linder et al., 2005; Linder, 2009). Tapping mode AFM imaging in liquid phase is a high-resolution technique, which enables visualization of assembled hydrophobin layers in the nanometer range. In order to prevent tip or sample degradation effects as a result of protein dislodgement from the substrate, tapping mode AFM rather than contact AFM is suitable for measuring soft and/or ‘sticky’ protein samples. Images of silicon wafers coated with hydrophobins via the drop-surface transfer method illustrate the formation of crystalline areas. For Ceg2-treated surfaces, small longitudinal structures with an average height of around 3 nm are detectable after a contact time between the solution and substrate of 1 min (Figure 3.1A). In contrast, a contact time of 10 min leads to the formation of unstructured areas with an average height of 6 nm (Figure 3.1B). Thereby, tubular structures were hardly identified.
AFM images of immobilized HFBI show the formation of small regular structures with an average height of around 3 nm after a contact time of 1 min (Figure 3.2A). These small substructures were also observed after a contact time of 10 min or longer; however, they seem to be arranged in a complete layer (Figure 3.2B).

Investigations by AFM confirmed the formation of homogeneous hydrophobin layers, when using the deposition methods. The layer thickness strictly depends on the deposition time.

**Figure 3.1: AFM topography images of class I hydrophobin Ccg2.** Hydrophobin films of Ccg2-HA (100 ng µL⁻¹) were generated by the drop-surface transfer method on a silicon wafer. The equilibration time was (A) 1 min and (B) 10 min, respectively. A JPK Nanowizard AFM was used to image the protein films in liquid under ambient conditions at 21 ± 1°C.

**Figure 3.2: AFM topography images of class II hydrophobin HFBI.** Hydrophobin films of HFBI-(R5P)₂ (100 ng µL⁻¹) were generated by the drop-surface transfer method on a silicon wafer. The equilibration time was (A) 1 min and (B) 10 min, respectively. A JPK Nanowizard AFM was used to image the protein films in liquid under ambient conditions at 21 ± 1°C.
3.1.3 Combination of ellipsometry and contact angle measurement

As shown in the previous section, AFM images revealed uniform hydrophobin coverage over a wide surface area. However, tapping mode AFM allows no reliable statement about the thickness of a protein layer. For that reason, a spectroscopic ellipsometry technique was used to quantify the thickness of assembled hydrophobin layers. Ellipsometry is a high precision method, known for the high accuracy when measuring very thin films with a thickness in the Ångström scale (JUNG et al., 2004; MCMLIAN et al., 2004). This technique measures the change of polarization of a laser beam upon reflection on a solid surface. Because hydrophobins possess distinct hydrophobic and hydrophilic patches, impact of hydrophobin-coating on surface wettability was also determined. The wettability of a surface can be estimated from the shape of water droplets, deposited on a solid surface by measuring their contact angles. Low contact angles represent pronounced hydrophilic coatings and vice versa.

The class I hydrophobin Ccg2 as well as two HFBI-based fusion proteins, each consisting of the class II hydrophobin and a different modified tag were used to coat prepared silicon surfaces, Parafilm M, and Teflon (see Section 2.7.2). Afterwards, thickness of the adsorbed protein films and hydrophobin-mediated wettability were determined at the same sample position. In Figures 3.3 and 3.4, the determined protein film thickness was related to the corresponding contact angle \( \theta \).

The thickness of the SiO\(_2\) layer on the silicon substrates was 17 ± 1 Å. The protein films on the Si/SiO\(_2\) substrates exhibited layer thicknesses between 7 and 45 Å for HFBI-treated silicon wafers and a maximum of 97 Å for Ccg2-treated silicon wafers. Overall, the measured contact angles \( \theta \) of protein-coated Parafilm M, Teflon, and Si/SiO\(_2\) substrates are in the range from 44 to 95°. Contact angles of Teflon, Parafilm M, and ethanol-cleaned silicon substrates were 121 ± 0.9°, 110.2 ± 1.5°, and 45 ± 2°, respectively. The contact angle of the ‘Piranha’-etched silicon wafers was too small to be determined accurately (\( \theta \leq 5° \)). Local inhomogeneity in the layers with submicrometer resolution could not be assessed with this method.

The data showed no linear correlation between the layer thickness and wettability. The measured values were fitted with regression functions as shown in Figures 3.3 and 3.4. This approach allowed the determination of local extreme values shown in Table 3.1. By arithmetically averaging the determined extreme values, the protein film thickness for a HFBI\(_{(aa23−97)-(R5P)_2}\) layer was calculated to be \( d_{\text{film}} = 14.0 \pm 0.2 \) Å, and that for the HFBI\(_{(aa23−97)-(XSR5P)_3}\) layer was calculated to be \( d_{\text{film}} = 13.6 \pm 0.7 \) Å. For the class I
hydrophobin Ccg2, a layer thickness of 27.4 ± 0.4 Å was determined, a contact angle
maximum of $\theta = 97 \pm 0.6^\circ$, and a contact angle minimum of $\theta = 45 \pm 1.2^\circ$.

**Table 3.1: Calculated data from Figures 3.3 and 3.4.** Extreme values of contact
angle measurements (in parentheses) and the corresponding layer thicknesses (in bold) for
protein-coated silicon wafers and ‘Piranha’-etched silicon wafers are given.

<table>
<thead>
<tr>
<th>Protein</th>
<th>1$^{\text{st}}$ layer</th>
<th>2$^{\text{nd}}$ layer</th>
<th>3$^{\text{rd}}$ layer</th>
</tr>
</thead>
<tbody>
<tr>
<td>HFBI-(R5P)$_3$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>on Si/SiO$_2$/ paraffin waxed Si-wafer</td>
<td>14.0 Å (55.7°)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>on ‘Piranha’-treated Si-wafer</td>
<td>13.8 Å (86.1°)</td>
<td>28.2 Å (50.9°)</td>
<td></td>
</tr>
<tr>
<td>HFBI-(XSR5P)$_3$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>on Si/SiO$_2$/ paraffin waxed Si-wafer</td>
<td>13.5 Å (58.4°)</td>
<td>27.5 Å (89.7°)</td>
<td>40.1 Å (65.6°)</td>
</tr>
<tr>
<td>on ‘Piranha’-treated Si-wafer</td>
<td>15.0 Å (86.0°)</td>
<td>26.2 Å (60.2°)</td>
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</tr>
<tr>
<td>Ccg2-HA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>on Si/SiO$_2$/ ‘Piranha’-treated Si-wafer</td>
<td>28.0 Å (45.1°)</td>
<td>55.6 Å (82.3°)</td>
<td></td>
</tr>
<tr>
<td>on paraffin waxed Si-wafer/Teflon</td>
<td>22.8 Å (97.0°)</td>
<td>55.5 Å (58.2°)</td>
<td>81.6 Å (79.2°)</td>
</tr>
</tbody>
</table>

**Figure 3.3: Correlation between the protein film thickness and surface wettability.** Contact angles of Ccg2-HA-treated surfaces were plotted versus corresponding layer thickness. Ccg2-HA on pure silicon (■) and on paraffin-waxed silicon or Teflon (●). Tangents connect extreme points. Two different methods were used for Ccg2-HA immobi-
lization on solid surfaces: the sessile drop and the drop-surface transfer methods. Paraffin wax, Teflon foil, and silicon wafers were treated with Ccg2-HA solutions in Tris buffer (50 mmol L$^{-1}$ at pH 8.5) at four different concentrations (10, 50, 100, and 200 ng µL$^{-1}$) for 15 to 60 min. Mean values ±sd are shown.
3.1 Characterization of assembled hydrophobin-layers

Figure 3.4: Correlation between the protein film thickness and surface wettability. Contact angles of HFBI-(R5P)$_2$-treated (upper graph) and HFBI-(XSR5P)$_3$-treated (lower graph) surfaces were plotted versus corresponding layer thickness. Hydrophobins on pure and paraffin-waxed silicon (■) and on ‘Piranha’-treated silicon (●). Tangents connect extreme points. Two different methods were used for protein immobilization on solid surfaces: the sessile drop and the drop-surface transfer methods. Paraffin wax and silicon wafers were treated with protein solutions in Tris buffer (50 mmol L$^{-1}$ at pH 8.5) at four different concentrations (10, 50, 100, and 200 ng µL$^{-1}$) for 15 to 60 min. Mean values ±sd are shown.

The influence of layer thickness on the surface wettability is documented by the alignment of tangents to the resulting curves by connecting the extreme values and the determination of the slope (Figure 3.3 and 3.4).

As shown in Figure 3.4, minima are characterized on average by a higher number of values reflecting low contact angles, which may indicate a preferential tag direction in the hydrophilic medium. The resulting reduction in hydrophobicity leads to the formation of ordered,
more homogeneous structures. The small number of structures, which expose the hydrophobic protein patch, seems to reflect an energetically unfavorable state in the aqueous media.
3.2 Development of a surfactant supported protein assembly method

In this section, an innovative method for a controlled ‘layer-by-layer’ assembly of surface active proteins is presented. This method relies on an in vitro organization of monolayered and bilayered surface active protein films on liquid/solid interfaces supported by surfactants. Enhanced reagent accessibility to tags of engineered proteins enables various applications, ranging from catalytic reaction engineering to biosensor systems and medicine.

Briefly, suspensions of functionalized proteins with added conventional ionic surfactants were deposited onto different substrates. This approach was applied using genetically modified Ceg2 and HFBI as representatives of class I and class II hydrophobins, respectively, and S13240, SbsC and SslA as members of the surface layer protein family, which were heterologously expressed in *E.coli*. The resulting surface assemblies were analyzed by contact angle measurement, ellipsometry, scanning electron microscopy (SEM) and atomic force microscopy (AFM). Independent of settling time and protein concentration, defined ratios between protein and surfactant concentrations control protein layer thickness on the substrate. This novel method provides a general strategy for the controlled organization of functional surface active proteins on solid surfaces.

Visualization of nanoscaled protein layer structures on solid surfaces is a challenging goal, mainly if interactions of protein subunits lead to the formation of close meshed lattice structures (~1 nm). By exploiting differences in refractive indices, ellipsometry served to determine thicknesses of protein layers. SEM was used to image the homogeneity of surface deposited layers. Finally, effects of the coating method on the nanoscale morphology of thin protein-films were analyzed by liquid AFM and atomic force acoustic microscopy (AFAM). As opposed to most surface analyses, for example ellipsometry or electron microscopy techniques, AFM can be operated in aqueous solutions allowing the investigation of biological samples under physiological conditions.

Besides detailed structural analysis of assembled protein films on solid surfaces, the influence of surfactants on the long-term stability of tRFP-tagged hydrophobins was investigated. The accessibility of protein tags to antibodies or reactants after protein assembly was verified by creating hydrophobin based HA-tagged and *Gaussia* luciferase-tagged protein chimeras.
3.2.1 Protein encapsulation by surfactants

Hydrophobins and S-layer proteins are surface active proteins (Sections 1.2 and 1.3), tend to form large aggregates in solution, resulting in the clogging of ultra porous structures (e.g. filters).

In order to observe the influence of ionic surfactants on the surface activity of proteins, SDS was added to fluorophore-tagged hydrophobins (Ccg2-tRFP and HFBI-tRFP) and tRFP, respectively. Hence, the effect of chaotropic agents on fluorophore protein chimeras can be easily monitored by measuring their fluorescence intensity. A fluorescence intensity variation allows certain conclusions regarding conformational changes, resulting from interactions with surfactant molecules.

Figure 3.5: Protein encapsulation by surfactants. Tris buffered (50 mmol L\(^{-1}\), pH 8.0) protein solutions (200 ng µL\(^{-1}\)) of HA-tRFP, Ccg2-tRFP and HFBI-tRFP (Reference) were treated with equal SDS concentrations (2 mM; SDS added). After filtration (0.22 µm), all samples were observed over a certain period of time. Fluorescence intensity measurements were done to quantify protein activity using excitation/emission wavelength of 525/574 nm and a gain of 100. Mean values ± sd are shown. After one week, 150 µL of the protein solutions were precipitated and separated on a 12% SDS-polyacrylamide gel (lane 2-5). Unfiltered and untreated protein solutions at 0 h were used as a control (lane 1). Proteins were immunostained with anti-tRFP antibodies (shown below).

To generate homogeneous mono- or bilayers of surface active proteins, it has to be guaranteed that protein solutions only contain protein monomers or predominant oligomers (\(n \leq 8\)).
Therefore, the protein solution was filtered (0.22 \( \mu \text{m} \)) after addition of surfactants. The effect of filtration as well as surfactant treatment was observed by fluorescence measurements. Results are summarized in Figure 3.5. As expected, filtration of untreated hydrophobin chimeras (Ccg2-tRFP and HFBI-tRFP) leads to a decrease in fluorescence intensity (37\% and 46\%), due to their surface activity. The untagged tRFP showed no significant decrease in its emission. A contrary observation was made for surfactant treated samples. Surfactant treated tRFP showed a decrease of 25\% in fluorescence intensity. Thereby, the filtration effect of surfactant treated tRFP was found to be equal to the untreated sample. In the case of hydrophobin chimeras, addition of surfactants to Ccg2-tRFP and HFBI-tRFP has nearly no effect on their fluorescence intensity as well as sample filtration. Long-term measurements showed no decrease in fluorescence intensity of the hydrophobin chimeras, which implies a positive stabilizing effect. In accordance with dynamic light scattering (DLS) experiments (Section 3.2.2), these data imply that formation of surfactant/protein complexes supports long-term stability of surface active proteins.

### 3.2.2 Self-assembly of surface active proteins in aqueous solution

As demonstrated above, the addition of small amounts of surfactants to solutions of surface active proteins can affect their assembly behavior and thus prevent clogging of fine-pored structures. Furthermore, it supports long-term stability of proteins in solution. The questions are: (1) Are there further hints for specific interactions between proteins and surfactants? If so, (2) to what extent do surfactants influence self-assembly of proteins? Finally, (3) can it be utilized for controlling protein film deposition?

DLS analysis offers the possibility to determine the size distribution profile of particles in solution. The obtained results can be used to calculate the hydrodynamic diameter \( d_H \) of a particle through the STOKES-EINSTEIN equation. Furthermore, DLS indicates the structural composition, which in turn provides useful information about the mechanistic process of (self-)assembly. Initially, solutions exclusively containing surface active proteins were examined. As expected, a large range of particle sizes (\( d_H = 5\text{-}200 \text{ nm} \)) was obtained. This broad range implies the existence of small protein monomers, medium sized oligomers as well as large multimers. In the next step, samples were (ultra-)centrifuged to remove large and heavy protein agglomerates. Hence, monomers and oligomers represent the primarily detectable protein structures. As time proceeds, the protein self-assembly process shifts the particle size distribution towards larger aggregates. In reference experiments, solutions with a
concentration above the CMC of SDS were analyzed. Uniform particles with an average size of 3.5 nm were observed. This value corresponds to the size of SDS micelles ($d_H \sim 3.5$ nm) (HASSAN et al., 2002). As expected, no particles were detectable under the CMC in pure SDS solutions in the absence of proteins. Now, surfactant concentration was gradually increased in pretreated protein solutions. After 30 min incubation, particles were detectable, which are slightly bigger than SDS micelles. It was observed, that particles in hydrophobin/surfactant solutions ($d_H = 5.5 \pm 0.9$ nm; Fig. B.2) are smaller than in S-layer/surfactant solutions ($d_H = 10.5 \pm 3.2$ nm). In principal, addition of simple electrolytes reduces the repulsion between similarly charged head groups of ionic surfactants, which decreases the CMC as electrolytes increases (ASWAL & GOYAL, 2000). It might be assumed that added proteins affect the aggregation behavior of ionic surfactants. However, detected particles in hydrophobin/surfactant solutions are only half the size of the particles in S-layer/surfactant solutions, while the percentage of charged amino acid residues is almost the same for both proteins (15-20%). Hence, an effect of salt on the aggregation behavior of ionic surfactants can be excluded. Data instead indicate the formation of protein/surfactant complexes, consisting of a protein core surrounded by a layer of surfactant. DLS measurements of protein solutions containing low concentrations of surfactant revealed the formation of larger protein aggregates between 10 to 30 nm (data not shown).

3.2.3 Thicknesses of assembled layers

To get an impression of how the addition of surfactants affects the layer thickness of protein assemblies, layer thicknesses of adsorbed protein films were determined after adding various concentrations of surfactant (0.002-10 mM) to protein solutions (100 µg/mL). Silicon wafers were coated using the drop-surface transfer method (Section 2.7.2.1). The determined thicknesses of adsorbed protein layer were plotted versus the negative logarithm of surfactant concentration (Figure 3.6).

At surfactant concentrations around the CMC (Fig. 3.6 (4)), a protein film thickness comparable to a characteristic monolayer was observed (PAANANEN et al., 2003; GRUNER et al., 2012). Interestingly, the synergism in the adsorption correlates with optimal detergent conditions. When decreasing the surfactant concentrations to about one-hundredth of CMC (Fig. 3.6 between (2) and (3)), the thickness of the adsorbed protein layer is systematically thicker than that observed for monolayers at an air/water interface. Larger standard deviations of the measured values imply a disordered deposition of the surface affine proteins on the substrate.
However, the addition of surfactant concentrations lower than one-hundredth of CMC (Fig. 3.6 (1)) leads to the formation of protein films, that are twice as thick as those with surfactant concentrations around the CMC; observed thicknesses are equivalent to self-assembled protein bilayers. As summarized in Table 3.2, similar results are obtained for eight different hydrophobin or S-layer protein chimeras. As a consequence, the thickness of assembled protein films seems to be individually adjustable in the nanometer range by the addition of defined surfactant concentrations. The following characterization of assembled protein films is consistently based on appropriate surfactant concentrations above or below obtained concentration limits. Protein assemblies corresponding to film thicknesses of a protein monolayer or bilayer of surface active proteins are from here on usually termed monolayer or bilayer.

Figure 3.6: Plot of SDS concentration versus corresponding thickness of HFBI-assemblies. (100 ng µL⁻¹). Differences in the protein layer thickness demonstrate the influence of surfactant concentration (cSDS) on layer formation of surface active proteins. At low concentrations of added surfactants, protein assemblies as thick as a bilayer of proteins (1). High surfactant concentrations lead to the assembly of a protein monolayer (4).

Generally, a prospective protein capping surfactant-layer with an average thickness of 24.7 ± 0.1 Å was observed, considering the molecular dimension of SDS (around 2.1 nm) and CTAB (around 2.6 nm) for a full cylinder formation at the surface. The expected height of a surfactant bilayer should be the surfactant length plus the distance of one hydrocarbon chain (0.6 nm) or 2.7 to 3.2 nm (Paruchuri, 2009). However, the observed cover layer is slightly thinner than reported (Xu et al., 1996; Naik et al., 2011). The height differences could be a result of the uneven underlayed protein films.
Table 3.2: Influence of surfactant concentration on thickness of protein films. Various hydrophobins and S-layer proteins were treated with different concentrations of surfactants. Silicon substrates were contacted with the surfactant/protein mixtures for 30 min. After incubation, surfactants were removed, and layer thicknesses (d) of assembled protein films were determined by nulling ellipsometry. At low surfactant/protein ratios, determined layers (bilayer) were twice as large as at higher ratios. Their corresponding contact angles (θ) and standard deviations are given. Each protein consists of a different number of amino acids (aa).

<table>
<thead>
<tr>
<th>Protein</th>
<th>aa</th>
<th>Monolayer d [Å]</th>
<th>θ [°]</th>
<th>Bilayer d [Å]</th>
<th>θ [°]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hydrophobins</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ccg2-HA</td>
<td>155</td>
<td>25.0 ± 0.5</td>
<td>43.8°± 0.5</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>HFBI-(R5P)₂</td>
<td>144</td>
<td>13.8 ± 0.9</td>
<td>56.4°± 0.7</td>
<td>25.7 ± 1.8</td>
<td>54.1°± 0.9</td>
</tr>
<tr>
<td>HFBI-(XSR5P)₃</td>
<td>134</td>
<td>13.5 ± 0.5</td>
<td>57.6°± 0.9</td>
<td>25.8 ± 0.8</td>
<td>56.5°± 1.4</td>
</tr>
<tr>
<td><strong>S-layer proteins</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S13240</td>
<td>1115</td>
<td>41.2 ± 0.7</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>sSbsC</td>
<td>673</td>
<td>28.0 ± 0.9</td>
<td>75.8°± 2.8</td>
<td>52.8 ± 1.0</td>
<td>n.d.</td>
</tr>
<tr>
<td>sSbsC-(R5P)₂</td>
<td>734</td>
<td>29.9 ± 0.6</td>
<td>76.4°± 2.1</td>
<td>55.7 ± 1.8</td>
<td>n.d.</td>
</tr>
<tr>
<td>sSbsC-(XSR5P)₃</td>
<td>724</td>
<td>29.5 ± 0.9</td>
<td>74.7°± 0.8</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>SsIA-HA</td>
<td>1145</td>
<td>42.8 ± 0.7</td>
<td>n.d.</td>
<td>79.8 ± 1.6</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

3.2.4 Wettability of assembled layers

Contact angle measurements were done to demonstrate the influence of immobilized proteins on the wettability of substrates. Furthermore, this method also provides an indication of the completeness and the polarity of the exposed protein side, which finally permits conclusions regarding the direction of single protein units. The results are depicted in Table. 3.2.

Water spreads readily (θ < 10°; data not shown) on surfaces, treated with a surfactant/protein solution. This is in agreement with contact angles for ionic surfactants (CTAB and SDS), which are typically in the range of 5-10° (Paruchuri, 2009). As a result, initial ellipsometric measurements (Section 3.2.3) and contact angle measurements revealed protein films covered by surfactant molecules. With respect to application, protein-bound surfactant molecules must to be removed. It was previously reported that cyclic oligosaccharides such as methyl-β-cyclodextrin (MCD) can include surfactant monomers primarily by a 1:1 stoichiometry,
although inclusion complexes with a 2:1 stoichiometry may exist by less than 10% (Lin et al., 2001; Otzen & Oliveberg, 2001). The inclusion complexation of surfactant monomers into the MCD cavity enables effective stripping of protein-bound ionic surfactants in the protein refolding process (Yamamoto et al., 2008). Hence, it was selected to replace ionic surfactants from assembled protein films.

When substrates were incubated in a MCD solution (7 mM), S-layer protein-coated surfaces showed contact angles of about 75°. These results imply the removal of surfactants. Furthermore, measured contact angles are larger than pure silicon (θ = 45 ± 2°) or ‘Piranha’-etched silicon (θ ≤ 5°), which indicates the presence of S-layer protein films.

Analogously, HFBI-monolayer assemblies revealed contact angles of 56.4 ± 0.7° (HFBI-(R5P)2) and 57.6 ± 0.9° (HFBI-(XSR5P)3). HFBI-bilayers are also characterized by goniometric values of 54.1 ± 0.9° and 56.5 ± 1.4°, respectively. In both cases obtained thicknesses of assembled hydrophobin layers and corresponding contact angles imply one preferred monomer orientation. For the class I hydrophobin Ccg2 (usually forming rod-like aggregates) (Linder et al., 2005), a contact angle of 43.8 ± 0.5° was obtained after surfactant removal. It also implies a preferred hydrophobic based protein/interface interaction.

### 3.2.5 SEM characterization of assembled layers

Low voltage SEM allows resolution of structures of approximately a few nanometer in size and enables surface morphological characterization of ultrathin polymer films or even self-assembled organic monolayers. For that reason, the influence of surfactant concentration on the homogeneity of protein coating was evaluated by this microscopic method. Figure 3.7 depicts SEM images of a surfactant/protein treated silicon substrate with surfactant concentrations around the CMC. The film exhibits a smooth and very homogenous surface (Fig. 3.7A). The local removal of surfactants is visualized by a sharp boundary line between the covered, more electron-rich, surfactant layer and the uniform microstructured protein layer (Fig. 3.7B). Compared to the protein/surfactant bilayer, the surface morphology of the surfactant stripped substrate appears more structured (Fig. 3.7C).
3.2.6 AFM imaging

The investigation techniques accomplished up to now are only restrictedly suitable to visualize nanoscaled structures. Therefore, AFM analyses were performed for a resilient evaluation of the completeness of the generated layers and its degree of order. Compared to SEM images, liquid AFM analyses provide better resolution of organic structures, which allow a more detailed statement about the assembly arrangement. Images of silicon wafers coated with surface active proteins via the drop-surface transfer method illustrate formation of crystalline areas. For Ccg2 treated surfaces, small longitudinal directed structures with an average height of around 3 nm are detectable (Fig. 3.8A). In contrast with such protein monolayers, surfactant/protein layers show a smoother topology with height variations of around 1 nm. Random weak contours indicate structures below a capping layer (Fig. 3.8B). After removal of the surfactant, clear protein-specific structures are visible. Assembled tubular-like structures (Fig. 3.8C and D) are similar to Ccg2-rodlets described in literature (LINDER et al., 2005). The liquid phase AFM images reflect a maximum hydrophobin layer thickness of around 3 nm, which is in sufficient correspondence with X-ray fiber diffraction studies (KISKO et al., 2008; KWAN et al., 2006).

For S-layer assemblies, an average height between 4 to 6 nm was determined. Figure 3.9 illustrates structural integrity of the isoporous S-layer assemblies. Surface coating was done
without substrate surface pre-coating with SCWP, poly-L-lysine or polyelectrolytes (see Section 1.1). Nevertheless, after surface treatment with surfactant/protein solution followed by the removal of the surfactant layer, formation of S-layer characteristic lattice structures were observed for SslA, SbsC and S13240 (MERTIG et al., 1999; JAROSCH et al., 2001; BLECHA, 2005). Results demonstrate no significant influence of surfactants on the formation of highly ordered S-layer lattice structures (Fig. 3.9B and Fig. B.3).

**Figure 3.8: Liquid AFM topography images of class I hydrophobin Ccg2.** Silicon substrates were treated with a solution of the class I hydrophobin Ccg2 (100 ng µL⁻¹) using the drop-surface transfer method (A) or sessile drop method (B-D). Solutions with (A) and without (B-D) SDS (4 mM) were applied. (A) shows a film of Ccg2. (B) reveals a Ccg2-monolayer, capped by a smooth SDS-layer. (C and D) show Ccg2-monolayer after removal of SDS with a maximum height of around 5 nm. The inset FFT image illustrates organization of protein assemblies in the longitudinal direction with a calculated length of approximately 62 nm. Proteinaceous rodlets are around 25 nm thick and up to 100 nm in length. A JPK Nanowizard AFM was used to image the protein films in liquid under ambient conditions at 21 ± 1°C.
Figure 3.9: Liquid AFM topography images of S-layer protein SbsC-(R5P)₂. Silicon substrates were treated with a solution of a N- and C-terminal truncated S-Layer protein SbsC-(R5P)₂ (100 ng µL⁻¹) containing 8 mM SDS using the sessile drop method. Topography image (A) reveals a monolayer of SbsC-(R5P)₂, capped by a smooth SDS-layer. (B) shows a monolayer of SbsC-(R5P)₂ after removal of SDS with a maximum height of around 7 nm. The N- and C-terminal truncated SbsC self-assembly products clearly exhibited the oblique (p2) lattice structure with lattice constants of a = 11.8 nm, b = 6.4 nm and γ = 83° (compare Section 1.1.1.1). A JPK Nanowizard AFM was used to image the protein films in liquid under ambient conditions at 21 ± 1°C.

3.2.7 AFAM imaging

To get detailed insight into the formation of ‘unstripped’ protein assemblies, a special AFM technique was used. It is based on analyzing longitudinal acoustic waves, which are emitted by the sample (Rabe et al., 1996). The measured cantilever vibrations can be processed to acquire cantilever vibration spectra or to take acoustic images. AFAM analyses in air of protein layers covered by a surfactant-layer are shown in Figure 3.10. In contrast to the smooth topology AFM image in Figure 3.10A, the AFAM amplitude scan in Figure 3.10B reflects the typical rodlet structure of assembled Ccg2 monomers (Linder et al., 2005). Analysis of the Fast Fourier Transformation (FFT) image indicates an oblique lattice formed by the proteins, which has two-dimensional lattice vectors of a = 18.7 ± 1.0 nm and b = 23.2 ± 2.8 nm and an angle of 31 ± 0.4°. This clearly shows that obtained rodlets are modularly structured by smaller subunits. This observation is in agreement with X-ray fiber diffraction studies (Kwan et al., 2006).
3.2 Development of a surfactant supported protein assembly method

Figure 3.10: AFAM images of SDS/Ccg2 bilayers. Silicon substrates were treated with a solution of the class I hydrophobin Ccg2 (200 ng µL⁻¹) containing 8 mM SDS using the sessile drop method. Topography image (A) reveals a monolayer of Ccg2 on a dried silicon surface, capped by a smooth SDS-layer. The AFAM-amplitude image (B) shows a structured monolayer of Ccg2 after removal of SDS. Protein ordering is clearly visible on the real space and on the inset FFT image in the upper right corner of (B). An Agilent 5600LS Scanning Probe Microscope was used to image the protein films in air under ambient conditions at 22 ± 1°C.

3.2.8 Determination of tag-accessibility

The application potential of self-assembly proteins can be extended by the generation of bifunctional protein chimeras. However, with regard to nano(bio)technological utilization (e.g. bioanalytical sensors, immunoassays or drug delivery), accessibility of fused protein/peptide domains has to be guaranteed. In order to ensure a proper accessibility, it must be guaranteed that self-assembly fusion proteins crystallize in a defined orientation of their subunits on solid supports. The following two sections will confirm that protein fused functional domains are definitely accessible, when applying the developed protein immobilization method.

3.2.8.1 Accessibility of antigens in protein chimeras

To evaluate the orientation of immobilized bifunctional protein monomers, HA-tagged Ccg2 was attached to a silicon substrate according to the method described in Section 2.7.2. This method enables assembly of a protein monolayer. Surface assembled proteins were immunostained with fluorophore-labelled antibodies (anti-HA, AlexaFluor® 488 Conjugate). Conjugates were detected by fluorescence microscopy using the FS38 GFP filter. A homogeneous
green fluorescent substrate surface was observed, which confirms accessibility of the Ccg2-fused HA-tag (Fig. 3.11A). In analogy, a silicon substrate was treated with bovine serum albumin (BSA). After antibody incubation, no green fluorescence signal was detectable (Fig. 3.11C). In addition, a silicon substrate was coated with Ccg2-HA using the drop-surface transfer method. Fluorescence microscopy images revealed non-fluorescent areas as well as the formation of protein aggregates (Fig. 3.11B).

The structure of the anti-HA, AlexaFluor® 488 antibody belongs to the large mouse immunoglobulin (Ig). The Ig molecule in monomeric form is a glycoprotein with a molecular weight of approximately 160 kDa. Therefore, it is around ten times bigger than the small HA-tagged class I hydrophobin (~16 kDa). The ability of class I hydrophobins to attach very strongly and almost without changing their conformation at interfaces complicates reorganization of protein monomers after immobilization. Insofar, the observed homogeneous green fluorescence of the surface, coated with a monolayer of Ccg2-HA implies outward facing HA-tag orientation.

Figure 3.11: Antigen accessibility confirmed by fluorescence microscopy. Silicon substrates were treated with equal concentrations (200 ng µL⁻¹) of Ccg2-HA (A,B) or BSA (C) using two different surface coating methods described in Section 2.7.2. After 30 min incubation with anti-HA, AlexaFluor® 488 Conjugate antibodies, samples were observed by fluorescence microscopy using FS38 GFP filter for green fluorescence and an exposure time of 1 s. In contrast to the drop-surface transfer method (B), the assembled monolayer of Ccg2-HA (A) is characterized by a homogeneous green fluorescence. BSA treated sample exhibits no fluorescence (C).

3.2.8.2 Accessibility of enzymatic domains in protein chimeras
To observe enzyme-substrate accessibility, the luciferase (GLuc) of *Gaussia princeps* was fused to HFBI. This luciferase catalyzes the oxidative conversion of luciferol by the simultaneous emission of light with a maximum peak at 475 nm. As controls, individual chimeric
subunits (HFBI-HA and GLuc) were used. The intensive luminescence reaction was quantified with the Infinite M200 microplate reader. Initially, dissolved proteins were mixed with reaction buffer according to the instructions of the BioLux® Gaussia Luciferase Assay Kits (NEW ENGLAND BIOLABS GMBH; Frankfurt am Main, Germany) to ensure bioluminescence activity. After addition of luciferol, the bifunctional chimera (HFBI-GLuc) as well as the GLuc domain showed a luminescence signal as expected. The catalytically inactive HFBI showed no luminescence. In a second experiment, proteins were attached to a polystyrene substrate according to the method described in Section 2.7.2, which enables assembly of ordered protein monolayer. Substrates were overlaid with reaction buffer, and luciferol was added to start the reaction. Only the immobilized bifunctional chimera provides a significant luminescence. Thereby, luminescence spreads concentrically along the vessel wall from that point, where luciferol was added (Fig. B.4). The results are summarized in Figure 3.12.

In conclusion, GLuc and HFBI-GLuc are catalytically active. However, only the latter is able to assemble at solid surfaces due to the fused surface active hydrophobin. Furthermore, short surfactant treatment of proteins does not significantly impair their structural integrity, which is required for bioluminescence activity.

**Figure 3.12: Assaying Gaussia Luciferase (GLuc) activity.** To ensure luminescence activity of a luciferase-tagged hydrophobin, solutions of HFBI-GLuc, GLuc or HFBI in a final concentration of 100 ng µL⁻¹ were prepared (Soluble form). Plastic substrates were treated with equal concentrations (100 ng µL⁻¹) of all three proteins (Immobilized form) using the surface coating method, described in Section 2.7.2. After addition of buffer and luciferol, luminescence reaction was quantified using a microplate reader with an emission wavelength of 475 nm and a gain of 200 (right). Mean values ±sd are shown.
3.3 Development of excitable oil droplets

In this section, requirements for the development of an artificial fluorescence sensor based on interfacial FRET with prospective application potential in various sensor systems were examined. Implementation of interfacial FRET mainly depends on an appropriate mediator, which stabilizes a two-phase system for one, and connects two FRET partners, for another.

In contrast to common protein families, hydrophobins are characterized by the formation of a distinct hydrophobic and hydrophilic patch. Because of their hydropathy pattern, hydrophobins are predestinated to serve as interfacial anchor domains at various interfaces. Moreover, interfacial adhesion of hydrophobin-based fusion proteins allows targeted surface functionalization. In the present study, the ability of those synthetic proteins to self-assemble along oil/water interfaces was exploited to generate hydrophobin-stabilized oil/water emulsions. Therefore, mature forms of class I and class II hydrophobins (Ccg2 and HFBI) were fused to the red fluorescent protein TurboRFP (tRFP) to visualize hydrophobin attachment to the oil droplet’s surface. Furthermore, the fused tRFP domain allows continuous photometric monitoring of the long-term stability of artificial oil bodies (AOBs). The red fluorescent protein is characterized by a high photostability and pH-stability (MERZLYAK et al., 2007).

Figure C.2 shows a typical fluorescence spectrum of a fluorescent hydrophobin chimeras. With average diameters of about 2.5 nm in aqueous solution, hydrophobins are indeed very small proteins (KISKO et al., 2009; MACINDOE et al., 2011). Hence, they are able to attach protein-fused domains very close to an oil/water interface. To avoid potential interferences between two protein domains, they are separated by a randomly structured (GGGGS)_{2} linker. When the flexible linker is completely stretched, it reaches a maximum length of 2.4 nm (MEGEED et al., 2006). Small-angle X-ray scattering experiments showed that this short linker is highly flexible (ARA'I et al., 2004). The distance between a fused fluorophore and the oil/water interface is in the range of possible FÖRSTER radius values, which is limited to 10 nm (FÖRSTER et al., 1948). In this context, it is only consequent to locate another fluorophore on the opposite side of the oil/water interface, which offers a suitable overlap in its emission or excitation spectrum. However, the composition of the fluorophore is limited to oil resistant components. For example semiconducting nanoparticles (QDs, see Figure C.3) are usually covered by a distinct hydrophobic ligand shell. QDs based on II-IV group semiconductors were found to have remarkable chemical and optical properties. Cadmium selenide (CdSe) QDs and cadmium sulfide/zinc sulfide (CdS/ZnS) core-shell QDs are representatives of this group.
3.3 Development of excitable oil droplets

3.3.1 Fluorescence microscopy imaging of excitable oil droplets

The ability of hydrophobin mediated two-phase systems to support FRET at an oil/water interface was examined. In the following section, an example of such an artificial hybrid system based on the excitation of CdSe QDs, localized in AOBs, is introduced. AOBs are surrounded and finally stabilized by tRFP-labelled class I or class II hydrophobins. Because the emission spectrum of the CdSe QDs overlaps the excitation spectrum of the tRFP (Fig. C.4), an energy transfer process from QDs to proteins might be detectable (Fig. 3.13). A summary of both fluorophores including important features is presented in Table C.1. Generation of oil/water emulsions was performed following the protocol described in Section 2.7.4.

Initially, fluorophore components were independently localized by fluorescence microscopy. Referring to their excitation/emission spectra, CdSe QDs and tRFP-labelled hydrophobins are detectable using the available FS49 DAPI or FS43 DsRed filters, respectively. The results are depicted in Figure 3.13. The fluorescence of the QDs was uniform and restricted in respective AOBs, suggesting homogenous distribution of QDs in the oil phase. Nevertheless, fluorescence intensity seems to be more concentrated at the oil/water interface. After a certain period of time, a pronounced fluorescent ‘corona’ was detectable at the oil/water interface missing the uniform fluorescence distribution in the oil droplet. It can be assumed that QDs accumulating at the oil/water interface may be due to interaction with protein chimeras. The tRFP fluorescence was exclusively localized to the oil/water interface. This ‘corona-like effect’ confirmed that fluorescently tagged Ccg2 and HFBI attached to the surface of AOBs. Especially, the ‘drop caps effect’ apparent in image B2.2 nicely illustrates the interfacial arrangement of the hydrophobin chimeras. This effect is a result of the applied AOB generation technique, which includes multiple sessions of ultrasonic treatment (Section 2.7.4). When using a series of short pulses, the formation of some drop-in-drop like structures cannot be prevented. Nevertheless, this method is more efficient than that with a single long pulse.

FRET requires a sufficiently large spectral overlap in the excitation/emission spectra of two fluorophores (donor and acceptor), which are localized within a favorable proximity of 1-10 nm. The visualization of the fluorophore interactions at the oil/water interface can be mainly resolved by confocal fluorescence microscopy using appropriate filters (Section 2.8.1.2). The specially adapted excitation/emission settings allow selective excitation of CdSe QDs as well as the simultaneous detection of the tRFP fluorescence emission. The fluorescence pattern of the ‘false-color’ images is identical to those resulting from protein specific excitation. In a control experiment, without QDs, a weak fluorescence signal can be observed.
Quantitative analysis confirmed that the signal occurs as a result of weak tRFP excitation in the range of QD excitation (see following sections).

Fluorescence microscopy images indicate a direct interaction between QDs and proteins. Because the assembly behavior of hydrophobins at oil/water interfaces is a dynamic process depending on various factors; such as (1) pH value, (2) critical assembly concentration of hydrophobins, (3) emulsifying solvent, (4) preparation method and (5) environmental conditions (e.g. temperature, ion composition, ionic strength) (de Vocht, 2001; Wang et al., 2005; Kisko et al., 2008); quantitative analysis provides information about the potential assembly mechanisms.

Figure 3.13: Visualization of protein/QD interactions at an oil/water interface using fluorescence microscopy. (A) shows excitation/emission spectra of QDs (left) and proteins (right). Colored insets schematically illustrate the applied filter sets to match spectral excitation/emission characteristics of CdSe QDs (upper section), tRFP (middle) and the FRET pair (lower section). (B) Images of 0.5% (v/v) oil-in-water emulsions stabilized by Ccg2-tRFP (300 ng µL⁻¹). Samples containing QDs in the oil phase were observed after 1 h (B1) and 3 h (B2) using three different filter sets, illustrated in (A). The lower images are ‘false-color’ images. (B3.1-3) is the negative control with no QDs added. Samples were monitored using a Zeiss ApoTome1 confocal microscope with an exposure time of 500 ms.
3.3 Development of excitable oil droplets

3.3.2 Influence of pH on the protein stability

The tRFP is a highly pH-stable variant (pI=4.4) of fluorescent proteins. It is known that the isoelectric point (pI) is characterized by a minimum in protein repulsion, which results in an increased self-aggregation of proteins (ARAKAWA & TIMASHEFF, 1985). In order to observe the influence of the pH value on protein assembly, fluorescence intensity was determined while using buffer solutions covering the pH range from 4.6 to 9.0. A plot of normalized fluorescence intensity versus pH of protein solution is presented in Figure 3.14. For the reference protein tRFP, an almost constant fluorescence intensity was determined at an emission wavelength of 574 nm. In comparison, both tRFP-fusion hydrophobins show in general a reduced intensity of the fluorescence emission. At pH 4.6 HFBI-tRFP exhibits a red-shift in the fluorescence maximum to an emission wavelength of 582 nm (data not shown), which could be a result of protein agglomeration. Ccg2-tRFP shows a red-shift in the emission wavelength from 574 to 584 nm from a pH of 5.4 to pH 4.6 (data not shown).

![Figure 3.14: Effect of pH value on fluorescence intensities.](image)

Figure 3.14: Effect of pH value on fluorescence intensities. Plots of normalized emission maxima of tRFP (●, dashed line), HFBI-tRFP (▲, dotted line) and Ccg2-tRFP (■) (concentration of 100 ng µL⁻¹) versus various pH values (4.6, 5.4, 6.8, 7.5, 8.0, 8.5, 9.0) were obtained by fluorescence intensity measurements using excitation/emission wavelength of 525/560-700 nm and a gain of 150. Mean values ± sd are shown.

3.3.3 Critical protein assembly concentration

Depending on their hydropathy pattern, a critical hydrophobin concentration is required to induce self-assembly. Generally, above this threshold concentration, hydrophobins start to
form compact, but well defined protein films semipermeable to small molecules (WANG et al., 2005; SZILVAY et al., 2006; Zao et al., 2007, BASHEVA et al., 2011). Only when this controlled assembly process is initiated, generation of protein stabilized oil/water spheres is possible.

To investigate the effect of protein concentration on the stability of oil spheres, experiments with various hydrophobin concentrations (60-400 ng µL\(^{-1}\)) were performed (Section 2.7.4). The oil concentration was kept constant at 0.5% (v/v). Freshly prepared emulsions, containing CdSe QDs in the oil phase, were observed by fluorometric analysis over a certain period of time. The acceptor (CdSe QD) was selectively excited using an appropriate excitation wavelength. Finally, the emission intensity of the donor (fluorescent proteins) was recorded (example is shown in Fig. 3.15). Fluorescence microscopy revealed that oil droplets containing CdSe QDs were covered by films of fluorescent proteins.

Generally, fluorescence intensity of the acceptor protein increases rapidly to a concentration-dependent maximum, followed by a decline. With increasing protein concentration the adjustment of a steady-state becomes obvious. Also the time frame of the stable steady state seems to be concentration-dependent.

![Figure 3.15: Stability of oil droplets at different protein concentrations.](image)

Figure 3.15: Stability of oil droplets at different protein concentrations. To observe the influence of the protein concentration on oil droplet stability, emulsions with different protein concentrations (in ng µL\(^{-1}\): 60, 120, 180, 240, 300) were prepared. The oil phase contains CdSe QDs. For each sample, total fluorescence of 100 µL aliquots was determined with a microplate reader using excitation/emission wavelength of 368/450-600 nm and a gain of 150. Graphical plots of yielded fluorescence maxima at 574 nm (due to acceptor) reveal time-dependent information about system stability.
3.3.4 Effect of QD concentration on fluorescence intensity

The phenomenon of FRET manifests itself through an increase in acceptor fluorescence emission, triggered by an increase in donor concentration. To evaluate FRET efficiency of the potential donor/acceptor system, hydrophobin stabilized octadecene (ODE)-in-water emulsions were prepared containing equimolar concentrated solutions of tRFP-tagged hydrophobins and different CdSe QD concentrations (0.5-2.5%(v/v)). Samples were incubated in a self-contained system at RT for four hours. Aliquots were analyzed by microplate reader-based fluorometry every 15 minutes (Fig. 3.16) to detect the fluorescence intensity of the tRFP-tagged protein chimera. Usually, increased QD concentration yielded increased maxima in emission of the tRFP-tagged hydrophobin. As a negative control, equimolar emulsions without QDs were prepared, which reveal a base signal. Detected fluorescence intensities of hybrid systems were usually higher than the negative control.

![Figure 3.16: Time-dependent fluorescence intensity](image)

**Figure 3.16: Time-dependent fluorescence intensity.** To observe the effect of an increasing CdSe QD concentration on the fluorescence intensity of Ccg2-tRFP, equimolar protein (300 ng µL⁻¹) emulsions with different CdSe QD concentrations (in % (v/v): 0.0, 0.5, 1.0, 1.5, 2.0) were prepared. The fluorescence intensity at the emission maximum of the protein (574 nm) was plotted versus time. For each sample, the total fluorescence of 100 µL aliquots was determined with a microplate reader using excitation/emission wavelength of 368/450-600 nm and a gain of 150. Graphical plots of obtained fluorescence maxima at 574 nm (due to acceptor) reveal time-dependent details about the system stability.

In the first hour, fluorescence emission of the tRFP-tagged hydrophobins was discovered to increase with time to a steady-state. This result might be explained by a time-dependent donor/acceptor assembly process. It is possible that QDs attach to the oil/water interface by QD/hydrophobin interactions as implied in Figure 3.16. Thereby, an increased donor to acce-
Ptpr ratio gives rise to higher maxima in fluorescence intensity, as accepted from FRET. However, an increased donor concentration seems to affect the stability of AOBs, which results in the decrease of acceptor emission after a certain period of time. Apparently, the stability of AOBs suffers if too many hydrophobins are involved in QD interactions. The surface activity of hydrophobins depends on a protein self-assembly process accompanied by a conformational change of the molecules at the interface (Wang, 2004).

### 3.3.5 Efficiency of energy transfer

FRET is the non-radiative transfer of energy from an energy donor to an acceptor. The fluorescence quenching efficiency of a FRET event $E$ indicates the percentage of the excited donor that contributes to FRET and is defined as:

$$ E = \frac{P_t}{P_t + P_r + P_{nr}} $$

in which $P_t$ and $P_{nr}$ are radiative or non-radiative relaxation processes, respectively. $P_t$ is the ratio of dipole-dipole interaction between the donor to the acceptor. $E$ is extremely sensitive to distance changes between donor and acceptor, and decreases as the sixth power of the distance separating the two.

 Principally, FRET efficiency can be determined from two points of view: (1) decrease of donor fluorescence induced by an increased acceptor concentration and (2) increase of acceptor fluorescence induced by an increased donor concentration. The former can be simply determined by comparison of fluorescence intensities $F_D$ and $F_{DA}$ of the donor (D) in the absence or presence of the acceptor (A), respectively:

$$ E = 1 - \frac{F_{DA}}{F_D} $$

FRET can also be estimated by comparing the ratio of emission from the acceptor to emission from the donor (Baneyx et al., 2002), or through the increase in acceptor emission $I$ from $F_A$ to $F_{AD}$ in the absence and presence of the donor (Lakowicz, 1999):

$$ I = 1 - \frac{F_A}{F_{AD}} $$

In this work, the fluorescence quenching efficiency (equation (3.2)) as well as the acceptor amplification plot according to equation (3.3) was exploited to determine FRET efficiency. The results are depicted in Figure 3.17.
3.3 Development of excitable oil droplets

Figure 3.17 shows the fluorescence quenching efficiency as a function of the tRFP-tagged hydrophobin concentration. This plot shows two phases - a linear phase followed by a plateau phase. The near linear correlation of the quenching efficiency over a wide concentration range of added tRFP-tagged hydrophobin implies a direct interaction between QDs and proteins. Following the increase of the quenching efficiency plot, values plateau at a certain QD-to-protein stoichiometry. In practice, fixed concentration of QDs limits the FRET efficiency and, as a result, surplus tRFP chimeras do not undergo FRET.

The fluorescence amplification of the tRFP chimeras (Figure 3.17B) increases rapidly in a more exponential way by the addition of small amounts of CdSe QDs. The steep slope of the emission intensity of the protein in the range of low QD concentrations implies high FRET sensitivity. An increasing QD concentration seems to lead to an effect of acceptor saturation, which results in a steady-state. Thereby all interfacial available tRFP chimeras are already excited and are not able to participate in the transfer process.

To sum up briefly, observed fluorescence quenching of QDs as well as the fluorescence amplification of the tRFP chimeras indicates the existence of a FRET effect. Therefore, the following section will give a detailed data evaluation concerning a possible FRET effect, which reinforces this assumption.

**Figure 3.17: Sensitivity of energy transfer.** FRET efficiency strictly depends on the donor-acceptor-ratio. (A) To determine the fluorescence quenching efficiency of the tRFP chimera to the fluorescence intensity of CdSe QDs, emulsions with different protein concentrations (in ng µL⁻¹: 60, 120, 180, 240, 300, 360) were prepared. Emulsions contained 0.5% (v/v) CdSe QDs. QD fluorescence was observed using excitation/emission wavelengths of 368/462 nm. The line show the best fit to guide the eye. (B) The increase in the emission of tRFP chimera was obtained by the preparation of equimolar protein (300 ng µL⁻¹) emulsions containing different CdSe QD concentrations (in % (v/v): 0.0, 0.5, 1.0, 1.5, 2.0, 2.5). Fluorescence of tRFP chimera was observed using excitation/emission wavelengths of 368/574 nm. Total fluorescence of each sample was determined using a microplate reader with a gain of 100. The straight line showes the logarithmic connection of acceptor emission with respect to the increased QD concentration.
3.3.6 Quenching effects

Ideally, the surface of a QD is totally and homogeneously covered with organic ligands. Identical functional groups of these surface ligands are exposed on the lattice in the same orientation. However, proteins are characterized by a set of equivalent and independent binding sites. In a mixture of both macromolecules, quenching effects can be observed as a result of direct interactions. Fluorescence quenching is the decrease in fluorescence intensity of a fluorophore induced by four types of interactions with a quencher: (1) hydrogen bonds, (2) electrostatic interactions, (3) VAN DER WAALS forces and (4) hydrophobic interactions (LECKBAND et al., 2000). Fluorescence quenching can be dynamic resulting from particle collisions, or static resulting from the formation of a ground-state complex between the fluorophore and the quencher (PAPADOPOULOU et al., 2005). To confirm the quenching mechanism, fluorescence data were analyzed with the well-known STERN-VOLMER equation (3.4).

\[
F_0/F = 1 + K_{SV}[P]
\]  

(3.4)

Whereby \( F_0 \) and \( F \) are the fluorescence intensities of the fluorophore in the absence and presence of quenchers, respectively; \([P]\) is the concentration of the quenching protein; and \( K_{SV} \) is the STERN-VOLMER quenching constant which can be obtained by the slope.

For static quenching interactions, the relationship between the fluorescence intensity of the fluorophore and a quencher can be described by a binding model (Fig. 3.17). When the overall amount of QDs (bound and unbound) is \([QD_0]\), the relationship between fluorescence intensity and unbound QDs \([QD]\) is given as:

\[
F_0/F = [QD_0]/[QD]
\]  

(3.5)

Depending on the protein concentration \([P]\), the apparent binding (or affinity) constant \( K_b \) between QDs and proteins and the binding sites \( n \) of the protein can be described by the following equation (MARTY et al., 1986).

\[
log[(F_0 - F)/F] = logK_b + n log[P]
\]  

(3.6)
3.3.6.1 Influence of protein concentration

Figure 3.18 shows the effect of increasing HFBI-tRFP concentration on the fluorescence emission spectrum of CdSe QDs. Addition of protein to an oil-in-water emulsion containing CdSe QDs in the oil phase resulted in the quenching of the QD fluorescence emission. The observed upward curvature in the STERN-VOLMER plot (Fig. 3.18) represents a special case, which can be described by the quadratic equation:

$$F_0/F = 1 + (K_D + K_S)[P] + K_DP[P]^2$$  \hspace{1cm} (3.7)

This modified form of the STERN-VOLMER equation accounts for the simultaneous occurrence of static and dynamic quenching processes. The dynamic portion ($K_D$) can be determined by lifetime measurements (dashed line in Fig. 3.18). From the linear relationship between the ratio $\tau_0/\tau$, where $\tau_0$ and $\tau$ is the lifetime in the absence/presence of quencher respectively, and the concentration of the quencher, the value of $K_D$ can be obtained by equation (3.8).

$$\tau_0/\tau = 1 + K_D[P]$$  \hspace{1cm} (3.8)

Afterwards, the diffusion-controlled dynamic quenching constant rate $k_q$ is calculated and compared to the maximum value possible for diffusion-limited quenching in water ($\sim 10^{10}$ L mol$^{-1}$ s$^{-1}$). Smaller values are usually indicative for dynamic quenching processes while larger apparent values of $k_q$ suggest some form of binding interaction (LAKOWICZ, 1999). At constant temperature (298 K), a $K_D$ value of $8.4 \times 10^4$ L mol$^{-1}$ was calculated, and for $k_q$, a value of $8.8 \times 10^{12}$ L mol$^{-1}$ s$^{-1}$ was obtained. Hence, the dynamic quenching constant rate implies the formation of a QD/protein complex.

According to equation (3.6) the apparent binding constant $K_b$ was calculated to $1.21 \times 10^{-6}$ L mol$^{-1}$, and the binding site number was obtained from the slope observed in Figure 3.18 to be $n = 2.3$. The high binding constant is evidence for strong interactions between CdSe QDs and hydrophobin fusion proteins. Data evaluation implies the accessibility of more than one binding site on the QD surface.
Figure 3.18: Influence of HFBI-tRFP on emission spectra of CdSe QDs. Fluorescence emission spectra (left) of CdSe QDs oil/water emulsions (concentration 0.5% (v/v)) ($\lambda_{ex}$=462 nm) in the presence of different concentrations of HFBI-tRFP (in ng $\mu$L$^{-1}$: (1) 60, (2) 120, (3) 180, (4) 240, (5) 300 and (6) 360) at 308 K. For the steady-state quenching of CdSe QDs by HFBI-tRFP in water, quenching parameters were determined using the STERN-VOLMER plot (middle) and the plot of the binding constant (right).

3.3.6.2 Characterization of protein/QD complexes

This section focuses on the general verification of accuracy of the measured results according to a reference system. Bovine serum albumin (BSA), as a common model protein, is capable of binding to fatty acids, amino acids and various metal ions such as Cu(II), Ni(II), etc. (KRAGH-HANSEN, 1981; ZHANG & WILCOX, 2002). In the case of biomedical applications of QDs, in particular drug delivery or receptor targeting, the conformational behavior of BSA into conjugation with ZnO nanoparticles was recently under investigation (BARDHAN et al., 2009; KATHIRAVAN et al., 2009). Because of previous studies, which allow a detailed insight in protein/QD interactions, the latter model experiment served as excellent reference to adjust measurement parameters. Therefore, the fluorescence emission spectra of equally concentrated ZnO QD solutions in the presence of different concentrations of BSA (10-1000 ng $\mu$L$^{-1}$) were obtained. Data evaluation was done as described above. Results of the detailed examination from the BSA/ZnO reference system are presented in the following text.

For the ZnO QD/BSA system, the STERN-VOLMER quenching constant, $K_{SV}$, was estimated from the plot (Fig. 3.19) to be $1.6 \times 10^4$ L mol$^{-1}$ at 306 K. This value indicates that the quenching mechanism is initiated by complex formation (PAPADOPOULOU et al., 2005). The binding constant $K_b$ and the number of binding sites $n$ of ZnO QDs with BSA were determined from the intercept and slope of Figure 3.19 ($K_b=0.91 \times 10^4$ L mol$^{-1}$; $n=0.938$) at 306 K, respectively. Both correlation coefficients $R^2$ are larger than 0.99. The obtained values
are in agreement with reported data (see Table 3.3; PAPADOPOULOU et al., 2005; BARDHAN et al., 2009).

Table 3.3: Binding constant, $K_b$, and number of binding sites, $n$, at different temperatures. Respective values were determined from Figure 3.19 (bolt) and compared to the reference (BARDHAN et al., 2009, grey).

<table>
<thead>
<tr>
<th>T [K]</th>
<th>$K_b$ [L mol$^{-1}$]</th>
<th>$n$</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>298</td>
<td>5.80 x 10$^4$</td>
<td>1.060</td>
<td>0.9999</td>
</tr>
<tr>
<td>303</td>
<td>5.20 x 10$^4$</td>
<td>0.994</td>
<td>0.9942</td>
</tr>
<tr>
<td>306</td>
<td>0.91 x 10$^4$</td>
<td>0.938</td>
<td>0.9918</td>
</tr>
<tr>
<td>308</td>
<td>0.75 x 10$^4$</td>
<td>0.903</td>
<td>0.9931</td>
</tr>
</tbody>
</table>

Figure 3.19: Influence of BSA on the emission spectra of ZnO QDs. Fluorescence emission spectra (left) of ZnO QDs (concentration 0.4 mmol L$^{-1}$) ($\lambda_{ex}$=300 nm) in the presence of different concentrations of BSA (in ng/µL: (1) 50, (2) 100, (3) 150, (4) 200, (5) 250 and (6) 1000) at 306 K. For the steady-state quenching of ZnO QDs by BSA in water, quenching parameters were determined using the STERN-VOLMER plot (middle) and the plot of the binding constant (right).

3.3.6.3 Customized zinc-finger protein

The binding between QDs and proteins mainly occurs by site specific intermolecular interactions between exposed functional groups. Due to their very active aromatic hydroxyl group, tyrosine residues are essential for forming ground state complexes in most cases (BARDHAN et al., 2009). Amines and thiols may also bind directly to the QD surface (MEDINTZ et al., 2005). For example, clusters of invariant histidine and/or cysteine residues are excellent metal ion chelating agents. In cells, such unique peptide structures preferentially complex zinc(II); therefore, they are called zinc-finger motifs (Zifs). Several studies demonstrated that Zifs are also able to chelate cobalt(II), copper(II) and cadmium(II) (PREDKI & SARKAR, 1992;
SCOTLAND et al., 1993). In this work, an artificial peptide sequence was designed, which contains a central ZiF flanked by six N- and C-terminal tyrosine residues. The derived sequence was fused to the C-terminus of HFBI. Like in the experiment of Section 3.3.6.1, fluorescence emission spectra of equal concentrated ZnO QDs solutions in the presence of different concentrations of the protein chimera HFBI-ZiF (10-300 ng µL⁻¹) were obtained. Data evaluation was done as described above.

![Figure 3.20: Influence of zinc-finger motif on the emission spectra of ZnO QDs.](image)

**Figure 3.20: Influence of zinc-finger motif on the emission spectra of ZnO QDs.** Fluorescence emission spectra (left) of ZnO QDs (concentration 0.4 mmol L⁻¹) (λ<sub>ex</sub>=300 nm) in the presence of different concentrations of HFBI-ZiF (in ng/µL:0, 10, 50, 100, 150, 200 and 240) at 306 K. For the steady-state quenching of ZnO QDs by BSA in water, quenching parameters were determined using the STERN-VOLMER plot.

In contrast to the reference (Section 3.3.6.3), the STERN-VOLMER plot of the HFBI-ZiF/ZnO QD system reveals no linear behavior (Fig. 3.20). In the range of low protein concentrations, the plot exhibits an upward trend, followed by a downward curvature. After a minimum is reached, the ratio of fluorescence intensities of the QDs (F₀/F) rises steeply with increasing protein concentration. A downward curvature of a STERN-VOLMER plot can be explained by different populations of the acceptor, one of which is inaccessible to the fluorophore. In proteins, more than one population of acceptor may be present. This is especially true for tryptophan residues, where some may be readily solvent accessible, and others may be buried (GEDDES et al., 2001). These findings are valuable with regard to the optimization strategies by modifying interface anchoring proteins (Section 4.3.2).
3.3 Development of excitable oil droplets

3.3.7 Lifetime measurements

To show the non-radiative energy transfer between the semiconductor QDs and the proteins, lifetime measurements were investigated. The lifetime expresses the averaged time range of the excited state of a fluorophore before photons are emitted, and the more stable ground state is reached. Only radiative processes are detectable. Non-radiative processes interfere during the measurement because they quench the radiative processes and so extend the measurement time. The decay of the excited state of CdSe QDs follows an exponential law and is normally in the range of nanoseconds. The relaxation process depends on the chemical surrounding of the fluorophore and so lifetime measurements can be used to detect different interactions between the fluorophore with the chemical environment.

The as-synthesized CdSe and CdS/ZnS QDs have a high quantum yield and follow biexponential decay on the scale of nanoseconds ($3.2 \times 10^{-8}$ s) as it is shown in Figure 3.21. The relaxation process of the QDs increases drastically by the addition of tRFP-tagged hydrophobins. The lifetime decreases three times of magnitude, which indicates a non-radiative relaxation or energy transfer to another fluorophore. If the QD acts as energy donor the emission intensity of the energy acceptor should increase. The exponential decays of the protein with and without QDs show the same results (not shown) which indicates that the relaxation process of both is the same. But a time-dependent measurement of both reaction systems visualizes the increased fluorescence of proteins in attendance of the QDs.

As a result energy must be transferred from the QDs in a non-radiative process to the proteins (FRET) because the fluorescence intensity of the protein increases while lifetime of the QDs decreases.

![Figure 3.21: Lifetime measurements. Emission decay traces of CdS/ZnS QDs (0.5% (v/v)) with and without Ccg2-tRFP (300 ng µL$^{-1}$) in an oil/water emulsion (left) was monitored. Lifetime of Ccg2-tRFP in the presence or absence of CdS/ZnS QDs was determined (right) using a 403 nm laser for excitation.](image)
3.3.8 Long-term measurement

With regard to applications of prospective interfacial FRET systems, a long durability must be guaranteed. Generally, the chart of a long-term observation reveals the typical profile, described in Section 3.3.4. The fluorescence intensity of the tRFP-tagged hydrophobin steadily declines over time. After three days observation, fluorescence of the tRFP-tagged hydrophobin in the hybrid system was approximately six times higher than in the negative control, without addition of QDs. Results of initial long-term measurements confirm functional FRET after several days. However, this system lifetime is not appropriate for technical large-scale applications. Lifetime extension can be achieved by the careful synchronization of the system components, which is discussed in detail in see Section 4.3.

Figure 3.22: Long-term fluorescence measurement. For long-term observation, emulsions of 0.5% (v/v) ODE in 300 ng µL⁻¹ Ccg2-tRFP solution were prepared. The fluorescence intensity at the emission maximum of the protein (574 nm) was observed over a given period of time. For each sample, total fluorescence of 100 µL aliquots was determined with a microplate reader using excitation/emission wavelength of 368/450-600 nm and a gain of 150.
IV. Discussion

4.1. Layer thickness of hydrophobin films leads to oscillation in wettability

Small globular hydrophobins have the ability to change the physicochemical nature of a surface after immobilization. The formation of mature hydrophobin membranes at the air/water interface and on hydrophobic solids is well studied (DeVocht \textit{et al.}, 1998; Linder \textit{et al.}, 2002; Kisko \textit{et al.}, 2009). However, there is a lack of understanding the mechanisms that allow hydrophobins to form hydrophobic coatings on hydrophilic surfaces. Furthermore, hydrophobins can be genetically modified and are conceivable as carriers for functional peptides and enzymes, which increase their application potential. It is known, that fusions between hydrophobins and other protein domains are possible at both the C- and N-termini of hydrophobins without affecting their structural integrity and high surface activity (Nakari-Setälä \textit{et al.}, 1996; Linder \textit{et al.}, 2002). Both termini are located within the hydrophilic protein patch (Kwan \textit{et al.}, 2006; Hakampää \textit{et al.}, 2006). Unfortunately, there are less valid studies so far that clearly proved the influence of fused domains on the hydrophobin orientation after assembly onto solid surfaces. Especially for biosensor applications, the challenge is to guarantee a directed hydrophobin immobilization, whereby the fused domain is exposed to the medium and thus accessible for reaction partners. As seen in Figures 3.1 and 3.2, AFM images as well as other high-resolution methods do not provide direct information about the orientation of hydrophobin monomers. This section will discuss a novel and simple method to determine the orientation of surface active proteins on solid surfaces. It is based on a comparison of film thicknesses considering their respective protein-mediated wettability.

It should be noted, that literature given contact angles of hydrophobin-coated surfaces vary widely. For example, contact angles of HFBI-modified hydrophobic surfaces (PCL, PDMS, or gold) range between 45.3 and 64° (Askolin \textit{et al.}, 2006; Zhao \textit{et al.}, 2009; Ahlroos \textit{et al.}, 2011; Zhang \textit{et al.}, 2011a). Contrary, HFBI-coated hydrophilic surfaces (e.g. glas) show a
contact angle of about 72 ± 15° (ASKOLIN et al., 2006). On the other hand, Kwan et al. (2008) reported a contact angle for Ceg2-coated Teflon of 80.4 ± 10.1. Therefore, each data imply a deviation of 33%. This deviation runs contrary to studies which report regular hydrophobin assembly based on an interaction of hydrophobin subunits (Kisko et al., 2009; Macindoe et al., 2012).

As described in the literature, the thickness of assembled hydrophobin layers at interfaces strictly depends on the protein concentration and time of incubation (Wang et al., 2004). To verify the impact of both parameters on the morphology of hydrophobin layers, high-resolution AFM images (Figure 3.1 and 3.2) were compared to the plots determined by ‘macroscopic’ analytical methods (Figure 3.3 and 3.4). Interestingly, wettability plots reveal a regular sinusoidal correlation. In accordance to the AFM observed uniform formation of hydrophobin layers, a minimum or maximum in the wettability plot represent a complete hydrophobin layer. In this regard, assembly of class I and II hydrophobin subunits must occur due to a ‘layer-by-layer’ formation. Hence, the formation of multilayers takes place in an ordered manner as evidenced by the sinusoidal correlation between the layer thickness and contact angle (Figures 3.3 and 3.4). Preferred orientations of hydrophobin subunits indicate directed diffusion and assembly. Furthermore, the results indicate that homogeneous layers are formed over a large area of the substrate by directed protein-protein interactions.

The comparison of minima and maxima in wettability and their corresponding film thicknesses in Figures 3.3 and 3.4 shows that assembled hydrophobins form well-defined layers with mean thicknesses of 14.0 ± 0.2 and 13.6 ± 0.7 Å for HFBI constructs and 27.4 ± 0.4 Å for Ceg2 derivatives. The thicknesses of the adsorbed layers are in good agreement with those formed at the air/water interface with mean thicknesses of 13 ± 2 and 25 Å (Paananen et al., 2003, Kwan et al., 2006). The difference in wettability of the determined monolayers implies a different orientation of the proteins on the two substrates. Because of their amphiphilic character, hydrophobins are able to interact with substrates by hydrophobic or hydrophilic interactions depending on the surface wettability of the substrate. The protein bilayers show an inversion in wettability compared to the monolayer, which implies that HFBI bilayers are probably arranged in the same basic dimer orientation as observed for crystal structures (Hakanpää et al., 2006).
4.1 Layer thickness of hydrophobin films leads to oscillation in wettability

4.1.1 HFBI assembly onto hydrophilic and hydrophobic solid surfaces

From X-ray measurements, it is known that the hydrophobic patch and the C- and N-termini flank the protein in the longitudinal direction (HAKANPÄÄ et al., 2006). Therefore, a maximum distance between both protein parts is guaranteed, which should prevent a direct interaction between protein-fused tags and the hydrophobic patch. For that reason, two HFBI fusion proteins were designed, differing in the amino acid composition of their fused tags (XSR5P and R5P). By the calculation of the Kyte-Doolittle hydrophobicity index (KD), it was determined that one domain (XSR5P; KD = -0.72) represents a more hydrophobic polypeptide chain than the other (R5P; KD = -1.80) (Kyte & Doolittle, 1982). Contrary the planar hydrophobic patch of HFBI is formed by solely hydrophobic aliphatic side chains (residues Leu12, Val23, Leu24, Leu26, Ile27, Leu29, Val59, Ala60, Val62, Ala63, Ala66, Leu67, and Leu68), which enables HFBI to interact with hydrophobic substrates (HAKANPÄÄ et al., 2006). The calculated KD index is 2.97. Both HFBI fusion proteins were deposited on surfaces of varying wettability.

For HFBI-coated ‘Piranha’-etched silicon wafers, a tag-independent contact angle of 86.0 ± 0.1° for the first minimum was identified. This contact angle suggests that the hydrophilic protein patches may serve as surface mediators whereas the hydrophobic patch is exposed to the medium. This mediating effect may be enhanced, due to the high content of tyrosine, serine and arginine residues in the amino acid sequence of the R5 peptide-based tags (76%). Polycationic peptide sequences with a high proportion of hydroxyl groups are ideally suited for ionic and hydrogen-bonding interactions with densified SiO₂ surfaces (Kröger et al., 1999).

In contrast, pure silicon and paraffin-waxed wafers represent a more hydrophobic structure. The contact angles displayed in Figure 3.4 from the first minimum thickness of coated silicon wafers indicate that the hydrophilic protein patch is exposed. However, the deviation in contact angle of approximately 3° may result from the different behavior of the exposed protein parts. This assumption is reinforced by the position of the first minimum in the inverted layer formation. Minima of different tagged HFBI proteins differ in Δθ by about 9.5°. It can be concluded that the fused tag is oriented into solution and directly influences the hydrophilic character of the adsorbed layer.

Depending on the assembled fusion protein, tangentially connected extreme values reveal a defined downward or upward trend (Figure 3.4). Hence, composition of the fused peptide
sequence directly affects the impact of hydrophobins on the surface properties. Depending on the hydrophobin chimer, the increase in layer thickness leads to an increasing or decreasing tendency in wettability. This impact is represented by the change in contact angles of HFBI-(XSR5P)$_3$ ($\Delta \theta = +3.9^\circ$) and HFBI-(R5P)$_2$ ($\Delta \theta = -4.7^\circ$) with increasing hydrophobin layer thickness.

The tag nature may also influence the thickness of the hydrophobin layer. Hydrophobin layers with embedded tags are slightly thinner than such, where the fused tag is exposed to the medium. Probably, surface assembly does not necessarily result in a strict perpendicular orientation of the monomeric hydrophobin subunits (PENFOLD et al., 2012), as previously reported (KALLIO et al., 2007). The results suggest that the protein subunits harbor a slightly tilted position, leading to a more surface-orientated tag direction. This effect may be enhanced by the HFBI fused R5 peptide sequences, which possess the ability to form self-assembled structures (KNECHT et al., 2003). Hence, the embedded fusion domains can directly affect the orientation of hydrophobin monomers in an assembled protein layer.

4.1.2 Ccg2 assembly onto hydrophilic and hydrophobic solid surfaces

The calculated layer thickness (27.4 ± 0.4 Å) of Ccg2 as a representative of class I hydrophobins is in good agreement with the film thickness of 25 Å determined by AFM. Similarly, the overall structure is in line with a high content of $\beta$-barrels (KWAN et al., 2006). The small deviation in layer thickness may be explained by the type of layer formation. A protein layer at the water/air interface represents a more flexible system than at the water/solid interface. Furthermore, the protein transfer to a solid interface via a dot-blotting method could lead to local defects. The determination of layer thicknesses by AFM also depends on the properties of the cantilever. As reported in the literature, when using the X-ray fiber diffraction of Ccg2 rodlets, a monomer size of 27 Å was determined (KWAN et al., 2006). Therefore, the developed calculation model represents a sufficient method to determine layer thicknesses of surface assembled proteins.

In the context of this work, the contact angles of Ccg2-assemblies were investigated for the first time. On hydrophobic surfaces, the observed contact angle of the minimum in Figure 3.3 was 45.1°. This is in good agreement with the literature value given for the exposed hydrophilic side of SC3, the first investigated representative of class I hydrophobins (SCHOLTMEIJER et al., 2002). In contrast to class II hydrophobins, the formation of an adequate inverted layer
was only observed on the very hydrophilic ‘Piranha’-treated silicon substrates. The contact angle of the hydrophobic patch of surface-assembled Ccg2 was determined to be $97.0 \pm 0.6^\circ$. This is approximately 83% in comparison with the most surface active protein SC3 ($\theta = 117 \pm 8^\circ$) (Wöstten & de Vocht, 2000).

It remains just one more question, why no tubular structures can be observed by AFM after long-term incubation in Figure 3B. It is proposed, that conformational changes of class I hydrophobins at interfaces lead to the formation of rodlet-patterned hydrophobin assemblies (De Vocht et al., 2002; Scholtmeijer et al., 2009). Lateral interactions of rodlets are required to form monolayers (MacIndoe et al., 2012). With respect to Wang et al. (2004), increased incubation time leads to the formation of multilayered hydrophobin assemblies. Hence, the absence of tubular structures in AFM images implies a non-directed interaction between hydrophobin monomers of the first and second hydrophobin layer. The absence of rodlet-like packing in multilayered hydrophobin films lead to an attenuation in the sinusoidal behavior of the wettability in relation to the thickness of the assembled hydrophobin as shown in Figure 3.3. Nevertheless, hydrophobin assembly on the first layer proceeds according to a predefined pattern so that every following layer is as thick as the one below.

### 4.1.3 Model for assembly of surface active proteins

The ‘layer-by-layer’ deposition of oppositely charged polyelectrolytes on surfaces allows a variation in layer thickness and its physical properties, which is affected by the number of charges (Decher, 1997). By analogy, amphiphilic hydrophobins also affect an alternation in surface properties from layer to layer. In contrast to polyelectrolytes, however, the alternating positioning of hydrophobin subunits follows a ‘domino-like’ pattern (Figure 4.1). Therefore, every following hydrophobin layer is inversely arranged to that below.

Considering the measurement deviation that results from the laser beam size ($2 \times 1 \text{ nm}^2$) and the drop size ($2 \mu\text{L}$), the applied methods produce only relative values. Because of a 6-fold repetition of selective measurements, ‘macroscopic’ results imply highly ordered areas. In agreement with the theory of protein crystallization (Rosenberger, 1986; Mropherson, 1990), single-crystalline domains take up a preferred orientation and transfer it to further growing particles. The formation of nucleation points strictly depends on the nature of the substrate, as shown by López et al. (2010) for surface layer proteins. Therefore, a model for the formation of partially homogeneous layers was created as shown in Figure 4.1. Depending
on the orientation of the hydrophobins, layers are formed with exposed or embedded fusion peptides. The oscillation in wettability results in an alternating influence of hydrophobic hydrophobin patches and the tagged domains, which implies an inverted positioning of the subsequent protein layer. KYTE-DOOLITTLE-based calculations show the existence of an additive effect, which finally leads to an increase or decrease in the wettability of two adjacent layers (KYTE & DOOLITTLE, 1982). Depending on the protein concentration and incubation time, the formation of a multilayer is initiated. The growing, inverted layer results in the ordered deposition of additional monomers or whole protein aggregates, which again serve as nucleation points for a next hydrophobin layer. In accordance to MACINDOE et al. (2012), formation of homogeneous layers generally follows a nucleation-dependent assembly mechanism. As a result, the model may provide an explanation for the reported variations in contact angles of hydrophobin-treated surfaces.

In summary, the developed method provides a determination of the orientation of hydrophobin monomers depending on the layer thickness.

In comparison to other available high-resolution surface-investigation methods such as AFM, scanning electron microscopy (SEM), or transmission electron microscopy (TEM), the ‘macroscopic’ method offers several advantages. It is simple and fast in preparation and handling. For example, protein samples observed by SEM and TEM have to be coated with a thin layer of heavy metal to increase the contrast, but effects on the morphology of the protein layer cannot be excluded. Furthermore, the combination of two cheap surface observation methods allows a sufficient evaluation of the layer thickness of immobilized hydrophobins over large-scale areas. We were able to show by AFM in liquid that ellipsometric and contact angle measurements of dried and reswollen hydrophobin-coated samples yield results that are identical to those under native conditions.

With regard to application of prospective hydrophobin-based biosensors, a qualitative statement about the orientation of the surface assembled fusion chimeras is essential. Considering applications in enzymatic and chemical processes, the position of fused tags is crucial for the accessibility of reactants. To ensure the maximal reactivity of modified and functionalized hydrophobins, directed monolayered protein deposition is necessary. As a consequence, the challenge is to control this ‘tricky’ assembly process in a target fashion. The following part will give a detailed insight about a novel method that enables directed deposition of surface active proteins on solid surfaces (see Section 4.2).
4.1 Layer thickness of hydrophobin films leads to oscillation in wettability

Figure 4.1: Model for hydrophobin-assembled monolayers. (A) Hydrophobic substrate–protein interaction allows the exposure of the C-terminal fused protein domain, which defines the surface wettability. (B) Protein-coated ‘Piranha’-etched silicon wafer resulting in the inverted assembly of protein monomers. The coupled protein domain is embedded in the protein layer.
4.2 Controlled assembly of protein layers supported by ionic surfactants

As discussed previously, surface active proteins are a promising tool to functionalize various solid surfaces. But, their adsorption and self-assembly properties are poorly characterized or understood (ZHANG et al., 2011b), which complicates targeted applications, e.g. in medicine or diagnostics. Based on the intention to develop a novel strategy for a controlled ‘layer-by-layer’ assembly of surface active proteins, this section discusses an innovative approach in combination with ionic surfactants. In order to study the influence of ionic surfactants on the assembly behavior of surface active proteins, two different families of amphiphilic proteins were applied. According to the presented results in Section 3.2, addition of surfactants to those protein solutions leads to the formation of protein/surfactant complexes. When a solid surface was contacted with the protein/surfactant solution, formation of an uniform protein coverage was observed over a wide surface area (Fig. 4.2). Furthermore, it could be demonstrated that functionalized protein chimeras maintain a high level of activity after assembly on solid surface. Hence, this novel method guarantees an excellent accessibility of reagents to protein fused domains, which is a result of ordered monomer orientation. In conclusion, a mathematical model was deduced, which reveals a direct dependency between the protein to surfactant ratio and the number of assembled protein layers.

Figure 4.2: Common protein coating methods versus developed method. A solution containing SbsC (200 ng µL⁻¹) without or with SDS (4 mmol L⁻¹) was contacted with a silicon wafer. When using common methods (left), without addition of ionic surfactants, liquid AFM images reveal a ‘hilly’ surface structure. It was found that functional, protein-
fused domains (balls) are partially embedded into the assembled protein layer (P). Hence, these embedded domains are not accessible for reagents. In contrast, the right image shows an uniform SbsC layer, which was generated with the novel surface coating method. Addition of ionic surfactants supports the formation of a well defined protein layer, where all protein-fused domains are exposed into the medium (scheme below).

4.2.1 Influence of surfactant concentration

Surface active proteins are structured in a large hydrophilic domain and a more or less distinct hydrophobic patch. Hence, they act as high molecular weight biosurfactants in physiological processes (Penfold et al., 2012). Synthetic surfactants are usually organic compounds that reveal structural analogy to biosurfactants. They commonly consist of both a small polar head group and a large hydrophobic tail. Hence, a surfactant molecule is generally formed by two parts with different affinities for a solvent. For that reason, they self-assemble in globular aggregates (called micelles) at a certain concentration. The intercalation of similar polarized components prevents repulsive interactions of surfactant molecules in solution. Nevertheless, at even higher concentrations, ionic surfactants such as SDS and CTAB induce protein unfolding (Zhang et al., 2011b).

As mentioned in Section 3.2.2, formation of free surfactant micelles in protein/surfactant solutions can be neglected, since particles of appropriate size were not detected in DLS investigations. Depending on the experiment, either the critical micelle concentration (CMC) was not exceeded or surfactant solution were added gradually to protein solutions. Wei et al. (2003) demonstrated that the latter option enables proteins to bind a high amount of free surfactant molecules, which apparently increases the CMC of added surfactants. In accordance with published studies, the CMC value of surfactants increases with increasing protein concentration (Ghosh & Banerjee, 2002; Wei et al., 2003). Detection of particles larger than surfactant micelles at a surfactant concentration lower than the CMC indicates that surfactant molecules adsorb on the protein surface and a surfactant/protein complex is formed. But take into account, that addition of surfactants does not automatically result in the formation of surfactant/protein complexes. This is because surfactant/protein interaction can just occur at a critical aggregation concentration (CAC). Considering that the existence of surfactant micelles at surfactant concentrations around the CMC induce protein unfolding, two extreme cases have to be focused on. (1) surfactant concentrations below the CAC do not affect the assembly behavior of surface active proteins. And (2), surfactant concentrations higher than the CMC finally induce an unfolding of proteins (Tofani et al., 2004; Ruiz-Peña et al., 2005; Chodankar et al., 2007). The region in between both extreme cases comprises a range where
proteins are complexed with surfactant molecules, but no significant unfolding of proteins occur (Zhang et al., 2011b).

In accordance with DLS investigation (Section 3.2.2), primarily large particles were observed, when low surfactant concentrations were added. It can be assumed that those large particles definitely represent protein aggregates. Because DLS measurements yield no information about structural composition, formation of surfactant/protein complexes cannot be excluded. Addition of higher concentrations of surfactant leads to a dramatic decrease of detected particle size. These detected particles are slightly larger than protein monomers. Considering, that aggregates of surface active proteins are in rapid equilibrium with monomers, it can be hypothesized that an increasing surfactant concentration leads to the disassembly of protein aggregates by forming surfactant/protein complexes, which are slightly larger than single protein subunits. Therefore, it can be assumed that the observed surfactant/protein interaction affects assembly behavior of surface active proteins.

Figure 3.6 exemplarily illustrates the direct effect of different deployed surfactant concentrations on the number and homogeneity of produced protein layers of the class II hydrophobin HFBI. Starting at low surfactant concentrations, the formation of protein bilayers is observed. Above a critical surfactant concentration protein layer thicknesses thinner than that expected for a bilayer were obtained. Apparently ideal protein monolayers were preserved if a certain surfactant concentration is exceeded. Hence, based on the determined data, a direct dependency between surfactant concentration and assembled protein layer thickness can be assumed. Zhang et al. (2011c) reported layer thicknesses thicker than that expected for an HFBII-monolayer when adding SDS concentrations between $2.5 \times 10^{-4}$ and $5.0 \times 10^{-4}$ mol L$^{-1}$ (negative logarithm of [SDS] ranges between -3.3 and -3.6; see Figure 3.6) to a 100 ng µL$^{-1}$ concentrated HFBII solution. In accordance to the high standard deviations in the given range, the described conditions lead to the formation of incomplete bilayers.

The effective size of a macroscopic particle can be sufficiently described by a theoretical hydrodynamic radius $R_H$. However, $R_H$ offers no conclusion about the shape of a particle. The three-dimensional structure of hydrophobins in solution was intensively studied and was reported to be compact and globular (Zangi et al., 2002; Hakanpää et al., 2004). Furthermore, at air/liquid interfaces the spherical monomers of class II hydrophobins are always arranged in pairs (Kisko et al., 2009). In contrast to hydrophobins, mature S-layer proteins are very flexible and elongated molecules (Pavkov et al., 2008). This elongated shape of S-layer proteins is a plausible explanation for the high standard deviation ($\pm 3.2$ nm) of the $d_H$ value.
in Section 3.2.2, because DLS simplifies the molecular shape to a rigid spherical body. In order to enable a qualitative assessment about the structure of surface assembled protein monomers, the determined film thickness of each protein monolayer (see Tab. 3.2) was plotted against the number of amino acids of the examined protein. The plot in Figure 4.3 reveals a linear correlation between both parameters. The correlation coefficient $R^2$ is 0.9982. A linear correlation indicates that the shape of used monomeric proteins, which are assembled on solid surfaces is almost spherical. On the basis of obtained results, class II hydrophobins and truncated S-layer proteins can be sufficiently described by a spherical model. Hence, the active protein surface area ($A_{O,\text{protein}}$) can be calculated via a simple spherical harmonic,

\[
A_{O,\text{protein}} = \pi \cdot d_{layer}^2
\]  

(4.1)

where $d_{layer}$ is the determined layer thickness of an assembled surface active protein. With sample preparation according to procedures described in the method section, the amount of surfactant to be added to the protein solution is calculated on the basis of this simplification.

In contrast to the observed linear dependency of class II hydrophobins and truncated S-layer proteins, the class I hydrophobin Ccg2 does not fit this simplification. The deviation of Ccg2 from the derived model can be explained by its ability to form complex protein superstructures. The self-assembly of some Ccg2 monomers results in the arrangement of rodlet-like unities at solid surfaces (shown in Figure 3.8). Hence, this finding strongly supports the assumed simplification.

![Figure 4.3: Thickness of protein monolayer versus amino acid residues.](image)

Layer thickness of eight different surface active proteins are plotted against their number of amino acid residues. This plotting method allows a clear classification into two protein structures. Different hydrophobins or S-layer proteins (100 ng µL$^{-1}$) were treated with calculated surfactant concentrations. Substrates were contacted with the surfactant/protein solutions for 30 min. After incubation, surfactants were removed and layer thicknesses of assembled protein films were determined by nulling ellipsometry. Mean values ±sd are shown.
4.2.2 Protein encapsulation supports uniform layer formation

In consideration of Figures 3.6 and 4.3 a mathematical model for the formation of surfactant/protein layers on solid surfaces was deduced, which represent four borderline cases; (1) A minimum concentration of ionic surfactants (c_{surf}) is essential for the formation of a protein bilayer:

\[
c_{surf} \geq \frac{A_{O,Liq}}{A_{O,SHG}} \cdot \frac{1}{N_A \cdot V_{Liq}} \quad (4.2)
\]

Generally, in the coadsorption of proteins with ionic surfactants there is very little surfactant absorption on the solid surface for surfactant concentrations below the CMC. After assembly of a protein film on the solid surface, an overlaid surfactant layer was observed. The result implies, that depending on the area of the liquid interface (A_{O,Liq}) a minimum of surfactant molecules seems to be necessary to prevent further immobilization of protein aggregates. The minimal surfactant concentration is indirectly proportional to the effective size of the surfactant head group (A_{O,SHG}). (2) Above a critical surfactant concentration, predominant protein oligomers will be partially cracked. Hence, the solution comprises a mixture of protein monomers and oligomers complexed with surfactant molecules. This mixture leads to an uncontrollable assembly of protein oligomers and monomers. The result is an indefinable protein layer formation, which is confirmed by high standard deviations from at a certain surfactant concentration in Figure 3.6 [2]. This break point reflects the CAC, above which the formation of surfactant/protein complexes is induced (Wei et al., 2003; Ruiz-Peña et al., 2005). Depending on the degree of surfactant/protein interactions, disassembly of protein aggregates passes three different stages. As a result, the total solvent accessible surface area (SASA) of proteins increases with an increasing surfactant concentration. Initially, the surfactant concentration correlates with the SASA of protein oligomers (A_{O,pol}). The total SASA of protein oligomers depends on the oligomer concentration c_{pol}:

\[
c_{surf} \leq \frac{A_{O,pol}}{A_{O,SHG}} \cdot c_{pol} \cdot a \quad (4.3)
\]

With increasing surfactant concentration, more and more oligomers are broken. (3) When a certain surfactant concentration is reached, mainly protein monomers are present. In contrast to equation (4.2), the surfactant concentration depends on the SASA of protein monomers (A_{O,protein}). The total available SASA of protein monomers depends on the monomer concentration c_{protein}. 

4.2 Controlled assembly of protein layers supported by ionic surfactants

\[ c_{surf} \geq \frac{A_{D,protein}}{A_{0,SHG}} \cdot c_{protein} \cdot b \]  \hspace{1cm} (4.4)

Finally, (4) an unfolding of protein monomers is induced, if an overcritical surfactant concentration is reached.

\[ c_{surf} \leq \frac{A_{D,protein}}{A_{0,SHG}} \cdot c_{protein} \cdot c \]  \hspace{1cm} (4.5)

Structure-dependent correction factors (a, b and c) consider structural equilibrium between multimers and monomers (Fig. 3.6 [2] and [3]), as well as the structural change of a spherical monomer into a random coiled structure by unfolding when adding a overcritical concentration of surfactant molecules (Fig. 3.6 [4]). Each correction factor is based on the ratio of two \( \rho \)-parameters.

The \( \rho \)-parameter of a structure is defined as the ratio of its hydrodynamic radius \( R_H \) and radius of gyration \( R_G \). It is a mathematical expression to describe molecular architectures (Burchard et al., 1980). For solid spheres, like the small, globular hydrophobins, the \( \rho \)-parameter is about 0.774 (Burchard et al., 2003). In contrast, random coiled, polydisperse macromolecules, like unfolded proteins, are characterized by a \( \rho \)-parameter around 2.05 (Burchard et al., 2003). Protein oligomers behave similar to random coiled polymers (Sunde et al., 2008).

4.2.3 Model for the formation of protein monolayers

The controlled influence of surfactants on size distribution of polymerization reactions is well studied (Jang et al., 2004; Chern, 2006). Assembly of biopolymers in solution represents a comparable kinetic controlled system, where protein monomers approach via Brownian motion. Finally, protein specific regions lead to association of monomers. Considering systems of proteins and ionic surfactants, electrostatic interactions drive the formation of protein/surfactant complexes. It was generally observed that surfactant molecules bind to proteins such as BSA and lysozyme to form mixed aggregates (Ruiz-Peña et al., 2005). In the first step, charged surfactant head groups bind to charged amino acid residues of proteins (Ruiz-Peña et al., 2005; Miller et al., 2008), and subsequently hydrophobic tails interact with hydrophobic protein domains (Miller et al., 2008; Zhang et al., 2011b).
The self-association of class I and class II hydrophobins is driven largely by hydrophobic interactions, which leads to the formation of predominantly dimeric and tetrameric structures (Kisko et al., 2008) or rodlets after assembly at interfaces (Wang et al., 2004). This process can be prevented by addition of surfactants. Zhang et al. (2011b) discussed masking of the hydrophobic patch of HFBII, which results from hydrophobic HFBII/surfactant interactions. Furthermore, in comparison to less tightly bound proteins such as BSA and lysozyme, the more compact and tightly bound hydrophobins show a higher surfactant tolerance. This results in higher stability of the native protein form (Zhang et al., 2011b). For these reasons, the very high affinity of surface active proteins for surfactants was used to hinder self-assembly (Linder et al., 2001).

On the other hand, low contact angle values (see Tab. 3.2) imply more weakly binding of surfactant molecules to proteins via hydrophobic interactions. This weakly binding allows a partial displacement of protein complexed surfactant molecules, when the surfactant/protein complex approaches to the solid surface. As a result, S-layer and hydrophobins always orient themselves with their more hydrophobic outer face against the interface (Pum et al., 1995). Hence, the amino- and carboxy-containing hydrophilic hydrophobin domain is exposed to the liquid (Sžilvay et al., 2007), which allows genetically or posttranslational protein modifications.

The applicability of the novel method was shown for various substrates, e.g. Teflon, paraffin waxed surfaces, polystyrene, and metal oxides. In other cases, silica wafer were treated with a surfactant/protein solution, which apparently supports the efficiency of the novel method, when using SDS. The adsorption behavior of ionic surfactants strictly depends on the surface charge. The point of zero charge for porous and non-porous silica is around pH 2.82, and silica surfaces possess significant dissociation of silanol groups at pH > 7 (Goyne et al., 2002). Below the CMC, the anionic SDS shows a low affinity for the negatively charged silica surface, and no detectable adsorption is observed (Penfold et al., 2002). Therefore, SDS is not significantly competing for available adsorption area. To densify silanol groups, individual silicon wafers were treated with ‘Piranha’ acid. The deprotonation of generated surface silanol groups can be achieved by treatment with water or Tris buffer (50 mM, pH 8.5). The pH value of the SDS/protein solution was adjusted to pH 8.5.

By including these results a model for the proposed mechanism of the assembly of protein monolayers on solid surfaces was developed (Figure 4.4). Oligomerization of native, surface active protein monomers (N) to structured aggregates (N_{2d}) is well established (Sleytr et al.,
Generally, the equilibrium of this aggregation is strongly shifted to the side of protein multimers. This aggregation process can be hindered by the addition of surfactants (S), which results in the formation of surfactant/protein complexes ([NS]x,y, Fig. 4.4a). At higher surfactant concentrations, the interaction between proteins and surfactants increases, which leads to the breaking of protein multimers. It was already shown that interaction of proteins and surfactants does not immediately lead to an unfolding of proteins (U), shown in Figure 4.4c.

At a certain time of incubation (approximately 30 min), a substrate can be contacted with the surfactant/protein solution. Zhang et al. (2011c) reported a preferred interaction between the class II hydrophobin HFBII and the surface, despite the presence of surfactant molecules. Hence, it could be hypothesized that weakly bound surfactant molecules at the hydrophobic protein part will be displaced, when a surfactant/protein complex approaches to the surface (Miller et al., 2008). As a consequence, the surfactant/protein complex attaches to the solid surface and serves as an initial point for further surface coating. The formation of an uniform protein/surfactant layer is driven by an ongoing displacement of surfactant molecules (Fig. 4.4c). However, the electrostatic interactions between surfactant molecules and proteins seems to be much more favored (Ruiz-Peña et al., 2005; Miller et al., 2008). As a result, the protein covering surfactant layer is not replaced, and acts as an inhibitor for a further assembly of surfactant/protein complexes.

\[
\frac{A_{O,protein}}{A_{SHG}} \cdot c_{protein} \cdot 0,516 \leq c_{surf} \leq \frac{A_{O,protein}}{A_{SHG}} \cdot c_{protein} \cdot 5,297
\]  

(4.6)

As demonstrated in Figure 3.6, the described deposition process occurs at a certain protein to surfactant ratio. Both borderline cases of the derived model can be described by a mathematic formula. Referring to equations (4.4) and (4.5), the ideal range of \( c_{surf} \) depends on the ratio of the \( A_{O,protein} \) to the effective surfactant head group area (\( A_{SHG} \)). Structural changes in consequence of either protein unfolding or the presence of multimers are arithmetically included via correction terms as included in equation (4.6).
Figure 4.4: Schematic drawing of interactions between surface active proteins and surfactant molecules. The upper part reflects the equilibrium between native protein monomers (N) and structured aggregates in solution without surfactant. The addition of surfactants (S) leads to the formation of surfactant/protein complexes (NS_x) and protein monomerization, as well as to unfolded proteins (U). Adsorbed NS_x ([NS_x]_ad) serves as nucleation point for growing of protein/surfactant layer. After stripping the surfactant layer, a homogenous and regular structured protein layer becomes obvious.

4.2.4 Model for the formation of protein bilayers

For the addition of very low proportion of surfactants, determined thicknesses of protein films are closely concordant with a calculated bilayer of the respective surface active protein. In comparison to an ideal bilayer, where protein monomers are positioned exactly on top of one another, hydrophobin assemblies showed a maximum deviation of 5%. The minor deviation might be explained by the globular structure of hydrophobins, which leads to a slightly tilted dimer orientation. This effect was also observed at an air/water interface (Kisko et al., 2009).
In contrast to investigated hydrophobins, S-layer proteins do not form a simple superimposed bilayer. Determined layers are 10% thinner than an expected ideal double layer. In all probability, the more porous structure of S-layers leads to the formation of an offset arranged protein layer (SÁRA & SLEYTR, 2000), which is set slightly lower.

The question now is; why should the addition of smaller amounts of surfactants leads to an increase in the thickness of observed protein films? In contrast to the formation of an uniform protein monolayer, the situation is slightly different in the case of bilayer assembly. To avoid a chaotic surface-assembly of protein monomers and oligomers, which leads to an uneven protein film, here, the formation of defined oligomer structures is desired. A vast number of studies and reviews deals with the complex impact of additives on the attraction of surface active proteins. For example, positive charged metal ions with high charge densities, could serve as aggregation mediators by compensating surface charges (PUM et al., 1994; 1995). Furthermore, the increase of ionic strength or a slow pH-shift near the isoelectric point of used proteins rises the probability of forming multimers (Section 3.3.2; LINDER et al., 2002). These charge masking effects were used to promote in vitro self-assembly of protein subunits, which increases the amount of structured protein assemblies in solution. Depending on activity and ionic strength of used additives, a direct dependency on the protein concentration is expected. Hence, the demand of additives can be calculated using an extended variant of the rule of three. Due to their distinct hydrophobic patches, hydrophobins preferentially form dimers or tetramers in aqueous solutions via hydrophobic interactions to conceal a large portion of their hydrophobic patch (HAKANPÄÄ et al., 2006). Those oligomeric assemblies represent building blocks of a surface assembled protein bilayer. Thereby it is useful, that the genetically modifiable C- and N-terminus are located in the hydrophilic protein region. Consequently, fused tags are exposed to the medium after immobilization onto solid surfaces. Measured contact angles of assembled HFBI (54.1° and 56.6°) and Ccg2 (56.4°) samples confirm this assumption. In analogy to the formation of a protein monolayer, addition of ionic surfactants suppresses a further oligomer assembly on the solid surface by capping the bilayer. Thereby, the very low concentration of added surfactant has to be directly proportional to the area of the coating surface. Furthermore, SCHUSTER et al. (1999) reported on the supportive effect of charged surfactants on the crystallization of S-layer lattices.

On the basis of self-assembly theories concerning hydrophobins (KISKO et al., 2008; LINDER et al., 2002) or S-layer proteins (PUM et al., 1994; 1995) as well as the obtained experimental results, supported formation of dimer and tetramer structures of hydrophobins and small assemblies of S-layer proteins seems to be essential for assembly of multilayered surface...
structures. Due to their high affinity for proteins, surfactant head groups interact with the exposed hydrophilic site of protein assembly, which leads to the formation of the protein capping layer. A low and imperfect surfactant surface coverage seems to be sufficient to hinder further superficial agglomeration of proteins (Fig. 4.4d).

$$c_{surf} \leq \frac{A_{O,Liq}}{A_{O,SHG}} \cdot \frac{1}{N_A V_{Liq}} = \frac{(2 \cdot A_{CA} + x \cdot A_{O,IF})}{A_{O,SHG}} \cdot \frac{1}{N_A V_{Liq}}$$  \hspace{1cm} (4.7)

According to equation (4.7), a maximum of added $c_{surf}$ was obtained, which depends on the $A_{O,Liq}$ and the degree of surface coverage of used surfactant. In primary, the effective $A_{O,SHG}$ seems to be the crucial factor for the agglomeration of surfactant molecules at interfaces. Depending on the wettability of the directly adjacent medium, two preferred orientations of ionic surfactants can be assumed. At hydrophilic/hydrophilic interfaces, like water/protein interfaces, surfactants form bilayers with exposed hydrophilic head groups to minimize electrostatic repulsions. Hence, at the contact area ($A_{CA}$) between liquid and solid substrate, the formation of a surfactant bilayer is preferred. In contrast, at hydrophilic/hydrophobic interfaces, comparable to water/gas interfaces ($A_{O,IF}$), surfactants tend to the formation of a surfactant monolayer. As a result, the correction factor $x$ recognizes that the bordering interface can be hydrophobic or hydrophilic as well.
4.3 Excitable oil droplets

This section discusses the ability of a resonance energy transfer from quantum dots to auto fluorescent proteins at an oil/water interface of AOBs. For the ‘proof of concept’, the number of ingredients was restricted to two main components. On the one side, CdSe QDs dissolved in an organic solvent were assigned as the fluorescent donor. On the other side, a fluorophore-tagged hydrophobin in aqueous solution served as the fluorescent acceptor. To create a homogeneous oil-in-water emulsion, oil was readily dispersed in the aqueous solution using ultrasonication (see Section 2.7.4). From the experimental data, an initial model concept was applied (shown in Fig. 4.5).

The obtained characteristics of the fluorescence intensity plots are found in Figure 3.3.3 and 3.3.4 indicate a time-dependent assembly process at the oil/water interface. In the first step, hydrophobins adsorb to the hydrophobic droplet surface, followed by a rearrangement of the protein subunits. In a second step, surface touching QDs interact with the proteins. These dynamic interactions lead to FRET with low efficiency. Increasing protein concentration finally favors the formation of protein/QD complexes. An increased presence of this species can explain the upward curvature in Figure 3.18, which is representative for a static fluorescence quenching. Depending on the protein concentration, phase separation of the oil/water emulsion occurred after a certain period of time.

![Figure 4.5: Schematic drawing of an interfacial FRET.](image)

The organic phase, containing the FRET donor (QDs), is stabilized by a mediator (hydrophobin) in an aqueous solution. If the mediator-fused acceptor (fluorophore) is in appropriate distance (1-10 nm) to the donor, a FRET event can occur.
4.3.1 Protein adsorption and rearrangement at oil/water interfaces

Hydrophobins are highly surface active proteins that adhere tightly to hydrophobic/hydrophilic interfaces and reverse their wettability (MACINDOE et al., 2012). Analogous to synthetic surfactants, hydrophobins are able to reduce the surface tension of aqueous solutions, which results in stabilization of immiscible binary phase systems (ASKOLIN et al., 2006). The denser the packing of hydrophobin subunits at the interface is, the larger the reduction in surface tension will be (ALEXANDROV et al., 2012). As a result of hydrophobin accumulation at the oil/water interface, phase boundaries tend to blur. This effect leads to a state of disorder, which extends the interface. Hence, the distance between donor and acceptor increases and energy transfer efficiency decreases. Furthermore, the construction of a stable and delimited two-phase system is essential to minimize side reactions (e.g. exchange of substances, interfusion, or degradation processes). In order to end up with a sharp oil/water interface, either the solutions, the composition of each phase boundary, or the protein design has to be adapted.

4.3.1.1 Effects of oil phase composition

1-Octadecene (ODE) was selected as the dispersant for QDs. It is an aliphatic hydrocarbon compound, which consists of a linear chain of 18 carbon atoms. Due to its chemical structure, the \( \alpha \)-olefin ODE leads to an increased electrostatic repulsion between the aliphatic carbon chains and water molecules at the oil/water interface. It is characterized by a very low surface tension coefficient (\( \sigma = 28 \text{ mN m}^{-1} \)) and a high viscosity (TROPEA et al., 2007). An increased repulsion between the oil and water phase results in a sharper interface. Therefore, molecules near the interface will form more ordered layers (VAN BUUREN et al., 1993). However, ODE already freezes at 17°C. To lower the operation temperature, ODE was replaced by mineral (\( T_m = -30°C; \sigma = 31 \text{ mN m}^{-1} \)) or olive oil (\( T_m = -6°C; \sigma = 33.1 \text{ mN m}^{-1} \)) (LIU et al., 1994; BERG, 2009). As a result, long-term stability of protein stabilized oil/water emulsions was increased (data not shown). A negative side effect is an increased surface tension coefficient which results in a decreased repulsion at the oil/water interfaces.

In addition to the selection of appropriate QD dispersants, the composition of the oil/water interface can be modified. For example, adding phospholipids (PL) increases structural integrity of the oil/water interface. These molecules consist of a hydrophobic tail and a hydrophilic phosphate group. Therefore, PL not only align side by side with aliphatic chains
to form structured layers at the interface of oil-droplets but also maintain the linearized order of molecules adjacent to the droplet surface (KAGANGER, 1999). Due to hydrophobic interactions between PL tails and surface ligands (e.g. trioctylphosphine oxide (TOPO)) of QDs (DUBERTRET et al., 2002), they could support interfacial arrangement of QDs. Initial experiments considering the applicability of phosphatidylcholine, the major PL found in plant oil bodies (TZEN & HUANG, 1992), were recently performed in this work. As a result, the long-term stability of aqueous emulsions against oil body coalescence was significantly improved by up to one month (data not shown).

4.3.1.2 Influence of interfacial anchoring domains Hydrophobins

Both tunable options, discussed above, are not able to prevent droplet unification, when they touch each other. In order to prevent this, droplets were surrounded with a hydrophobin layer. It should be noted that the wrinkled hydrophobin monolayer prevents OSTWALD ripening and provides spheres with longevity (BASHEVA et al., 2011). However, the stability of the oil/water interface depends critically on the conformational stability of the hydrophobin layer and structural flexibility of the hydrophobin monomers attached to the interface (DE VOCHT et al., 2002; HAKANPÄÄ et al., 2006). As it was calculated in Section 3.3.6, at least two hydrophobin molecules apparently participate in the formation of a stable QD/protein complex. Such QD-interacting hydrophobin molecules are not completely available for the formation of a stable interfacial monolayer (DE VOCHT et al., 2002). Consequently, the resistance of the hydrophobin film is strongly reduced by the formation of QD/protein complexes at the oil/water interface at a certain QD/protein ratio. Hence, it can be hypothesized that high QD concentrations significantly hinder the ability of hydrophobins to attach to the oil/water interface, and to stabilize oil droplets. This results in a decreased long-term stability of highly concentrated emulsions, as seen in Section 3.3.4. High resolution observation methods, for example freeze fracture electron microscopy or neutron reflectivity studies, can provide detailed information about spatial arrangement of proteins and QDs in the oil/water interface.

As obtained in Section 3.3.2, hydrophobin domains have an impact on the fluorescence intensity of the fused tRFP domain. Therefore, class I hydrophobin-based Ccg2-tRFP shows a more reduced fluorescence intensity compared to the class II hydrophobin-based HFBI-tRFP. It was found that a decreasing fluorescence intensity correlates with a decrease in surface tension from BSA (54 mJ m$^{-2}$) as a standard model protein to HFBI (42 mJ m$^{-2}$) and finally to SC3 as a representative of class I hydrophobins (27 mJ m$^{-2}$) (AMARAL et al., 2002; ASKOLIN
The surface tension as a measure for surface activity of proteins, mainly depends on the ratio and distribution of hydrophilic and hydrophobic amino acids that are surface exposed (Bull & Breese, 1973). This supports the hypothesis that the more surface active the anchoring domains, the more intense is the unwanted interaction with the fused tRFP in solution (Lumsdon et al., 2005). However, it also compromises the structural integrity of the fluorophore and finally affects the FRET efficiency.

Alternative proteins

To optimize the stability of AOBs, the interface anchoring domain can be replaced by suitable surface active proteins or peptide sequences. As previously mentioned, S-layer proteins belong to the group of surface active proteins (Section 1.2). Nevertheless, because even N- and C-terminal truncated S-layer proteins are 5-11 nm in height, S-layer proteins do not seem to be a suitable alternative for interfacial FRET applications (Debabov, 2004; Kepplinger et al., 2009).

In the 1990’s isolated oleosins are alkaline plant proteins, which stabilize cellular lipid droplets. Each oleosin molecule comprises of three structural domains: amphiphilic N- and C-terminal domains and a central hydrophobic domain. The latter is an oil-body anchoring domain, which penetrates the oil/water interface into the oil droplet (Tzen et al., 1998). It was recently shown, that N- and C-terminal truncated oleosins retained their ability to stabilize AOBs (Peng et al., 2007). Therefore, a synthetic peptide sequence, derived from a central oleosin domain, was fused to the C-terminus of the tRFP-HFBI fusion protein. Preliminary long-term measurements confirm a significantly prolonged total lifetime of AOBs when treated with this fusion protein. This indicates an increased stability of AOBs in the presence of proteins containing the hydrophobic oleosin domain (data not shown).

Customized oil-anchoring domains

Polyhistidine (His)-tags were recently used for immobilizing vesicles and lipid bilayers (Stora et al., 2000; Gizeli et al., 2004). Originally developed for protein purification, Histags allow reversible protein/peptide chelation mediated by Ni\(^{2+}\) complexation (Hochuli et al., 1987). Due to the interaction of a His-tag and modified amphiphiles, which are integrated into the oil/water interface of an oil droplet, specific protein coupling becomes possible (Kepplinger et al., 2009). Use of streptavidin opens another substrate specific immobilization technique. Contrary to the Ni\(^{2+}\)-affinity of a His-tag, streptavidin allows stronger non-
covalent chelation of biotin (GONZÁLEZ et al., 1999). Such a strong interaction is necessary for the precise positioning of vesicles on surfaces (CHRISTENSEN & STAMOU, 2007).

4.3.1.3 Effect of aqueous phase composition

Besides architectural and structural optimization of oil/water interface attaching proteins, composition of the aqueous phase has a strong impact on the protein activity and, thereby, the stability of oil-in-water emulsions. As demonstrated in Section 3.3.2, pH has a significant influence in fluorescence intensity of hydrophobin-tagged fluorophores. Due to the charge neutrality of proteins at the isoelectric point (pI), the pI is characterized by a minimum in protein repulsion, which results in an increased self-aggregation of proteins (ARAKAWA & TIMASHEFF, 1985). The used hydrophobins Ccg2 and HFBI have a theoretical pI of 4.28 and 5.74, respectively (GASTEIGER et al., 2005). Hence, the decrease in fluorescence intensity of the tRFP chimeras near the pI may be a result of uncontrollable agglomeration.

Previous studies have also demonstrated that the ionic strength plays an important role in the hydrophobin oligomerization. As observed for both class I (SC3, HGFI) and class II (HFBI) hydrophobins, high ionic strength suppresses self-assembly of surface active proteins (WANG et al., 2004; WANG et al., 2010b).

4.3.2 Interactions between proteins and QDs

Intermolecular energy transfer can generally be caused either by dynamic or static interactions between donor and acceptor fluorophores. Dynamic energy transfer occurs when the excited fluorophore, which is sensitive to its environment, collides with a particle that can facilitate non-radiative transitions. It is a diffusion controlled process. On the contrary, static energy transfer occurs when at least two fluorophores are involved in the formation of a stable ground-state complex. With regard to the results of the dynamic quenching constant rate and lifetime measurements (see Sections 3.3.7 and 3.3.8), amphiphilic hydrophobins directly interact with the QDs forming protein-QD complexes. Principally, the hydrophobic protein patch interleaves with the hydrophobic side chains of the QD stabilizing ligands while the hydrophilic end extends out into the water aiding in solubility. Because of relatively weak anchoring of the hydrophobins, these complexes are not generally stable under aqueous conditions (YU et al., 2006). However, if QD stabilizing ligands can be displaced, a direct attachment of proteins to the QD surface becomes more attractive. By the addition of a strong
amphiphilic ligand to QDs, their coordinating surface ligands (e.g. TOPO) can be replaced (Walling et al., 2009). Hydrophobins possess a distinct hydrophobic patch, which could displace TOPO from the QD surface.

Irrespective of the complexing mechanism, QDs seem to accumulate at the oil/water interface. The calculated number of binding sites ($n = 2.3$, Section 3.3.6.2) implies simultaneous interaction with two or three protein subunits.

The attachment of QDs at an oil/water interface could be supported by either modifying the QD’s surface or the primary structure of the mediator protein. The first and most common method is the chemical modification of the QD ligand surface by introduction of functional groups (e.g. thiols, carboxylates or amines). These appended functional groups are readily able to conjugate with biomolecules (Zhang & Clapp, 2011).

Site-directed mutagenesis is a versatile tool used to generate customized proteins or peptides, which allows the introduction or exchange of individual amino acid residues. Such minimally invasive modifications support bioconjugate formation. Examples include metal-affinity driven coordination of polyhistidine appended proteins to the Zn atoms of QDs or dative thiol bonding of cysteine residues to the QD surface (Sapsford et al., 2006). For example, Gaponik et al. (2002) investigated the effect of thiol-capping of CdTe QDs, which represents the formation of a naturally protective sulfur-capped surface. In this context, site directed mutagenesis offers the possibility to introduce thiol groups at the oil/water attaching protein patch. Hence, modified proteins act as pronounced QD anchoring points.

In an elegant combination of both methods, Sapsford et al. (2006) showed that an engineered maltose binding protein expressing a positively charged leucine-zipper domain enables electrostatic interactions with the negative surface of dihydrolipoic acid functionalized QDs. In this thesis, the interaction between an engineered zinc-finger motif and ZnO-QDs, a representative of the II-VI semiconductors group, has been analyzed (see Section 3.3.6.3). Comparable to helix-turn-helix or leucine-zipper motifs, zinc-finger motifs are distinct DNA-binding peptide sequences, which have been identified in eukaryotic transcriptional regulatory proteins. Due to their characteristic formation of four invariant cysteine and/or histidine residues, zinc-finger motifs are able to coordinate one or more zinc ions (Klug, 2010). Several studies demonstrated that the chelation of radioisotopic zinc(II) can be quantitatively inhibited by cobalt(II), copper(II) and cadmium(II) (Predki & Sarkar, 1992; Scotland et al., 1993). The latter is a main component of CdSe QDs. In addition, six tyrosine residues were appended
to the N- and C-terminus of the zinc-finger motif. These amino acid side chains offer stabilization through aromatic groups. Therefore, a complex between proteins and surface exposed metal ions of QDs should form preferentially at this artificial peptide sequence. As demonstrated in Section 3.3.6.3, this artificial peptide sequence affects the emission intensity of ZnO QDs, which is seriously dependent on the QD/protein ratio. From calculation models, it can be concluded that the first concentration maximum in Figure 3.20 corresponds approximately to a protein monolayer, which completely covers the accessible surface of the ZnO QDs. This implies a targeted complex formation between QDs and proteins. In future studies, the influence of the artificial zinc finger motif on CdSe QDs has to be studied in detail.

4.3.3 The FRET event

In a model approach, the signal transduction across an oil/water interface was successfully demonstrated, using inorganic QDs and organic fluorescent proteins. Both components obviously exhibit a sufficient spectral overlap for a non-radiating energy transfer. Briefly, the spatial arrangement of hydrophobins at the oil/water interface was visualized using high resolution fluorescence microscopy. As mentioned in Section 3.3.1, a pronounced red fluorescent ‘corona’ was obtained for the discussed model approach, when energy donor was excited. When the donor/acceptor pair was interchanged, oil droplets were apparently not surrounded by a red fluorescent ‘corona’ (see Fig. C.5.). Both findings were initial indices for a functional FRET. Efficiency of energy transfer was determined by the variation of the relative concentration of donor and acceptor fluorophores. Finally, lifetime measurements were evidence for a successful FRET. However, turbidity of the oil-in-water emulsions complicated measurement of transmitted light.

In an ideal scenario, 100% of QDs are excited by incident light. Regarding the applied setup, the oil/water interface represents an excellent reflective surface, because ODE ($n=1.444$) has a higher refractive index than water ($n=1.333$). For this reason, incident light is partially scattered at the oil/water interface, and therefore single QDs are not transferred into the excited state. Nevertheless, despite successful proof of concept, numerous spectral parameters still need to be optimized to realize prospective implementation in diagnostics, medicine or technique.
4.3.3.1 Increase of FRET efficiency

The performance of a FRET system is reflected by the FRET efficiency, which is the number of acceptor-transferred photons per donor-absorbed photon. FRET efficiency encompasses parameters such as Förster radius of the FRET pair, spectral overlap, donor quantum yield, and the orientation of the transition dipoles (Lakowicz, 1999) and depends on the immediate environment of the donor.

Förster radius

The Förster radius is the distance between the donor/acceptor pair at which the rate of energy transfer is half that of the natural decay rate and depends on the spectral overlap between donor and acceptor. It should be noted that FRET efficiency decreases by the inverse sixth power of the distance. Hence, FRET efficiency can be significantly increased when the donor/acceptor pair converges. In addition, an increase in the stability of the donor/acceptor conjugates also affects the FRET efficiency. Optimization methods are described in detail in Sections 4.3.1 and 4.3.2.

Spectral overlap

The spectral overlap integral can be evaluated from the overlap area of the donor emission spectrum and the acceptor absorption spectrum (Dexter, 1953). On one hand, choosing FRET pairs that feature a large degree of separation between donor emission peak and acceptor excitation peak reduces photobleaching effects. In this case, photostability increases. Otherwise, a strong spectral overlap between donor and acceptor maximizes the rate of energy transfer. FRET measurements should be a compromise between both parameters.

Donor quantum yield

The lower the photoluminescence quantum yield of the donor is, the more photon energy will undergo non-radiative decay, and therefore, it is no longer available for FRET events. Considering a QD/protein conjugate, either QDs or proteins can act as FRET donors. In QD/protein conjugates where QDs act as the FRET donor, the quantum yield of QDs can be increased by selecting reaction conditions that minimize surface disorder and surface degradation, enabling good passivation of the QDs. Recently, detailed investigations of these factors enabled reproducible production of high quantum yield (50-85%) CdSe QDs (Donegá et al., 2003). Initially, these QDs were applied in the first experiments for studying the basic
system functionality. In order to reduce surface defects, QDs can be passivated with a thin inorganic layer (ZHANG & CLAPP, 2011). However, first investigations with CdS/ZnS core/shell QDs show no significant impact on FRET efficiency (ČAPEK et al., 2009).

4.3.3.2 Advantages and perspective applications of excitable oil droplets

As already mentioned, the excitable oil droplet (EOD) setup consists of three adjustable components that provide several advantages in comparison to the common setups. Applications in biological systems generally require water-soluble QDs. Therefore, a variety of techniques, often sufficiently complex, had to be developed to transfer QDs into the aqueous phase. Unfortunately, this usually leads to a dramatic decrease in their fluorescence quantum yield. A phase transfer becomes unnecessary, when both donor and acceptor can be kept in their favored medium. The new setup introduced here allows the tailoring of EODs to the individual needs, e.g. by exchanging components to increase selectivity. Hence, tailored EODs can be exploited as effective signal transducers for the dynamic monitoring of processes or environmental conditions (e.g. nutrition concentration, pH value, or content of salts). With regard to applications in bioanalytics, the possibility to interchange donor and acceptor could be applied for the quantification of micronutrients. For example, all known living cells contain adenosine triphosphate (ATP). ATP is a coenzyme, which is used as an energy carrier for metabolic processes, cell signaling or maintaining cell structures. However, the total quantity of ATP in living cells ranges from 1 to 10 µmol g\(^{-1}\) cell wet weight (BEIS & NEWSHOLME, 1975). ATP can be assayed with the ATP-dependent oxidation of luciferine, catalyzed by luciferases while producing light (TURMAN & MATHEWS, 1996; KENNEDY et al., 1999). This assay is extremely sensitive. Although the assay is very sensitive, it is not sensitive enough to measure extracellular ATP levels. The extracellular ATP level of bacterial cells (5-10 pmol g\(^{-1}\) cell wet weight) is barely one thousandth of the intracellular ATP level (IVANOVA et al., 2006). This is harmful in the case of even smallest degree of microbial contamination of water, food or pharmaceuticals. Thereby, EODs might offer a possibility to detect ATP levels in this molar range. The ability of QDs to absorb a broad spectral range and emit in a narrow and tunable range, can be used to transform a wide spectrum of incoming radiation into amplified intense signal of narrow bandwidth. This amplifier effect of QDs could be exploited for the signal transformation of a luciferase, that is fused to an oil-anchoring protein. Such an EOD setup enables in vitro detection of ATP in aqueous solution.
Another key advantage of EODs is their long-term stability, which is necessary for near-line and long-term monitoring applications. Zampieri et al. (2010) reported on the formation of highly stable coatings by hydrophobins, which can be used to improve the biocompatibility of materials. At the same time, encapsulation of EODs in hydrophobin matrices inhibits the leakage of QDs from the oil phase, which is advantageous when using EODs in an aqueous surrounding (see Section 1.5). More importantly, toxicity of QDs is locked away from the environment. For this reason, it is in principle conceivable to apply EODs in biological systems, e.g. for a wide range of medical applications. Considering applications in biolabelling, this novel technique represents an efficient tool for photothermal cancer therapy (Dreaden et al., 2011; Choi et al., 2012). For a targeted use of a perspective ‘cell laser’, the fusion protein can be extended by a cell type specific antigen. Hence, a tight contact between cells and EODs can be achieved, which increases the efficiency of the developed amplifier.

Moreover, EODs represent microstructured two-phase systems, which offer further advantages. The high surface-to-volume ratio of EODs is advantageous in terms of high mass and heat transfer rates, as well as a narrow residence time distribution (Mills et al., 2007). A faster system response time improves process control and product yields. Small-sized systems are also characterized by lower material and energy consumption (Moharana et al., 2011), which is important for technical applications in industry.

One further example for the realization of the developed setup is given by a bidirectional electron exchange, based on a Dexter electron transfer. The biological pentose phosphate pathway for the conversion of chemical energy from glucose to NADH, the preliminary stage for hydrogen, is very complex. In contrast, the energy conversion efficiency of natural photosynthesis is comparatively low (Kalyanasundaram & Graetzel, 2010). Development of artificial systems is an appealing strategy for producing sustainable fuels (Listorti et al., 2009). Unfortunately, biomimetic alternatives possess several disadvantages: First, synthetic electron mediators are often based on precious and water-unstable metal compounds (Cogdell et al., 2010). Secondly, many synthetic materials have low electron transfer rates, which lead to relatively low efficiencies (Zhao et al., 2012). The lower the efficiency the larger the surface area required for light harvesting. EODs can offer a promising alternative for efficient, cell-free hydrogen production. Based on artificial oleosins, size-optimized oil-anchoring domains can provide a near-zero distance between donor and acceptor. Such customized setups enable relative short electron transfer fluxes from oxidoreductases in aqueous solution to synthetic catalysts, located in the oil phase, and vice versa. In the first
reaction, light could be harvested and concentrated by QDs of different size (Makhal et al., 2010). The captured radiant energy enables the separation of charges across the oil/water interface, whereby an excited electron is transferred to a catalytic center (Cogdell et al., 2010). The following reaction uses the accumulated positive charges of QDs to oxidize water and the last uses the remaining negative charges of the catalytic center for reductive chemistry to generate sustainable fuels.

Hence with such versatility and wide ranging applicability this new methodology has the potential to impart a significant and positive impact on many of today’s important technological challenges.


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Werner, C.; Eichhorn, K.-J.; Grundke, K.; Simon, F.; Grahlert, W.; Jacobasch, H.J. Insights on structural variations of protein adsorption layers on hydrophobic fluorohydrocarbon


Appendix A

Supplemental data for the characterization of assembled protein layers

Figure A.1: Schematic illustration of used hydrophobins in Section 3.1. Mature hydrophobin Ccg2 from *N. crassa* was fused to a 3x HA-tag (upper part). Mature hydrophobin HFBI from *T. reesei* was fused to a R5 peptide sequence (R5P) from *C. fusiformis* (above) or a truncated version (XSR5P, below). Hydrophobin and fused domain were separated by a $(GGGGS)_2$-linker. For protein purification via metal chelate affinity chromatography, all protein chimeras were fused to a N-terminal His-tag (H).
Figure A.2.: Nickel-chelate affinity chromatography for Ccg2-HA. For protein purification via nickel-chelate affinity chromatography, almost all protein chimeras were fused to a N- or C-terminal His-tag. The image exemplarily shows the individual steps of the protein purification of the 15.7 kDa Ccg2-HA. According to Section 2.5, gene expression was induced at an appropriate cell density (lane 1). Cells were harvested (lane 2) after 6 h incubation. At the end of the cell disruption procedure, targeted proteins were found in the insoluble fraction (lane 3), which was washed at least three times (lane 4-6). Ni-IDA column was loaded using three protein extracts (lane 7-9). The flow through was captured (lane 10) and column was washed with Wash buffer A, B and C (lane 11-13). Finally, targeted protein was eluted (lane 14-18). To restore the sepharose matrix, column was washed with SDS and EDTA (lane 19 and 20).
Appendix B

Supplemental data for the development of a surfactant supported protein assembly method

Figure B.1: Schematic illustration of used hydrophobins and S-layer proteins in Section 3.2. The upper part exhibits the hydrophobins Ccg2 from *N. crassa* and HFBI from *T. reesei*, which are fused to a 3x HA-tag, two R5-based peptide sequences, tRFP or GLuc, respectively. The hydrophobin or S-layer protein and fused domain were separated by a (GGGGS)$_2$-linker. Used S-layer proteins are presented in the lower part. Mature S-layer proteins S13240 from *G. stearothermophilus* and SslA from *S. ureae* were fused to a 3x HA-tag. In contrary, a N- and C-terminal truncated version of SbsC from *G. stearothermophilus* was fused to two R5-based peptide sequences. For protein purification via metal chelate affinity chromatography, most protein chimeras were fused to a N-terminal His-tag (H).
Figure B.2: Typical dynamic light scattering analyses of surface active proteins. The diagram reveals the particle size distribution (by intensity) from two protein solutions, containing the class I hydrophobin Ccg2-HA (100 ng µL⁻¹). In a dialyzed protein solution, Ccg2-HA was highly aggregated (red). The amount of Ccg2-HA monomers with a diameter of 6 nm was significantly increased after addition of 2 mmol L⁻¹ SDS (black).

Figure B.3: Liquid AFM topography image of S-layer protein SslA. Silicon substrates were treated with a solution of SslA (100 ng µL⁻¹) and 4 mM SDS using the sessile drop method. Topography image shows a monolayer of SslA after removal of SDS with a maximum height of around 7 nm. The SslA assembly products clearly exhibited the squared (p4) lattice structure with lattice constants of a = 12.8 nm, b = 12.2 nm and γ = 89°. A JPK Nanowizard AFM was used to image the protein film in liquid under ambient conditions at 21 ± 1°C.
Appendix B

Figure B.4: Localized luminescence of assembled *Gaussia* Luciferase (HFBI-GLuc). A well of a 96-well microplate was incubated with a SDS/HFBI-GLuc solution (100 ng µL⁻¹) for 30 min using the surface coating method, described in Section 2.7.2. After addition of buffer and luciferol, luminescence reaction was observed with an Olympus Camedia C-5060 Wide Zoom camera. Only the wall of the 96-well microplate exhibited obvious luminescence. The luminescence intensity first increased and then remained constant.
Appendix C

Supplemental data for the development of excitable oil droplets

Figure C.1: Schematic illustration of used hydrophobins in Section 3.3. Mature class I hydrophobin Ccg2 from *N. crassa* was fused to a tRFP-tag (upper part). The mature class II hydrophobin HFBI from *T. reesei* was C-terminally fused either to eGFP, tRFP, an artificial oleosin (ArtOLEO) or an artificial zinc finger motif (ZiF). Furthermore, HFBI-ArtOLEO was N-terminally fused to tRFP. For protein purification via metal chelate affinity chromatography, all protein chimeras contain a N- or C-terminal His-tag (H).
Figure C.2: Fluorescence spectra of tRFP. The obtained fluorescence excitation (dashed line) and emission (solid line) spectra of Ccg2-tRFP in Tris buffer (100 mmol L$^{-1}$, pH 8.5) is exemplarily shown. Inset illustrates the corresponding energy diagram, also known as JABLONSKI diagram. Excitation/emission spectra are similarly colored as the corresponding state transition in the JABLONSKI diagram.

Figure C.3: TEM image of CdSe/ZnS core-shell QDs. QDs were synthesized according to the one-pot synthesis by BAE et al. (2008). Sample was monitored at 89 kV.
Figure C.4: Schematic illustration of a FRET. Principally, FRET can occur when the emission spectrum of the donor fluorophore (QD, blue line) significantly overlaps the absorption spectrum of the acceptor (P, grey dotted line). FRET also depends upon the distance between both fluorophores, which can typically be up to 10 nm. The sketch on the right site suggests that there is significant energy transfer via a ground state complex [QD…P_n] formation. Excitation/emission spectra are similarly colored as the corresponding state transition in the inset sketch.

Figure C.5: Visualizing of protein/QD interactions at an oil/water interface using fluorescence microscopy. (A) shows excitation/emission spectra of proteins (black) and QDs (grey). Colored insets schematically illustrate the applied filter set to match spectral excitation/emission characteristics of tRFP. (B) Images of 0.5% (v/v) oil-in-water emulsions stabilized by HFBI-tRFP (300 ng µL⁻¹) were recorded using a Zeiss Axio Observer.Z1 microscope with an exposure time of 500 ms. Samples containing CdSe-QDs in the oil phase were observed after 3 h (a) and 72 h (b) using the FS43 DsRed filter set. After 3 h no red fluorescence ‘corona’-like structure was obtained in all focal planes. Even after a certain period of time (72 h), when complete protein accumulation occurred, an extended red fluorescence ‘corona’ becomes obvious. As a negative control, image (c) exhibits a typical oil droplet, surrounded by the tRFP-tagged hydrophobin.
Table C.1: Characteristics of CdS/ZnS core-shell QDs, CdSe QDs and tRFP. Size, excitation and emission maxima, quantum yield, molar extinction coefficient and relative photostability are listed in the table (ORMÖ et al., 1996; YU et al., 2003; MERZLYAK et al., 2007; BAE et al., 2008).

<table>
<thead>
<tr>
<th></th>
<th>CdS/ZnS QD</th>
<th>CdSe QD</th>
<th>TurboRFP</th>
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</thead>
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<tr>
<td>Size [nm]</td>
<td>~ 7.0</td>
<td>~ 3.9</td>
<td>4.2 x 2.4</td>
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<tr>
<td>Excitation max [nm]</td>
<td>372</td>
<td>368</td>
<td>553</td>
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<tr>
<td>Emission max [nm]</td>
<td>488</td>
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<td>574</td>
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<tr>
<td>Quantum yield</td>
<td>0.42</td>
<td>~ 0.05</td>
<td>0.67</td>
</tr>
<tr>
<td>Excitation coefficient ($10^{-3}$ L mol$^{-1}$ cm$^{-1}$)</td>
<td>1933</td>
<td>58</td>
<td>92</td>
</tr>
<tr>
<td>Photostability</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
</tr>
</tbody>
</table>
Authorship Declaration

I, Leopold Gruner, confirm that this dissertation and the experiments presented in it are my own achievement. The dissertation has not yet been presented in this or similar form to any other authority.

Where I have consulted the published work of others, this is always clearly attributed. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this dissertation is entirely my own work. I have acknowledged all main sources of help. If my research follows on from previous work or is part of a larger collaborative research project, I have made clear exactly what was done by others and what I have contributed myself.


Dresden, September 2012

Leopold J. Gruner