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Self–assembling fungal proteins and their biotechnological applications

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1. Summary

Hydrophobins are small secreted proteins playing diverse roles in the life cycle of filamentous fungi. They are capable of self–assembling at hydrophilic–hydrophobic interfaces, resulting in the formation of amphipathic films. This film can make hydrophobic surfaces of a liquid or a solid material wettable, while a hydrophilic surface can be turned into a hydrophobic one. These properties, among others, make hydrophobins of interest for medical, bio–technological and technical applications.

The class I hydrophobin Vmh2 from the basidiomycete fungus *Pleurotus ostreatus* seems to be the most hydrophobic hydrophobin characterized so far. Structural and functional properties of the protein as a function of environmental conditions have been determined. The self–assembled film has been characterized and tested as masking material in the KOH wet etch of crystalline silicon. Because of the high persistence of the protein film, the hydrophobin–coated silicon surface is fully protected during the standard KOH micromachining process. The Vmh2 film has also been exploited as coating material of a commercial steel sample–plate of a MALDI–TOF mass spectrometer. The presence of self–assembled Vmh2 on MALDI wells allowed us to perform on plate desalting of protein and peptide samples, and to immobilize trypsin to perform on–plate digestion of protein mixtures.

Key words: fungal proteins, self–assembling, hybrid surfaces, amyloid fibrils

2. Introduction

Hydrophobins are proteins produced by filamentous fungi. Their functions are mainly based on their capability to self–assemble into a highly surface active film at a hydrophilic–hydrophobic interface [1,2]. The presence of hydrophobins is not associated with a specific microbial lifestyle — they are produced by saprophytic fungi, pathogenic fungi and fungi that establish a mutually beneficial symbiosis. They play a key role in growth and morphogenesis in the majority of these fungi, as a coating/protective agent, in adhesion, surface modification, or other types of function that require surfactant–like properties [3]. Hydrophobins can be secreted out in the surroundings or retained in the fungal structures, such as fruiting bodies or mycelium. Their biological functions seem to be diverse, but always in some manner related to interactions with interfaces or surfaces. They allow fungi to escape an aqueous environment, confer hydrophobicity to air–exposed fungal surfaces, mediate the attachment of fungi to hydrophobic solid substrates such as the surface of a host, facilitate dispersion of spores and contribute to invasion of abiotic and biotic substrates [4].

Aerial growth is mediated by hydrophobins that are secreted by the hyphae into the moist substrate. Through a process of self–assembly, these structures reduce the water surface tension of the substrate–air interface, which eliminates this physical barrier and thereby allows the hyphae to grow into the air. The aerial hyphae continue to secrete hydrophobins, which assemble at the hyphal surface. The hydrophilic side of the amphipathic film is positioned next to the cell wall, whereas the hydrophobic side is exposed. As a result, aerial hyphae are water repellent. Similarly, water–soluble hydrophobins assemble at the surfaces of spores that have developed from differentiated aerial hyphae. The coating of spores by hydrophobins facilitates their dispersal by wind [5].

The intriguing properties of these proteins make them of significant interest to biotechnologists, as they have potential for numerous applications, ranging from medical and technical coatings to the production of proteinaceous glue and cosmetics [6].

Although hydrophobins show differences in their primary sequence, they share eight conserved cysteine residues that form four disulphide

bridges [7]. The Cys residue pattern has a striking symmetry and can be easily recognized in the primary structure. The second and third Cys and the sixth and seventh Cys residues follow each other immediately in sequence, forming two pairs, the rest of the Cys residues does not have other Cys residues as near neighbours. Based on the spacing of the cysteine residues and their biophysical properties, hydrophobins can be divided in two classes. The members of the group named class I share a functional similarity since the aggregates that they formed are highly insoluble in aqueous solution, whereas the members of class II form aggregates that are much easier to dissolve. So far, class II hydrophobins have been observed only in Ascomycetes, whereas class I hydrophobins are produced both in Ascomycetes and Basidiomycetes.

3. Class I hydrophobins

In this paper we will focus our attention on class I hydrophobins. These proteins self–assemble at hydrophilic–hydrophobic interfaces into an amphipathic membrane that consists of a mosaic of amyloid–like fibrils known as rodlets [8].

Amyloid fibrils have historically been associated with pathology in a class of degenerative diseases including Alzheimer's disease and Creutzfeldt–Jakob disease. Amyloid fibrils share a structural motif — the cross–β structure — which indicates that these fibrils have common properties [9]. This structure consists of β–sheets that are stacked in the direction perpendicular to the fibril axis, with hydrogen bonds parallel or perpendicular to it. The similarity in structure implies a common mechanism of fibril formation and that the fibrils themselves have common properties. Indeed, all amyloids increase the fluorescence of the dye thioflavin T, exhibit green–gold birefringence on binding the dye Congo red, and cause a red–shift in the absorbance spectrum of Congo red.

Recent data have shown that amyloid fibril formation can also provide biologically functional molecules [9,10]. Functional amyloids have been identified on the surfaces of both fungi and some bacteria (i.e. curli). The deployment of amyloid hydrophobins contributes to the presence of several hundred, and sometimes thousand, fungal

spores per cubic metre of air. These spores can attach onto the surface of a plant or an animal, or can enter the body by inhalation, a lesion in the skin or an implant to which spores have adhered. There is experimental evidence that the amyloid layer forms a protective "coat" to allow microorganisms to evade the immune system of the host [11].

Class I hydrophobin, SC3 from *Schizophyllum commune* has been extensively studied from the point of view of structure/function relationships. It spontaneously self–assembles via an α–helical intermediate state into a stable β–sheet end configuration at a water–air interface. In contrast, upon contact with hydrophobic solids (e.g., Teflon) in water, SC3 is arrested in the intermediate α–helical configuration. The transition to the stable β–sheet end form is promoted by high protein concentration, the presence of cell wall polysaccharides, and the combination of heat and detergents [12].

The most detailed structural work on a class I hydrophobin has been obtained for the EAS hydrophobin from *Neurospora crassa* [13]. The monomer consists of a four–stranded β–barrel core, an additional two–stranded β–sheet and two sizeable disordered regions (Fig. 1). EAS is cross–linked by the four disulphide bridges connecting C1–C6, C2–C5, C3–C4 and C7–C8. Notably, the charged residues are localized at one side of the surface of the protein. This strongly suggests that the water–soluble form of EAS is amphipathic. The largest disordered region of EAS (M22–S42) is contained between the third and the fourth cysteine residue. This part is the least conserved portion of class I hydrophobins in terms of both size and makeup. Importantly, the disordered regions of EAS do not seem to be important in the self–assembly process. Mutated EAS, in which half of the largest disordered region was deleted, was still able to self–assemble. More recent mutagenesis studies identified the F72–N76 segment of EAS as the critical amyloidogenic segment [14]. These results demonstrate that the F72–I75 region directly forms the core of the cross–β structure in EAS hydrophobin functional amyloid rodlets. Moreover a peptide encompassing the sequence of this region can form amyloid fibrils on its own. Therefore Macindoe *et al.* [14] postulate that a conformational change in the protein monomer leads to expose a previously buried segment that is prone to cross–β stacking, leaving the bulk of the native fold of the protein largely unchanged.

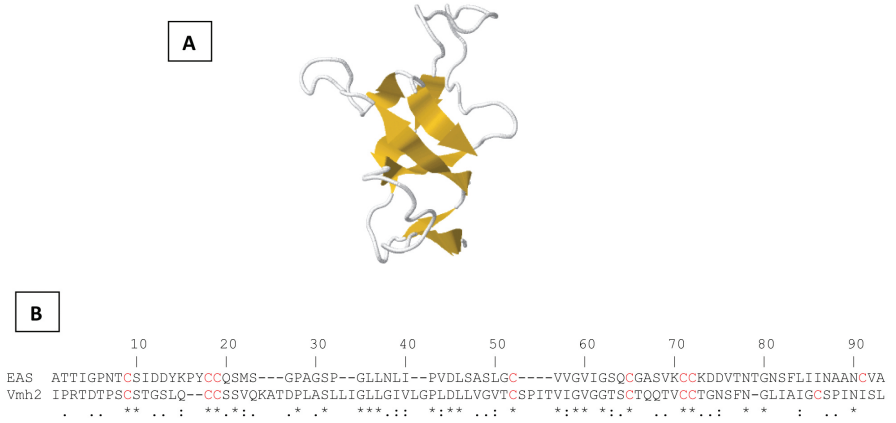

Figure 1. a) 3D structure of the Class I hydrophobin EAS; b) alignment of EAS and Vmh2 class I hydrophobin sequences. In red the eight Cys residues, highlighted in gray the F72–N76 segment.

This conformational change model is analogous to those designed to describe the behavior of proteins that "switch" to an amyloidogenic state due to a localized conformational change under appropriate conditions.

4. The class I hydrophobin Vmh2 from the basidiomycete fungus *Pleurotus ostreatus*

A class I hydrophobin secreted by the basidiomycete fungus *Pleurotus ostreatus* has been purified and identified as Vmh2. According to Peñas *et al.* [15], this protein is specific to vegetative mycelium, is produced throughout the culture time, and is found both as cell wall–associated protein and in the bulk medium. The pure protein is not soluble in pure water, but in ethanol solution, whereas complexes formed between the protein and glucans, produced in culture broth containing amylose, are soluble in water. Vmh2 seems to be the most hydrophobic hydrophobin characterized so far because both SC3 and EAS can be dissolved in water up to 1 mg/mL [16]. It has been verified that glucose is also able to solubilize the hydrophobin in water. Interaction between Vmh2 and glucose has been verified

by the finding that both molecules elute in a unique peak by gel filtration. The aqueous solution of the protein, in the presence of glucans, showed propensity to self–assembly, while the pure protein dissolved in less polar solvent (60% ethanol) is not prone to self–assembly [17].

Structural and functional properties of the protein as a function of the environmental conditions have been determined. By increasing the pH of the solution (pH $\geq$ 6), Vmh2 undergoes a conformational change, forming a self–assembled β–sheet rich state (a significantly increased association at alkaline pH has also been demonstrated for SC3). Analogous behavior was observed in the presence of Ca^{2+}, while a monovalent cation, Na^{+}, has quite an opposite effect, inhibiting the conformational change and self–assembly occurring at pH 6. Another class I hydrophobin has showed propensity to self–assembly in the same conditions [18]. It has been suggested that the formation of large agglomerates can be triggered by bivalent cations bridging and prevented by charge screening with monovalent cations. However, it is worth noting that in the case of Vmh2 we do not observe conversion into the β–sheet rich, assembled form, triggered by migration to hydrophobic/hydrophilic interfaces, as demonstrated for the other known hydrophobins.

In summary, the following events occur starting from the Vmh2 helical structure observed in low polarity solvents:

a) Irreversible conformational change toward a β–structure followed by self–assembling when the pH increases above 6, or in the presence of Ca^{2+} ions.
b) Increased tendency to reach hydrophobic/hydrophilic interfaces when the solvent polarity increases, with no detectable conformational change.
c) Reversible conformational change toward a disordered structure and reversible aggregation at high temperature.

A schematic representation of the behavior of Vmh2 by changing the environmental conditions is shown in Fig. 2.

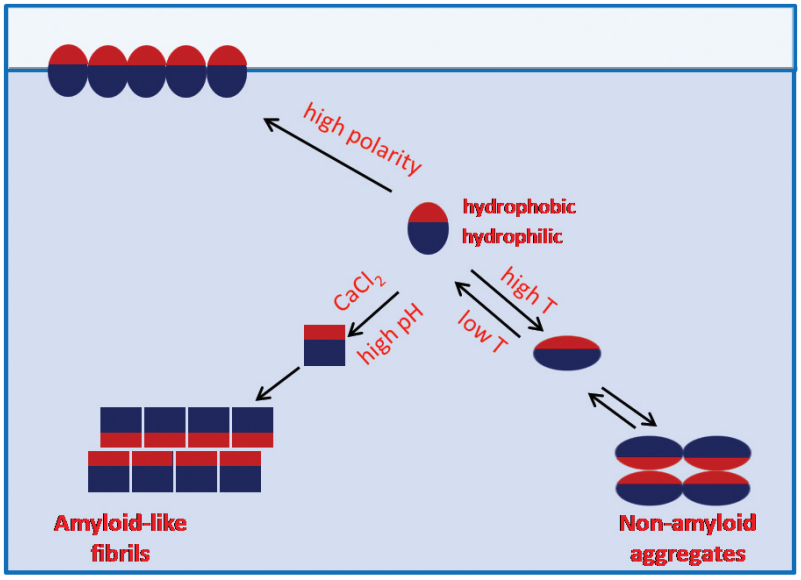

Figure 2. Schematic representation of the Vmh2 behavior in different conditions.

5. Functional surfaces based on Vmh2 biofilms

Silicon is the most used solid support in all micro– and nanotechnologies developed for the integrated circuits industry. For this reason, silicon is also used in many commercial technological platforms for biomedical and biosensing applications. The anisotropic wet micromachining of silicon, based on a water solution of potassium hydroxide (KOH), is a standard fabrication process that is extensively exploited in the realization of very complex microsystems such as cantilevers or membranes.

Vmh2, deposited on crystalline silicon forms a chemically and mechanically stable layer of self–assembled proteins. The Water Contact Angle (WCA) of the silicon surface after the Vmh2 deposition falls down from 90° to 44°; so that the dramatic increase in the surface wettability is well evident [19]. Atomic Force Microscopy (AFM) highlighted the presence of nanometric rodlet–like aggregates on the biofilm surface (Fig. 3).

This biomolecular membrane has been tested as masking material in the KOH wet etch of the crystalline silicon. Because of the high

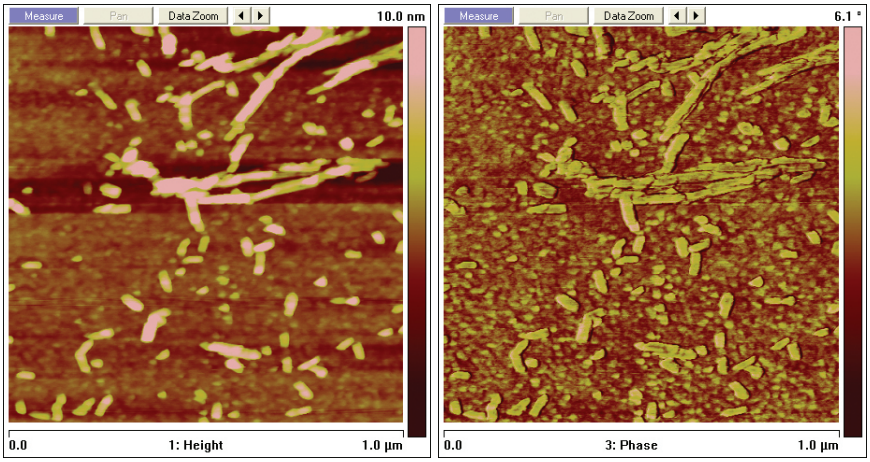

Figure 3. AFM images of Vmh2 film on silicon. On the left topography image and phase image on the right.

persistence of the protein biofilm, the hydrophobin–coated silicon surface is perfectly protected during the standard KOH micromachining process [19].

We have found that Vmh2–glucose complexes forms a chemically stable biofilm on silicon, containing 35% of glucose. The wettability of a silicon surface, covered by the organic layer of Vmh2–glucose, strongly changed: WCA decreased from 90° down to 17°, a decrease of about 27° higher than the pure protein–coated surface [20].

The protein modified silicon surface is suitable for immobilization of other proteins. Two different proteins were successfully immobilized on the HFBs–coated chips: bovine serum albumin and an enzyme, a laccase, which retains its catalytic activity after binding to the chip. Moreover enzyme immobilization on the hydrophobin layer improves the enzyme stability [21].

The Vmh2 self–assembled layer has also been exploited as a coating of a Matrix–Assisted Laser Desorption Ionization (MALDI) steel sample–loading plate, aimed at developing lab–on–plate platforms focused on proteomic applications [22].

The Vmh2 film coating on steel was homogeneous and compact, as shown by Scanning Electron Microscopy (SEM) (Fig. 4), even if it appears less smooth than that formed on crystalline silicon. Its thickness, fitted by Variable–Angle Spectroscopic Ellipsometry (VASE)

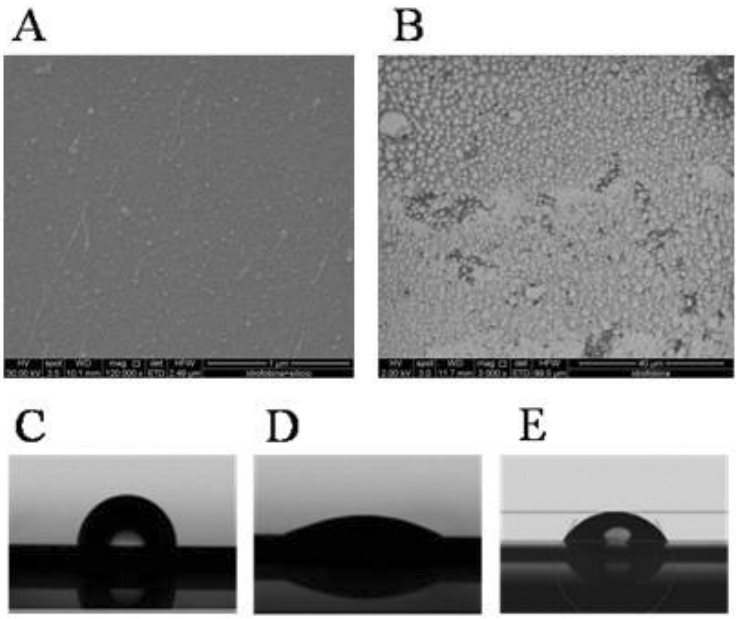

Figure 4. SEM images of Vmh2 coating on silica (A) and on steel (B); WCA of a water drop on steel surface (C), on Vmh2–coated silicon (D) and on Vmh2–coated steel (E).

measurements, was about 10 nm, higher than that found on silicon (about 3 nm). Analysis of the WCA of Vmh2 coated steel compared to the bare surface, showed a change of surface wettability (from 96° to 75°). remarkably less than that in the case of Vmh2 coating on silicon. Therefore the nature of surface affects the characteristics of the Vmh2 film: since hydropathy of bare surfaces (silicon and steel) is almost comparable, the observed differences could be ascribed to other characteristics, such as their different roughness. As a matter of fact, crystalline silicon wafers have a mean roughness of 1–2 nm, whereas steel surface shows roughness values that could be several orders of magnitude greater. However, the self–assembled film showed a strong adhesion to both surfaces.

Vmh2 coating of MALDI plates allows for a very simple and effective desalting method, suitable for development of lab–on–plate platforms focused on proteomic applications [22]. Mixtures of standard proteins, as well as tryptic peptides, in the nanomolar–femtomolar

range, can be analysed in the presence of salts and denaturants. As evidence on a real complex sample, crude human serum has also been analysed and high–quality spectra over a wide mass range have been acquired.

Trypsin has also been immobilized on Vmh2–coated MALDI wells in order to perform on–plate digestion of protein mixtures. The immobilized trypsin is active and able to perform the complete hydrolysis of substrate more quickly than the free enzyme.

The ability of Vmh2 to coat a wide range of different surfaces makes it applicable to several biotechnological fields, from lab–on–plate to biosensing. The Vmh2 self–assembly can serve as intermediate layer for secondary protein immobilization, thus creating hybrid devices applicable in proteomics as well as environmental monitoring, food quality control, etc.

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