Insight into the interfacial self-assembly and structural changes of hydrophobins

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CHAPTER 6

Pore formation of hydrophobin SC3 on a phospholipid bilayer

X. Wang, H. Miedema, and G. T. Robillard
Abstract

The role of hydrophobin SC3 in stabilizing/destabilizing phospholipid bilayers was studied systematically. A small fraction of SC3 incorporated into a DOPC/DOPE (molar ratio 7:3) liposomal membranes within a few hours at room temperature. The structure of SC3 does not change significantly upon interaction with DOPC/DOPE and DPPC liposomes, as determined by both Thioflavin T fluorescence and Circular Dichroism spectroscopy, but an increased level of \( \beta \)-sheet structure was formed when the same samples were incubated at temperatures above 50°C. The incorporation of SC3 into the liposomal bilayer caused enhanced release of calcein. In the first 6 hours of incubation, calcein release was proportional to the amount of SC3 added, whereas after 6 hours the release rate remained constant for all the SC3 concentrations tested. By using a planar lipid bilayer system consisting of DOPC/DOPE, we could show that SC3 forms an ion permeable pore in the lipid bilayer. The pore formation was observed after 10 min of SC3 addition, protein and salt concentration dependent, but voltage independent. The SC3 pores showed obvious heterogeneous conductance states, suggesting that they have various sizes, probably caused by the formation of different oligomeric states of SC3.
Introduction

Hydrophobins are small secreted fungal proteins that have similar hydropathy patterns and eight conserved cysteine residues that form intramolecular disulfide bridges (Wösten et al., 1993; 1994a; van Wetter et al., 1996, 2000a). Two different types of hydrophobins, class I and class II, have been distinguished on the basis of differences in their hydropathy patterns and biophysical properties (Wessels, 1994). Class I hydrophobins self-assemble and form a water-insoluble amphipathic membrane at hydrophobic/hydrophilic interfaces, which can resist 2% SDS at 100°C and proteolysis. Class II hydrophobins self-assemble into a membrane which is less stable in hot detergent. Class I hydrophobins have been identified in both ascomycetes and basidiomycetes, but class II hydrophobins have so far only been found in ascomycetes.

SC3, secreted by Schizophyllum commune, is the best-characterized class I hydrophobin. Its disulfide bridges are believed to stabilize the soluble-state of SC3 in solution and account for its controlled assembly at hydrophilic/hydrophobic interfaces (de Vries et al., 1993; de Vocht et al.,1998; 2000). The o-type glycosylated N-terminal part of SC3 is exposed at the hydrophilic side of assembled SC3 and, therefore, is expected to determine the surface properties of this side (de Vocht et al.,1998). Once self-assembled spontaneously at an air/water interface, SC3 forms a robust, electron-microscopically identifiable monolayer with a rodlet structure and a thickness of around 10 nm (Wösten et al., 1993, 1994a, 1994c). The hydrophobic side of assembled SC3 is composed of a mosaic of 5-12 nm wide rodlets, each of which consists of two tracks of 2-3 protofilaments with a diameter of about 2.5 nm each (Wösten and de Vocht, 2000). The rodlets mimic amyloid fibrils in as much as both bind the dyes Thioflavine T and Congo red (Wösten and de Vocht, 2000; Mackay et al., 2001; Butko et al., 2001). Therefore, hydrophobins have been suggested to represent biologically functional amyloid proteins that facilitate the emergence of fungal aerial hyphae or spores from the medium or their aerial dispersal (Wösten et al., 1994a, 1999).

Soluble-state SC3 in solution was found to consist of various oligomeric states, with the dimeric state being dominant (about 70% in total). The monomeric form of SC3 only exists transiently in pure water. Dimeric SC3 is proposed to be a building block for both solution aggregation and interfacial assembly of SC3 (Wang et al., chapter 3). Soluble-state SC3 can assemble into a so-called β-sheet II state (increased β-sheet structure) via the β-sheet I state, either at an air/water interface or on a hydrophobic surface, such as Teflon spheres, upon heating and adding detergent. The formation of β-sheet II state structure therefore has become a characteristic for hydrophobin SC3 self-assembly and rodlet structure formation. The assembled
SC3 can only be dissolved with neat trifluoroacetic acid (TFA) or formic acid. When the heat/detergent treatment is omitted, an intermediate so-called α-helical state (increased α-helical structure) can be trapped on Teflon and visualized (de Vocht et al., 1998, 2002; Wang et al., 2002). The C-terminal end of SC3 is solvent exposed when SC3 assembles on a Teflon surface in the β-sheet state. A portion of the predicted 2nd loop (C39-S72) not only binds to a Teflon surface, but this region is also responsible for the formation of the α-helical-state structure (Wang et al., chapter 4).

Hydrophobins benefit fungal growth and development in various ways. The water surface tension can be largely decreased, that is from 72 to 30mJ/m², by the self-assembly of SC3 at the air/water interface, which allows the escape of hyphae from the moist aqueous solution into the air (Wösten et al., 1999). Hydrophobins secreted by aerial hyphae can self-assemble outside the cell wall once they encounter the air and form an amphipathic film, with the hydrophilic side interacting with the cell wall, possibly through a lectin-like interaction, and the hydrophobic side facing the air (Wösten et al., 1999, 2001). For example, the air channels in the fungal fruiting bodies and lichens are coated by a hydrophobin film, conferring hydrophobicity to the air channels, and therefore, preventing the channels from being soaked with water (Lugones et al., 1996, 1999). The coated hydrophobin films can also protect against possible bacterial infection of fruiting bodies, or protect some emergent structures against adverse environmental conditions such as water evaporation (Wösten et al., 1993). The amphipathic hydrophobin membrane can also function as a “glue” to combine two incompatible materials together. For instance, in pathogenic fungi, the secreted hydrophobins first assemble on the host surface (hydrophobic), making this surface hydrophilic, after which a water-based glue combines the hyphae or spores to the target surface. Alternatively, the hydrophobin film formed outside the cell wall of pathogens can provide hydrophobicity in attaching a hydrophobic host surface via hydrophobic interaction (Wessels, 1994). Besides serving as a attachment mediator, hydrophobins are also supposed to contribute to the virulence of pathogens. The injection of class II hydrophobin CU of O.novo-ulmin and O.ulmi into the host white elm caused wilting, reduction in transpiration, and electrolyte loss, etc (Takai, 1974). The toxicity of CU was suggested to be the result of plugging xylem vesicles by CU-coated air bubbles or increased host plasma membrane permeability (Setvenson et al., 1979; Russo et al., 1982).

The remarkable biophysical properties of hydrophobins also make them attractive for medical and industrial applications (Wessels, 1997; Scholtmeijer et al., 2001). The hydrophobin SC3 membrane formed at an oil/water interface has been recently characterized to have unidirectional
permeability, with the hydrophobic side permeable to molecules up to 10,000 Da, but the hydrophilic side impermeable to all the molecules tested down to 300 Da (Wang et al, chapter 5). This finding is significant not only for the elucidation of some biological functions of hydrophobins, but also for some innovative industrial applications.

The present investigations addresses the interaction between hydrophobin SC3 and various phospholipids, and the influence of SC3 on the stability of liposomes.

**Results**

*Interaction of hydrophobin SC3 with phospholipid bilayer*

Liposomes (400 nm) were prepared from DPPC (gel phase), DOPC, DOPC/DOPE (7:3 molar ratio), DOPC/DOPS (7:3 molar ratio), and DOPC/DOPE-PEG (94:6 ratio). Mixtures of dansyl-labeled SC3 (dansyl-SC3) and liposomes were incubated for 3 h at room temperature, examined by light (phase contrast) microscopy. The micrographs showed liposomes with a similar size-range distributions for each of the lipid mixtures. The largest liposomes migrated slowly under the microscope and, therefore, were easier to image. When the same samples were illuminated by fluorescence light, only the sample with DOPC/DOPE liposome clearly showed a green fluorescent layer around the large liposomes (Fig. 1B). Overnight incubation of the sample did not increase the fluorescence very much, indicating that the interaction between SC3 and liposome had reached maximal level within 3 h. The dansyl fluorescence from the liposomes was much weaker than that from dansyl-SC3 coated oil-droplets in buffer (see chapter 5).

![Fig. 1](image)

**Fig. 1** Fluorescence micrographs of DOPC/DOPE (molar ratio 7:3) liposomes in the presence of dansyl-SC3. The sample was incubated for 3 h at room temperature before being imaged. A, the sample illuminated with normal light. B, the sample illuminated with fluorescence light. Pictures were recorded with 100 times zoom, and 5 seconds exposure for fluorescence light. Bar indicates 10 μm.
The amount of SC3 that interacts with a DOPC/DOPE liposome was estimated by Sephacryl S-1000 gel filtration chromatography. Dabcyl-labeled SC3 (dabcyl-SC3) was used for these experiments. Previous work has shown that the labeling of SC3 at the N-terminus with either a dansyl or dabcyl group does not change its biophysical properties (Wang et al., 2002). The SC3/liposome mixture was incubated for a given time before being injected onto the gel filtration column. By comparing the elution patterns of the dabcyl-SC3/liposomes after 10 min of incubation at room temperature with the dabcyl-SC3 alone, one observes very small differences, indicating that SC3 hardly interacts with the liposome within this short period of time (Fig. 2 A and B). An additional peak appeared at the elution volume of 15-20 ml when the dabcyl-SC3/liposome sample was incubated for 3 h at room temperature, indicating that a significant amount of dabcyl-SC3 was bound (Fig. 3C solid line). The same sample measured at 660 nm showed a major peak at a volume of about 19 ml, confirming that the 460 nm absorbance peak at 15-20 ml consists of liposomes that are associated with dabcyl-SC3, whereas the peak following it mainly contained free dabcyl-SC3 (Fig. 3C dotted line). The amount of incorporated dabcyl-SC3 was estimated to be about 30% of the total material. An overnight incubated sample showed almost the same result, indicating that no more dabcyl-SC3 was incorporated after 3 h.

Fig. 2 Determination of the amount of dabcyl-SC3 that was incorporated into DOPC/DOPE LUV using sephacryl S-1000 gel filtration chromatography. A, dabcyl-SC3 in the absence of liposomes. B, mixture of dabcyl-SC3 and DOPC/DOPE liposomes, incubated for 10 min at room temperature before being loaded onto the column. C, mixture of dabcyl-SC3 and DOPC/DOPE liposomes, incubated for 3 h at room temperature before being loaded onto the column. Solid line, the absorbance of the eluate measured at 460 nm (absorbance of dabcyl-SC3). Dotted line, the absorbance of the eluate measured at 660 nm (scattered light from the liposomes)
Structural changes of hydrophobin SC3 upon interaction with phospholipid bilayer

It has been known that soluble-state hydrophobin SC3 converts to a β-sheet structure when it self-assembles at a hydrophobic/hydrophilic interface such as air/water or oil/water (de Vocht et al., 1998). The possible structural change of SC3 upon interaction with liposomes of different lipid compositions was examined by both CD and Thioflavin T (ThT) fluorescence (Wösten and de Vocht, 2000; Butko et al. 2001). SC3 was mixed with liposomes as described in the previous section. The ellipticity of CD could only be recorded from 200 to 250 nm due to the interference of lipid. The CD spectrum of SC3 did not change after it was mixed with either DOPC/DOPE liposomes, or DPPC (liquid-crystalline phase) liposomes, irrespective of whether the mixtures were incubated for 3 h or overnight at room temperature. The CD signals for the liposomes in the absence of SC3 are negligible in this experiment (Fig. 3A thick solid line). When the same samples were mixed with Thioflavin T at room temperature, only a small fluorescence increase could be detected for SC3/DOPC/DOPE and SC3/DPPC samples, compared to the sample of SC3 in the absence of lipids (Fig. 3A). The addition of ThT to a liposome sample in the absence of SC3 did not result in fluorescence increase (data not shown). Compared to the same samples after vortexing, in which the maximal level of β-sheet structure has been achieved, the β-sheet structure formation in the SC3/DOPC/DOPE sample was less than 6%, whereas it was less than 2% in the SC3 sample in the absence of lipids. Since the gel filtration chromatography experiments indicated that more than 30% of SC3 interacts with DOPC/DOPE liposome, the data suggest that the lipid-associated SC3 does not change its structure significantly. Interestingly, vortexing of the SC3/DPPC sample resulted in a lower level of ThT fluorescence than that of SC3/DOPC/DOPE (see Fig. 3B, vortex samples), suggesting that the interaction between SC3 and DPPC liposomes might stabilize the SC3 structure and prevent β-sheet structure formation upon vortexing. The ThT fluorescence in both of the SC3/lipid samples, especially for SC3/DPPC, increased significantly after the samples were treated at 65°C for 30 min, and the measurements done at room temperature, indicating that more β-sheet structure had formed, or, more SC3 in the same structure had associated with the liposomes (Fig. 3B). Perhaps due to the presence of saturated acyl carbon chain, DPPC liposomes above the lipid transition temperature function more efficiently in associating with SC3 and converting its structure than the DOPC/DOPE liposomes. The formed β-sheet structure in the SC3/liposomes after heating could be diminished by the presence of 0.1% Tween 80. This differentiates the characteristic of SC3 assemblage formed at an air/water interface or on a Teflon surface. It is likely that
liposome-bound SC3 did not assemble into a β-sheet structure-characterized and detergent-insoluble form in this case, but remained a loose aggregated form.

![Graph of SC3 structural changes upon interaction with various liposomes.](image)

**Fig. 3** Structural changes of SC3 upon interaction with various liposomes. A, Circular Dichroism (CD) spectra of the samples incubated at 65°C for 5 min. Thick solid line, DOPC/DOPE liposomes. Thin solid line, SC3 alone. Dotted line, SC3 mixed with DOPC/DOPE (molar ratio 7:3) liposomes. Dashed line, SC3 mixed with DPPC liposomes. B, β-sheet-state structure determination using Thiofavin T (ThT) fluorescence. Protein/liposome mixtures were incubated for 3 h before being subjected to ThT fluorescence measurements. Black column, SC3 alone. Gray column, SC3 mixed with DOPC/DOPE liposomes. White column, SC3 mixed with DPPC liposomes.

**Destabilization of liposomes upon SC3 incorporation**

Calcein is widely used as a model compound in determining the permeability properties of lipid vesicles. The release can be quantified by determining the fluorescence dequenching of the encapsulated calcein. In this study, calcein release was utilized to determine the liposome stability following incubation with SC3. The liposomes with calcein encapsulated at a concentration of 100mM, were mixed with soluble-state SC3, and the calcein release was determined immediately after the calcein-loaded liposomes were separated from the free calcein by gel filtration chromatography. Fig. 4A shows the time-dependent calcein release from DOPC/DOPE (7:3) liposomes upon SC3 addition to a final concentration of 100 µg/ml. It is clear that SC3 made the liposome more leaky as compared to the control sample. The leakage
was fast at the beginning but became slower after 5 to 6 h of incubation. The slow leakage after 5 h of incubation may be due to liposomes without SC3 incorporated, because the rate of calcein release in DOPC/DOPE liposomes in the first 5 h was SC3 concentration-dependent, but the same after this time period (Fig. 4B). The concentration of SC3 that was added to the DOPC/DOPE liposomes varied from 10 μg/ml to 1 mg/ml. After 6 h of incubation, more than 60% calcein was released with 1 mg/ml SC3, and only 30% with 10 μg/ml of SC3. Obviously, there is a strong non-linear protein concentration dependence in the extent of release (see Fig. 4C). After 5 hours, the SC3-mediated liposomal leakage had largely stopped and the intrinsic leakage from non-SC3 incorporated liposomes dominated.

![Figure 4](image.png)

Fig. 4 Calcein release from SC3-incorporated DOPC/DOPE (molar ratio 7:3) liposomes. SC3 final concentration was 100 μg/ml. A, Calcein efflux was determined immediately after mixing SC3 (to a final concentration of 100 μg/ml) and the sephadex G-50 chromatographed liposomes. B, SC3 concentration-dependent calcein release from DOPC/DOPE (molar ratio 7:3) liposomes. ■, no SC3 added. ●, 0.01 mg/ml SC3. ▲, 0.05 mg/ml SC3. ▼, 0.15 mg/ml SC3. ♦, 0.5 mg/ml SC3. □, 1 mg/ml SC3.

When DPPC was used instead of DOPC/DOPE the liposomes did not leak any (Fig. 5). This indicates that the DPPC lipid bilayer is too rigid to allow calcein to escape and SC3 to incorporate into the membrane. However, when the same samples (in the presence of SC3) were incubated at 65°C for 30 min, and then cooled down to room temperature, a large amount of calcein was released, with almost 100% release for SC3 at 0.5 and 1 mg/ml, and 60% for the control sample without SC3.
Obviously, DPPC liposome becomes more leaky around and above the transition temperature, and this leakage is significantly enhanced by the incorporation of SC3 molecules. This is in agreement with the observation of SC3 structural change observed by CD and ThT fluorescence.

**Possible mechanism of SC3-mediated liposomal destabilization: pore formation on the phospholipid bilayer**

The possibility that SC3 caused lysis of liposomes upon incorporation could be excluded, because the light scattered by the sample did not change upon SC3 addition (data not shown). The possibility of pore formation of SC3 in the phospholipid bilayer was then studied using a planar phospholipid bilayer membrane (black lipid membrane) composed of DOPC/DOPE. After the planar bilayer membrane was formed, SC3 was added from one side of the bilayer and the current through the membrane was recorded at a constant voltage of -50 mV (Fig. 6). The sampling and filtering frequency were 5 and 1 kHz, respectively. The channel activity as shown in Fig. 6 A was typically detected 10 min after SC3 addition. With increasing time, more and more SC3 incorporated into the membrane, resulting in a shift of the current trace further away from the baseline and the disappearance of individual channel openings (compare Fig. 6 A and C). The current profile showed heterogeneous conductance states, indicating that the pores formed might vary in size. The channel formed by SC3 was found to be voltage-independent. The application of both positive and negative voltage (50mV) caused similar ionic current profiles. The channel formation, however, was protein concentration and salt concentration dependent. At low salt concentration (100mM KCl), low protein concentration (7.5 μg/ml) could already cause clear pore formation, whereas at high salt concentration (1M KCl), the lowest
protein concentration that elicited pore formation was about 75 µg/ml. It seems that high salt affects the efficiency of SC3 to form channels. The channel formation was an irreversible process, because the current trace remained after perfusion with fresh buffer (Fig. 6C).

**Fig. 6** SC3 pore formation in a planar lipid bilayer (PLB). A, current profile after 10 min of SC3 addition. B, current profile after 12 min of SC3 addition. C, after the recording of B, the chamber containing SC3 was perfused with 20 volumes of fresh buffer, which took about 20 min.

**Discussion**

Fluorescence microscopy using dansyl-labeled SC3 provides an easy and direct way to determine the interaction between hydrophobin SC3 and unilamellar liposomes. DOPC/DOPE-PEG liposomes have PEG tails at their surface, preventing the adhesion of SC3. DPPC has a saturated 16-carbon chain and the liposomes in the liquid-crystalline state are probably too rigid
to allow SC3 to incorporate. Both DOPC and DOPE lipids have unsaturated acyl-chains, but SC3 only incorporated into DOPC/DOPE liposome, forming a green fluorescent layer on the outside. DOPC has a cylindrical shape and is a bilayer-forming lipid, whereas DOPE has a cone shape and is non-bilayer forming. Thus, the presence of DOPE must have destabilized the bilayer structure of DOPC/DOPE liposome in such a way that SC3 molecules could be incorporated into the bilayer. As a proof, SC3 had no effect on DOPC liposome alone as well as DOPC/DOPS (molar ratio 7:3) liposome. How SC3 resides in the bilayer is not clear, but one can imagine that the hydrophobic core of SC3 binds to the hydrophobic acyl-chains of the lipids, whereas its hydrophilic parts turn inwards to form a channel. It is unlikely that SC3 forms a stable membrane outside the DOPC/DOPE liposome as it does at an air/water interface, because only a limited amount of SC3 was involved in the interaction with lipids, as judged by the weak fluorescence from dansyl-SC3/DOPC/DOPE liposomes and the low amount of β-sheet-state structure formed (see next section).

SC3 does not undergo significant structural changes upon interacting with DOPC/DOPE and DPPC liposomes, as revealed by both CD and ThT fluorescence, but high temperature treatment enhanced the structural change to β-sheet state or to increase the amount of incorporated SC3. Obviously, the lipid bilayer became more fluid upon heating, either increasing the hydrophobic interaction between SC3 and lipids, or facilitating the incorporation of more SC3 molecules. Interestingly, detergent treatment of SC3/liposome eliminated the heat-induced β-sheet structure, suggesting that dissolution of the lipid bilayer also liberates the incorporated SC3 and/or converts its β-sheet-state structure back to the soluble-state. This is in contrast with the well-documented observation that the SC3 membrane formed at an air/water interface or on a Teflon surface is detergent-resistant. It seems to support the model that SC3 incorporated into a lipid bilayer involves loosely associated SC3 oligomers, rather than a rodlet enriched membrane. The facts that SC3 does not convert its structure upon oligomerization in solution (data unpublished), that dansyl-SC3 forms a relatively weak fluorescent layer outside the liposome, and that SC3 forms pores of different sizes in the bilayer (see next section), strongly support this hypothesis.

The incorporation of SC3 into a lipid bilayer caused significant leakage of calcein from the liposome. The reason that hydrophobin SC3 caused more leakage is probably due to pore formation in the lipid bilayer. This was confirmed in the black lipid membrane experiment. The data indicate that SC3 forms ion-conducting pores in the lipid bilayer. Within the time period (about 1 h) of measurement, more and more SC3 incorporated into the lipid bilayer. This observation is consistent with the calcein release experiment, in which the addition of more SC3
caused more calcein efflux from the liposomes. The conductance induced by hydrophobin pores is heterogeneous, indicating that the pores formed might have different sizes. Our recent study of soluble-state SC3 has shown that it exists in various oligomeric states (data not published). It is possible that these different oligomeric states could contribute to the variable pore sizes.

Hydrophobin and amyloid proteins/peptides share many similar properties. The oligomeric forms of an amyloid protein, α-synuclein, have been shown to form highly size-selective channels in a lipid bilayer. The monomeric form, however, can lyse the lipid vesicle through a detergent-like property (Volles and Lansbury, 2002). The pores formed by SC3 have electrophysiological properties that differ from those formed by amyloid peptides/proteins. For instance, Amyloid β protein forms a Zn$^{2+}$-sensitive channel, whereas hydrophobin SC3 does not (data not shown) (Rhee et al., 1998; Lin et al., 1999). The amyloid peptide, amylin, forms voltage-dependent channels, whereas SC3 forms voltage-independent channels (Mirzabekov et al., 1996). The formation of ion permeable channels by amyloid peptides/proteins is proposed to be involved in the pathogenesis of amyloidogenic diseases (Mirzabekov et al., 1996; Volles and Lansbury, 2002). Although a class II hydrophobin, CU, showed high toxicity after being injected to the host white elm, no evidence so far indicates that class I hydrophobins, even from pathogenic fungi, are toxic to their hosts. In contrast, it has been shown that neither soluble-state nor assembled hydrophobin SC3 is toxic for human cell growth (Janssen et al., 2002.). The present study, however, showed that hydrophobin SC3 can destabilize, or damage, the phospholipid bilayer by forming ion permeable pores.

**Materials and Methods**

**SC3 and fluorescent labeled SC3**

SC3 was purified as described previously (Wösten et al., 1993). The purified SC3 was stored after being lyophilized. Before use, a portion of lyophilized SC3 was treated with TFA, dried with a flow of nitrogen gas, and the dried material was dissolved in a desired buffer. The N-terminus of SC3 was specifically labeled with amine-reactive probes, 6-((5-dimethylaminonaphthalene-1-sulfonyl)amino)hexanoic acid, succinimidyl ester (dansyl, Molecular probes) and 4-((4-(dimethylamino)azo) benzoic acid, succinimidyl ester (dabcyl, Molecular probes). The labeling was done according to the published procedures (Wang et al., 2002).
Liposome preparation

All the lipids used in this study were purchased from Avanti Polar Lipids. Each lipid (powder) was weighted and dissolved in chloroform at a concentration of 10 mM, and this stock solution was stored at -80°C. Before use, an aliquot of the lipid stock was dried in a glass tube with a flow of nitrogen gas. For lipid mixtures, different lipid stocks were mixed at a given molar ratio in a glass tube, and the mixture was dried with nitrogen gas. The dried lipid was then hydrated with a desired buffer to a final concentration of 5 mM. For the calcein release assay, a buffer containing 25 mM Tris-HCl, 100 mM calcein, 1mM EDTA-Na, pH 8.0, was used to hydrate the lipids, whereas for the other studies, 50 mM sodium phosphate, pH 7.0, was used. One ml of lipid solution was then extruded through a 400 nm polycarbonate filter. The extrusion of DPPC liposomes was done at 55°C (above the transition temperature of DPPC), and the mixture was cooled down to room temperature after extrusion. All other procedures were the same for all lipid mixtures.

Fluorescence microscopic study on liposomes incorporated with dansyl-SC3

A 180 μl aliquot of freshly prepared liposomes (5 mM of lipid) in 50 mM sodium phosphate, pH 7.0 was mixed with 20 μl of 1 mg/ml dansyl-SC3 in the same buffer, and the sample was incubated for 3 h or overnight at room temperature. 20 μl of the incubated sample was dropped on a glass slide and covered with a coverslip, followed by fluorescence microscopy measurements using an Olympus Provis AX70 microscope (Japan). The pictures were taken with a 3.3MegaPixel-camera Color View II and analyzed with AnalySIS docu software by Soft Imaging System (Münster, Germany).

Sephacryl S-1000 gel filtration chromatography

A 0.7 × 25 cm gel filtration column packed with 15 ml of Sephacryl S-1000 resin (Pharmacia) was equilibrated with 5 column volumes of 50 mM sodium phosphate, pH 7.0. A 180 μl aliquot of prepared liposomes (5mM of lipid) was mixed with 20 μl soluble-state dabcyl-SC3 in 1 mg/ml in 50 mM sodium phosphate, pH 7.0, and after being incubated for a given time, the mixture was carefully loaded onto the column. The flow speed used was 0.3ml/min, and the eluate was collected at 0.5 ml per tube. The absorbance at 460 nm (dabcyl-SC3) and 660 nm (liposomes) was measured for each collected fraction, and plotted against the elution volume. A control experiment with dabcyl-SC3 alone was done under the same conditions.
Circular Dichroism Spectroscopy (CD)

A 450 μl aliquot of liposome (5mM of lipid) in 5 mM sodium phosphate, pH 7.0, was mixed with 50 μl of 1 mg/ml soluble-state SC3 in the same buffer, and the sample was incubated either 3 h or overnight. CD spectra of the incubated sample were recorded from 200-250 nm on an Aviv 62A DS CD spectrometer. The temperature was controlled at either 25°C or 65°C, and the sample compartment was continuously flushed with nitrogen gas. The final spectra were obtained by averaging 5 scans, using a bandwidth of 1nm, a stepwidth of 1 nm, and a 5-s averaging per point.

ThT fluorescence

The sample of liposome/SC3 was prepared in the same way as that for the CD measurements. A 5 μl aliquot of ThT stock solution (300 μM) was then added to each sample (500 μl), and the mixture was subjected to the fluorescence measurement using a SPF-500C spectrofluorometer (SLM Aminco). The excitation wavelength used was 450 nm, and the fluorescence emission at 500 nm was measured. The data collected for each sample was corrected for the background signal that was collected before the addition of ThT. As a control for the determination of the maximal level of β-sheet structure formation, the same liposome/SC3 preparation was vortexed for 5 min in the presence of ThT, and the fluorescence was measured under the same conditions.

Calcein efflux assay

Liposomes were prepared in 25 mM Tris-HCl, 100 mM calcein, 1mM EDTA-Na, pH 8.0. After extrusion through a 400 nm polycarbonate filter, the solution was immediately loaded onto a sephadex G-50 desalting column to remove the free calcein. The column was equilibrated with 25 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA-Na, pH 8.0. The calcein-loaded vesicles collected from the column were mixed with a given amount of soluble-state SC3. A small portion of the mixture was taken at stated times and diluted to 2 ml of column buffer in a cuvette, which was immediately subjected to fluorescence measurement using a SPF-500C spectrofluorometer (SLM Aminco). The excitation wavelength for calcein was set at 490 nm and the emission of fluorescence was at 520 nm. The slitwidth for both excitation and emission was set at 4 nm. After the measurement, Triton X-100 was added to the same sample to a final concentration of 0.5% to determine the maximal level of calcein release.
Planar Lipid Bilayer (PLB) set up and SC3 pore formation

The lipid bilayer was formed between two electrically insulating compartments, i.e., cis and trans compartment. The cis compartment was connected to the measuring electrode and to the pre-amplifier in the head stage of the patch-clamp amplifier, while the trans compartment was electrically grounded. In most of the cases, ionic currents through the ion channel in the PLB were measured at a constant voltage (V), but in some cases, corrections had to be made for a voltage changed as a result of the current flow arising from channel opening. By convention, potentials are referenced to the potential of the cis compartment and a positive current (upward deflection) is defined as a net flux of positive charge from cis to trans.

A lipid bilayer could be formed in the hole that separates the two compartments. This was done by dipping a ‘brush’ in a solution of lipids in organic solvent (mixture of phosphatidylethanolamine and phosphatidylcholine in a 8:2 ratio, dissolved in n-decane at 10 mg/ml) and ‘painting’ this solution over the aperture that is below the surface of the surrounding solution. In a few seconds, the lipid film thins out and this results in the formation of a lipid bilayer. The process can be followed by monitoring the electrical capacitance of the bilayer, since the specific capacity of a decane containing bilayer is around 0.4 μF/cm², about half the specific capacitance of a solvent free bilayer. Furthermore, after thinning the capacitance is a direct measure of the surface area of the planar lipid bilayer, which is an important parameter because the larger the membrane surface area, the better the chances for ion channel reconstitution. A typical bilayer has a surface area of 0.005 mm² and a capacitance of 200 pF. Note that just part of the aperture (with a total surface area of 0.02 mm²) is covered by a bilayer but that most of the surface area is taken up by the so-called lipid annulus. In this study, the two chambers were filled with buffer containing 25 mM Hepes, pH 7.4, supplemented with either 100 mM or 1 M KCl. Soluble-state SC3 in the same buffer was added to the trans compartment while stirring, until the concentration reached a given level, and the channel activity was recorded after turning off the stirring device.
Summary and perspectives

Hydrophobins are functionally important proteins in fungi and filamentous bacteria. Unlike most proteins, fungal hydrophobins function by interfacial self-assembly forming a chemically- and proteolysis-stable membrane either outside the fungal aerial hyphae or at an air/water (medium) interface. In the case of aerial hyphae, the hydrophobin coating confers hydrophobicity to the surface and, therefore, facilitates both pathogenic and symbiotic surface attachment of fungi to their hosts. For fruiting bodies and lichens, the hydrophobic lining of the air channels may prevent the channels from being soaked by water and secure the air exchange, while for conidiospores, the hydrophobic coating may facilitate both the surface attachment and the dispersion by wind. The assembled hydrophobin membrane also lowers the air/water surface tension and, therefore, facilitates the emergence of fungal aerial hyphae from the aqueous medium into the air (chapter 1). The membrane formed by the self-assembly of hydrophobin SC3 on both aerial hyphae and on a hydrophilic solid surface is composed of a rodlet-network, with a with of approximately 10 nm and a membrane thickness of 5-10 nm. The rodlets mimic amyloid fibrils in as much as both consist of protofilaments with a diameter of 2.5 nm, and in both cases their formation is accompanied by a characteristic structural change from a “soluble state” to a β-sheet enriched “β-sheet state”. In addition, hydrophobins and amyloid proteins/peptides also share some common biochemical properties; both can specifically bind the dyes, Congo-red and Thioflavin T (ThT). In most cases, however, amyloids are toxic to their hosts whereas hydrophobins are not (chapter 1).

The hydrophobin (SC3) membrane formed at a hydrophobic/hydrophilic surface is asymmetric. The rodlet structure found on the hydrophobic side of the membrane is not found on the hydrophilic side. Furthermore, the SC3 membrane formed at an oil/water interface has unidirectional permeability. Marker molecules up to 10,000 Da can pass through the membrane from the hydrophobic side to the hydrophilic side, whereas molecules from 300 to 70,000 Da are all excluded from the other direction. This finding suggests that the hydrophobin membrane, covering fungal aerial structures, may allow nutrients in the environment to be efficiently taken into the cells and to prevent their escape. This might be significant for the fungal growth (chapter 5).

One might expect, on the basis of these results, that SC3 also would form a layer at a lipid/water interface and thereby stabilize the lipid bilayer. This does not occur. Indeed, SC3 significantly destabilizes a phospholipid bilayer, especially DOPC and DOPE mixture, as judged
by the time-dependent release of calcein encapsulated in DOPC/DOPE liposomes. SC3 does not cause similar calcein release from DPPC liposomes, which have a higher gel/liquid crystalline transition temperature and are in the gel phase at room temperature. The destabilization effect is protein-concentration dependent, and the experiments indicate that self-association of the protein causes leakage through the liposome membrane. This is supported by electrophysiological data using planar lipid bilayers. SC3 incorporates and forms an ion permeable pore in DOPC/DOPE lipid bilayers. The pores vary in size, accumulate in time, and are voltage-independent (chapter 6). Whether or not the pore formation has any physiological consequences is not known.

The amino acid sequences of hydrophobins are diverse, however, they all contain eight cysteine residues yielding four loops which are separated by four disulfide bridges. Class I hydrophobins are distinct from class II hydrophobins in hydropathy patterns and biophysical properties. In vivo complementation studies indicate that they are not able to functionally substitute each other. From a structural point of view, class I hydrophobins have many more residues between cys3 and cys4 (2nd loop) than the class II hydrophobins. Whether or not the structural differences determine the differences in their biophysical properties or biological functions is not known.

The structure/function relationships of SC3 have been well investigated. Soluble-state SC3 is found to be primarily a stable dimer; the monomer exists only transiently and is probably unstructured (chapter 3). Dimeric SC3 is most likely the building block for both aggregation in solution and interfacial self-assembly. It is partially unstructured and elongated in shape. Molecules within different oligomers exchange with each other, as revealed by fluorescence resonance energy transfer (FRET). Dimerization, as well as disulfide formation, might be the factors that stabilize SC3 in the soluble-state, protecting the nascent molecules from proteolytic degradation and/or preventing premature assembly (chapter 3).

Self-assembly/β-sheet-state structure formation of SC3 can only be achieved by exposing the protein to a hydrophobic/hydrophilic interface, such as an air/water or oil/water interface, or a solid Teflon surface. A α-helical-state intermediate is found on the path from soluble-state SC3 to the β-sheet state. It exists transiently when the β-sheet-state structure forms at an air/water interface, but it remains “trapped”, on a solid Teflon surface, and can only proceed via the β-sheet I to the β-sheet II state when both heat and detergent are applied. The molecules in α-helical state on a Teflon surface do not move and stay in a dissociated, probably monomeric form (chapter 2). The β-sheet content in the assembled SC3, either at an air/water interface or on a Teflon surface, increases as a function of time. In both cases, the dynamic formation of the β-
sheet I and II state structure takes place within 5 to 10 hours, with a very fast change in the first 1 hour and a slow change in the following hours. On a Teflon surface it is accompanied by a spatial movement toward each other, indicating a clustering process leading to the formation of the rodlets (chapter 2).

Which amino acids are crucial for the formation of different structural states of hydrophobin SC3 has been investigated by MALDI-TOF and electrospray mass spectrometry in combination with proteolysis of the cysteine-modified protein. The soluble-state SC3 cannot be fully digested, even with the non-specific protease pepsin, unless the disulfide bridges are reduced and the cysteines are modified. The modified SC3 shows identical coating properties and structural changes on a Teflon surface as the unmodified protein. The digestible part of unmodified SC3 is mainly unstructured, as seen by the fast hydrogen-deuterium exchange (H/D exchange). The first half of the 2nd loop of modified SC3 binds with high affinity to the hydrophobic surface, as revealed by MALDI-TOF analysis of hydrophobin-digestion-mixture covering a Teflon target. This conclusion is supported by the low H/D exchange in this region. A chemically synthesized peptide corresponding to this region also shows binding and a typical α-helical-state structure on a Teflon surface, indicating that this part of the molecule is responsible both for binding and for the α-helical state formation. This peptide, however, does not form a typical β-sheet-state structure, suggesting that the β-sheet structure formation probably involves other parts of the molecule, which agrees with the result of molecular dynamic simulations (chapter 4).

The studies presented in this thesis have answered some questions, and have opened up prospects for applications of hydrophobins in the future. On the basic side, future work will have to be focused on resolving the structures to high-resolution. X-ray scattering and X-ray 2D crystallography have been used to study the structures of soluble-state and assembled class II hydrophobin HFBI and HFBII, respectively. Although the resolution still needs to be improved, this approach may reveal the structure of class I hydrophobins in various states in the future. Alternatively, Nuclear Magnetic Resonance spectroscopy (NMR) might also be a promising approach. Although the structure of a class I hydrophobin EAS in its soluble-state has been investigated by high-resolution NMR, the study of its assembled form is still a challenge. The high propensity of class I hydrophobins to aggregate in aqueous solution at high concentrations is probably the main obstacle for both X-ray crystallography and NMR spectroscopy. Solid-state NMR, using solid spheres with hydrophobins either covalently-linked or noncovalently-coated on the surface, might be an alternative to overcome this obstacle. The approaches we have established and the knowledge we have obtained in the study of hydrophobin SC3 should be
useful for high-resolution structure studies in the future. The one-dimensional $^1$H NMR study on dimeric SC3 that is described in chapter 3 shows a stable, structure-representative signal, suggesting that more detailed structural information would be available if multi-dimensional NMR were performed in the future.

The large-scale production of hydrophobins is obviously important for the future applications, but heterologous gene expression did not produce a satisfying level in our previous studies. A possible pore formation by hydrophobins in the cell plasma membrane might explain such a low heterologous protein expression. Studies in this direction should be done in the future using a planar lipid bilayer and various lipid compositions, e.g. the composition that mimics fungal cell membranes.

Site-directed mutagenesis of hydrophobins will be important not only for various applications but also for obtaining more structure information, for example, the introduction of tryptophans or lysines into SC3 (there are no Trp and Lys in SC3) can be useful in defining changes in their microenvironments by following the change in Trp fluorescence or fluorescently labeled Lys. Such an effort has already been made, but was limited by the low amount of mutant protein produced in *S. commune*. More work should be done in the future to solve this problem by trying to use other hosts, such as *T. reessei*. On the other hand, the production of some functional domains of hydrophobins, e.g. the 2$^{nd}$ loop of SC3, might be an alternative to the intact proteins for some applications. As pointed out in chapter 4, such a “mini hydrophobin” retains the surface-coating property of native SC3, but its smaller size might be an advantage for manipulation, gene expression and protein purification.

The finding of unidirectional permeability of SC3 membrane assembled at an oil/water interface not only suggests a new biological function of hydrophobins for fungal growth and development, but also illuminates a new type of application. For instance, such a property can be utilized in refining oils, as a single step to remove the impurities by the introduction of a water phase which is separated from the oil by a hydrophobin membrane. Such a procedure would be safe, clean, and economical compared to the traditionally used methods based on heating and chemical extractions. Of course, the realization of such possibilities, or, dreams needs a huge amount of work, but will also bring to light more exciting applications.
Samenvatting en perspectieven

Hydrophobinen zijn belangrijke eiwitten in het paddestoelenrijk; eiwitten met overeenkomstige functies worden ook in andere organismen gevonden, zoals de draderige bacterie, streptomyces. Zij zijn dus waarschijnlijk alom aanwezig in de natuur. Schimmel hydrophobinen associëren door zelfassemblage om een chemische- en proteolyse-stabiel membraan te vormen zowel aan de buitenkant van de schimmel luchthyphae als bij een lucht-/watergrensvlak. Het geassembleerde hydrophobine membraan vermindert de lucht-/wateroppervlakte spanning en kan daarom de totstandkoming van schimmel luchthyphae uit het waterige milieu in de lucht vergemakkelijken (hoofdstuk 1). De hydrophobinelaag resulteert in hydrofobiciteit aan de oppervlakte en vergemakkelijkt daarom zowel pathogene als symbiotische oppervlaktehechting van de schimmel aan hun host. De hydrophobine coating van de luchtkanalen kan voor fruiting van organismen en korstmossen verhinderen dat de kanalen door water worden doorweekt, wat de luchtuitwisseling beveiligd. Terwijl de hydrophobine coating voor conidiosporen zowel de oppervlaktehechting als de verspreiding door wind kan vergemakkelijken. Het membraan dat zowel op luchthyphae als op een vaste oppervlakte wordt gevormd is samengesteld uit rodlets, stafjes van hydrophobine moleculen met een breedte van ongeveer 10 nm, een dikte van 5-10 nm en een lengte van honderden nanometers. Rodlets boot sen amyloïde fibrillen na, in zoverre dat ze allebei bestaan uit protofilamenten met een diameter van 2.5 nm (de diameter van een enkele molecuul). In beide gevallen wordt hun vorming begeleidt door een kenmerkende structurele verandering van een oplosbare vorm naar een β-sheet vorm. Bovendien delen hydrophobinen en amyloïde eiwitten/peptiden ook sommige biochemische eigenschappen; beiden kunnen de kleurstoffen Congo-red en Thioflavin T (ThT) specifiek binden. In de meeste gevallen zijn amyloïden echter giftig voor hun gastheren (hoofdstuk 1).

Het hydrophobine (SC3) membraan dat aan een hydrofoob/ hydrofiel oppervlakte wordt gevormd is asymmetrisch. De rodlet-structuur die aan de hydrofobe kant van het membraan wordt gevonden, wordt niet gevonden aan de hydrofiele kant. Verder heeft het SC3 membraan dat bij een olie-/watergrensvlak gevormd wordt, alleen in een richting permeabiliteit. Marker moleculen tot 10,000 Da kunnen het membraan passeren van de hydrofobe kant naar de hydrofiele kant; terwijl passage van moleculen tussen 300 en 70,000 Da van de andere richting geblokkeerd is. Deze eigenschap resulteert in een diffusie van marker moleculen uit de oliefase en een ophoping in de waterfase. Het suggereert dat schimmel luchtstructuren bedekt met een
hydrophobine membraan, voedingsmiddelen uit het milieu kunnen opnemen en accumuleren. Dit zou significant kunnen zijn voor de schimmelgroei (hoofdstuk 5).

Men zou, op basis van deze resultaten, kunnen verwachten dat SC3 ook een laag vormt bij een lipide-/watergrensvlak en daardoor de lipide bilaag zou stabiliseren. Dit gebeurt echter niet. SC3 destabiliseert namelijk de fosfolipide membraan significant, vooral het mengsel van DOPC en DOPE, zoals bewezen door de tijdsafhankelijke release van calceine, aanvankelijk ingesloten in DOPC/DOPE liposomen. SC3 kan geen gelijkwaardige calceine release veroorzaken uit DPPC liposomen, welke een hoger gel/vloeibare kristallijnen overgangstemperatuur hebben, mits bij temperaturen boven 50°C. Het destabilisatie-effect is eiwitconcentratie afhankelijk en komt beduidend meer voor in de eerste 5 tot 6 uren, suggererend dat de zelf-associatie van SC3 een rol speelt in het veroorzaken van lekkage in het liposoom-membraan. Dit wordt gesteund door elektrofysiologische gegevens gebruik makend van vlakke lipidebilagen (Black lipid membranes); SC3 wordt opgenomen en vormt een ionen permeabele porie in een DOPC/DOPE lipide bilaag. De poriën variëren in grootte, accumuleren in tijd, en zijn voltage onafhankelijk (hoofdstuk 6). Of een dergelijke porievormende eigenschap enige fysiologische gevolgen heeft, is nog niet duidelijk, maar dit is de eerste keer dat een "negatief" effect van SC3 is gevonden.

De aminozuurvolgordes van hydrophobinen zijn divers, zij bevatten echter allen acht cysteine residu's die alle vier loops vormen, elk met een disulfidebrug. Klasse I hydrophobinen verschillen van klasse II hydrophobinen in hydropathie patronen en biofysische eigenschappen van de assemblage. Aanvullende in vivo studies wijzen erop dat zij niet in staat zijn elkaar functioneel te vervangen. Vanuit het structuurstandpunt gezien heeft klasse I hydrophobinen opmerkelijk meer residu's tussen cys3 en cys4 (de 2e loop) dan klasse II hydrophobinen. Het is nog niet duidelijk of de structurele verschillen al dan niet de verschillen in hun biofysische eigenschappen of biologische functies bepalen.

De structuur-/functieverhouding van SC3 is intensief onderzocht. Oplosbaar SC3 werd hoofdzakelijk gevonden als een stabiele dimeer; het monomeer bestaat alleen vluchtig en is waarschijnlijk ongestructureerd (hoofdstuk 3). Dimeer SC3 is het meest waarschijnlijk bouwsteen voor zowel aggregatie in oplossing (geen structurele verandering) als voor zelfassemblage op een grensvlak. Het is gedeeltelijk ongestructureerd en heeft een langwerpige vorm. Moleculen binnen verschillende oligomeren wisselen met elkaar uit, zoals bewezen door de fluorescentie resonantie-energieoverdracht (FRET). Dimerisatie, net als disulfidevorming, zou de factor kunnen zijn die SC3 in de oplosbare vorm stabiliseert, de ontluikende moleculen
bescherm tegen proteolytische degradatie en/of voorbarige assemblage verhindert (hoofdstuk 3).

Zelf-assemblage/β-sheet structuurvorming van SC3 kan alleen bereikt worden door de aanwezigheid van een hydrofoob/hydrofiel grensvlak, zoals een lucht/water of olie/water grensvlak, of een vast Teflon oppervlak. Een α-helix tussenvorm wordt gevonden op de weg van oplosbaar SC3 naar de β-sheet vorm. Het bestaat vluchtig wanneer de β-sheet structuur wordt gevormd bij een lucht/water grensvlak, maar het wordt gestabiliseerd op een Teflonoppervlakte, en kan slechts naar de β-sheet vorm overgaan, wanneer zowel hitte als detergens worden toegevoegd. De moleculen in α-helix vorm op een Teflonoppervlakte bewegen zich niet en blijven in een gescheiden, waarschijnlijk monomer-achtig vorm (hoofdstuk 2). De β-sheet signatuur in geassembleerde SC3, ofwel bij een lucht/water grensvlak ofwel op een Teflonoppervlakte, neemt toe als een functie van tijd. In beide gevallen vindt de dynamische vorming van de β-sheet structuur binnen 5 tot 10 uren plaats, met een zeer snelle verandering in het eerste uur en een langzame verandering in de volgende uren. Op een Teflonoppervlakte vindt er tevens een beweging naar elkaar toe plaats, wat tot de vorming van rodlets leidt (hoofdstuk 2).

Welke aminozuren essentieel zijn voor verschillende structurele staten van SC3 is onderzocht met MALDI-TOF en electrospray massaspectrometrie in combinatie met proteolyse van het eiwit. Oplosbare SC3 kan niet volledig worden afgebroken, zelfs met de niet-specifieke protease, pepsine, mits de S-S bruggen chemisch gereduceerd worden tot vrije SH groepen die daarna gestabiliseerd worden via een chemische omzetting. Gemodificeerd SC3 toont identieke coating eigenschappen en structurele veranderingen op een Teflonoppervlakte als het ongemodificeerde eiwit. Snelle H/D uitwisseling toont aan dat het afbreekbare gedeelte van ongemodificeerd SC3 hoofdzakelijk ongestructureerd is. MALDI-TOF analyse van hydrophobine fragmenten heeft bewezen dat de eerste helft van de 2e loop van gemodificeerd SC3 met grote affiniteit bindt op Teflon. Deze conclusie wordt gesteund door de lage H/D uitwisseling van dit gebied. Een chemisch gesynthetiseerde peptide die overeenkomt met dit gebied bindt en vormt een typische α-helix structuur op Teflon, dit geeft aan dat dit gedeelte van de molecuul zowel voor het binden als voor de α-helix signatuur verantwoordelijk is. Deze peptide vormt echter geen typische β-sheet structuur, dit suggereert dat de vorming van de β-sheet structuur waarschijnlijk andere delen van de molecuul impliceert. Dit komt overeen met het resultaat van moleculaire dynamische simulaties (hoofdstuk 4).

De studies die in dit proefschrift worden gepresenteerd hebben enkele vragen beantwoord en vooruitzichten voor toepassingen van hydrophobinen in de toekomst geopend. Aan de
fundamentele onderzoekskant, zullen toekomstige werkzaamheden worden gefocussseerd op het oplossen van de hoogresolutie 3D structuur. X-ray verstrooing en X-ray 2D kristallografie zijn gebruikt om de structuren van, respectievelijk de oplosbare en geassembleerde klasse II hydrophobine, HFBI en HFBII te bestuderen, zoals die in hoofdstuk 1 wordt beschreven. Hoewel de resolutie nog verbeterd moet worden, kan dezelfde benadering worden verwacht om de structuur van klasse I hydrophobinen in diverse vormen in de toekomst te openbaren. Als alternatief zou Nuclear Magnetic Resonance spectroscopie (NMR) ook een veelbelovende benadering kunnen zijn. Hoewel de structuur van de oplosbare klasse I hydrophobine, EAS, door NMR is onderzocht, is de studie van haar geassembleerde vorm nog een grote uitdaging. De grote neiging van klasse I hydrophobinen om te aggregeren in een waterige oplossing bij hoge concentraties is waarschijnlijk de belangrijkste hindernis voor zowel X-ray kristallografie als NMR spectroscopie. NMR in vaste toestand zou een benadering kunnen zijn om deze hindernis te overwinnen. Aan de andere kant, de één-dimensionale 1H NMR data van dimeer SC3 in hoofdstuk 3 toont een stabiel, structuur-representatief signaal, suggererend dat meer gedetailleerde structurele informatie beschikbaar zou zijn als multidimensionale NMR in de toekomst werd uitgevoerd.

De productie op grote schaal van hydrophobinen is duidelijk belangrijk voor de toekomstige toepassingen, maar heterologe eiwit expressie heeft niet tot bevredigende niveaus geleid. Een mogelijke porievorming door hydrophobinen in het plasmamembraan van de cel zou een dergelijke lage eiwitexpresse kunnen verklaren. “Black lipid membrane” met SC3 en diverse lipiden, die de samenstelling van de schimmelcelmembranen nabootst, zou waardevolle informatie hieromtrent leveren.

Plaatsgerichte mutagenese van hydrophobin zal waardevol kunnen zijn voor diverse toepassingen maar ook voor het verkrijgen van structuurinformatie. De introductie van een tryptofaan of een lysine in SC3 zou bijvoorbeeld nuttig kunnen zijn in het bepalen van veranderingen in de microomgeving door de verandering in Trp fluorescentie of de fluorescentie van een gelabeld Lys te volgen. Een dergelijke inspanning is reeds gemaakt, maar werd gestaakt wegens de lage hoeveelheid gemuteerd eiwit geproduceerd in S.commune. In de toekomst zal meer werk gedaan moeten worden om dit probleem op te lossen, door andere gastheren zoals T.ressei te gebruiken. Aan de andere kant, de productie van functionele domeinen van hydrophobin, e.g. de 2ᵉ loop van SC3, zou een alternatief kunnen zijn voor het intacte eiwit bij sommige toepassingen. Zoals aangehaald in hoofdstuk 4, behoudt een dergelijke "minihydrophobin" de oppervlakte-coating eigenschap van natuurlijke SC3, maar is het eiwit kleiner. Dit zou een voordeel kunnen zijn bij genmanipulatie, eiwit expressie en zuivering.
Het vinden van eenrichting permeabiliteit van het SC3 membraan die bij een olie-/ water grensvlak wordt geassembleerd suggereert niet alleen een nieuwe biologische functie van hydrophobin in de schimmelgroei en ontwikkeling, maar verlicht ook een nieuwe toepassingsmethode. Bijvoorbeeld een membraan met een dergelijke eigenschap kan gebruikt worden voor het zuiveren van water oplosbare verontreinigingen uit olie. Zo’n procedure zou veilig, schoon en economisch zijn in vergelijking met de traditioneel gebruikte methodes die op verwarmen en chemische extracties worden gebaseerd. Natuurlijk vergt de realisatie van dergelijke mogelijkheden een reusachtige hoeveelheid werk, maar zal ook meer spannende toepassingen aan het licht brengen.