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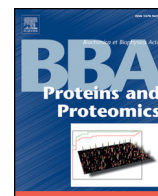


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Spontaneous self-assembly of SC3 hydrophobins into nanorods in aqueous solution



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ABSTRACT

Hydrophobins are small surface active proteins secreted by filamentous fungi. Because of their ability to self-assemble at hydrophilic–hydrophobic interfaces, hydrophobins play a key role in fungal growth and development. In the present work, the organization in aqueous solution of SC3 hydrophobins from the fungus *Schizophyllum commune* was assessed using Dynamic Light Scattering, Atomic Force Microscopy and fluorescence spectroscopy. These complementary approaches have demonstrated that SC3 hydrophobins are able not only to spontaneously self-assemble at the air–water interface but also in pure water. AFM experiments evidenced that hydrophobins self-assemble in solution into nanorods. Fluorescence assays with thioflavin T allowed establishing that the mechanism governing SC3 hydrophobin self-assembly into nanorods involves β -sheet stacking. SC3 assembly was shown to be strongly influenced by ionic strength and solution pH. The presence of a very low ionic strength significantly favoured the protein self-assembly but a further increase of ions in solution disrupted the protein assembly. It was assessed that solution pH had a significant effect on the SC3 hydrophobins organization. In particular, the self-assembly process was considerably reduced at acidic pH. Our findings demonstrate that the self-assembly of SC3 hydrophobins into nanorods of well-defined length can be directly controlled in solution. Such control allows opening the way for the development of new smart self-assembled structures for targeted applications.

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1. Introduction

Self-assembly biological processes at various length scales are ubiquitous in diverse natural systems [1]. Elucidation of the fundamental mechanisms governing self-assembly is crucial to understand the structure formation in living systems and to control them. These spontaneous processes allowing the assembly of complex supramolecular structures from small building blocks, like viruses or proteins, are mediated by weak noncovalent interactions [2]. However, more complex inter-protein interactions coming from the anisotropic character of proteins are involved in the self-assembly of proteins. These interactions depend also significantly on solution conditions, such as pH and the concentrations of other ions [3]. In the last ten years, due to their wide implications in living systems and their complexity, numerous self-assembly mechanisms of proteins were investigated *via* theoretical and computational tools [4–6]. Indeed, the ability to control the self-assembly became a subject of considerable importance both from a fundamental point of view and for its potential applications allowing *e.g.* self-assembly into a given target structure or the development of

novel biomaterials based on self-similarity at multiple length scales [1, 7,8]. However, self-assembly of proteins still raises many questions: our understanding of how the self-assembly mechanism actually works is still incomplete and many of the basic principles governing this type of organization are unclear.

Class I hydrophobins are ideal candidates to study the self-assembly of proteins due to their particular ability to spontaneously organize through β -sheet interactions. They constitute a class of surface active proteins that play a key role in fungal growth and development [9], and are found associated on the surfaces of different fungal structures, such as conidia, aerial hyphae, and fruiting bodies [9–11]. The most important feature of class I hydrophobins is that they are able to self-assemble at different hydrophobic–hydrophilic interfaces into regularly arranged nanoscale rods known as rodlets (*e.g.* air–water, oil–water, and hydrophobic solid–water) [12–15]. Because of their amphiphatic nature, hydrophobins are able to coat hydrophobic or hydrophilic surfaces and to reverse their hydrophobic character [12–14]. In fungal conidia and aerial hyphae, hydrophobins thus provide a water-repellent hydrophobic coating, which facilitates fungal escape from the growth medium to the dispersion in air currents [11,12].

Amongst class I hydrophobins, SC3 hydrophobins from the wood-rotting fungus *Schizophyllum commune* are probably the most widely

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studied and are often considered as “model proteins” [16]. SC3 are involved in the formation of aerial hyphae and in the attachment of hyphae to hydrophobic interfaces [11,13]. Several works have already been conducted on the structural changes and organization of SC3 hydrophobins at different interfaces [12–15]. At the air–water interface, the organization of SC3 hydrophobins was shown to involve several conformational changes. Indeed, protein proceeds through an intermediate α -helical structure (α -helical state) to an amorphous film of a β -sheet signature (β -sheet I state), which reorganizes after prolonged incubation to the final β -sheet II state consisting of amyloid-like fibrils, called rodlets [16]. On the contrary, very few works were devoted to understand the assembly of SC3 hydrophobins directly in solution. However, the soluble-state of hydrophobins remains crucial since it is considered as the starting point for interfacial assembly [15]. Stroud et al. [17] have proposed a model for the assembly process of SC3 hydrophobins, which involves three distinct types of protein assemblies. Two of these assemblies were suggested to be structured in solution: (i) monomers or multimers, which are organized in loose micelles and (ii) irregular insoluble structures. The third assembly was formed at the interface [17]. Two types of SC3 assemblies in solution were also evidenced by Corvis et al. [18]. Indeed, dynamic light scattering data indicated that SC3 hydrophobins exist in solution both in monomeric and in aggregated forms.

The present paper proposes to get further insight into the SC3 hydrophobins organization in a bulk solution, through the investigation of protein assembly by Dynamic Light Scattering (DLS), Atomic Force Microscopy (AFM) and fluorescence spectroscopy. Specifically, DLS experiments were performed in order to directly follow in solution the protein organization by measuring the mean size of assembled proteins. AFM imaging was then used to investigate the morphology of protein assemblies formed in solution. Fluorescence assays with thioflavin T (ThT) allowed characterizing the structure of SC3 assemblies. These complementary approaches allowed us to show for the first time that SC3 hydrophobins spontaneously self-assemble in aqueous solution into nanorods and not only at the air–water interface. In addition, fluorescence assays allowed establishing that the mechanism of SC3 nanorod formation involves β -sheet stacking. The effects of ionic strength and solution pH on protein assembly were also evaluated.

2. Material and methods

2.1. Materials

SC3 hydrophobins and ThT dye were purchased from Sigma-Aldrich and were used as received.

2.2. SC3 hydrophobin self-assembly in water at pH 5.5

The protein self-assembly was studied in ultrapure water (pH 5.5). SC3 hydrophobins were dissolved by gentle head-over-tail mixing at a concentration of 0.2 mg/mL. Special care was taken to not introduce air bubbles during protein dissolution. SC3 was then incubated for 30, 60 and 120 min under ambient conditions and no agitation was used. Immediately after the incubation, the core of the protein solutions was carefully sampled in order to be diluted 200 times into ultrapure water (pH 5.5) to quench the concentration-dependent aggregation process. The glass coverslip was cleaned with a piranha treatment before washed in ultrapure water. Finally, the glass substrate was dried in a stream of nitrogen gas. A tiny droplet of the diluted protein solution was deposited onto the glass coverslip heated at 25 °C to promote a rapid drying (within 2 min) while avoiding the formation of concentration gradients on the substrate. The sample was then immediately imaged by AFM.

2.3. Study of SC3 hydrophobin self-assembly at different pH and ionic strength values

In the same manner, the influence of pH on protein self-assembly was studied in aqueous solutions, for the same protein concentration of 0.2 mg/mL, at three pH values: pH 3 (pH adjusted with 0.1 M HCl), pH 5.5 (ultrapure water) and pH 9 (pH adjusted with 0.5 M NaOH) in the presence of 0.5 mM NaCl. The effect of ionic strength on protein self-assembly was also investigated in aqueous solutions of 0.5 mM and 10 mM NaCl at pH 5.5 (ultrapure water). For each condition, model protein was incubated for 60 min under ambient conditions and no agitation was used. At the end of incubation time, a tiny droplet of a bulk solution was carefully sampled before being diluted and deposited onto a glass coverslip prior to AFM observations.

2.4. Dynamic light scattering

The mean particle size in solution was determined with Zetasizer Nano ZS (ZEN 3600) from Malvern (Malvern Instruments, Malvern, UK) using Non-Invasive Back Scatter (NIBS) technology with a light source from 633 nm He–Ne laser. Measurements were performed at a scattering angle of 173° and a constant temperature of 25 °C. The kinetic of SC3 self-assembly was followed in ultrapure water (pH 5.5) at a concentration of 0.2 mg/mL. The hydrodynamic diameter was determined from the autocorrelation function of the intensity fluctuation of the scattered light. These measurements yielded to the diffusion coefficient, D , used to calculate the particle hydrodynamic diameter, d_H , using the Stokes–Einstein equation: $D = kT/3\pi\eta d_H$, where k , is the Boltzmann constant, T , is the absolute value, and η , is the viscosity of the continuous phase (water with $\eta = 8.9 \times 10^{-4}$ Pa s at 25 °C). DLS data were analysed with Malvern Zetasizer Nano software.

2.5. Atomic force microscopy

All AFM images were recorded with a NanoWizard® Atomic Force Microscope (JPK, Germany) operating in intermittent contact mode under ambient conditions. A standard rectangular cantilever (Nanosensors NCL-W) was used for imaging, with a free resonance frequency of 165 kHz and a typical spring constant of about 40 N/m. The radius curvature of the tip was ~10 nm. In order to ensure the reproducibility of the observed morphology, all samples were scanned at least on three different zones. Each sample was investigated using fresh tips previously cleaned by UV-ozone treatment. The measurement of nanorods length (typically 50 measurements per sample) was performed using JPK Data Processing software (JPK, Germany).

2.6. Fluorescence spectroscopy

Fluorescence assays were performed with SC3 hydrophobins dissolved in ultrapure water (pH 5.5), 0.5 mM NaCl (pH 5.5) and 10 mM NaCl (pH 5.5) at a concentration of 0.2 mg/mL in the presence of 40 μ M ThT. The protein solution and dye were carefully mixed in order to not introduce any air bubbles. Fluorescence was measured using a spectrofluorimeter FluoroLog® (HORIBA Jobin Yvon). Samples were excited at 435 nm and fluorescence was recorded between 450 and 600 nm with 5 nm slit widths and 1 s integration time. Intensity of 485 nm was used and the results are the mean of two replicates.

3. Results and discussion

3.1. Spontaneous self-assembly of SC3 hydrophobins in water

SC3 hydrophobins from *S. commune* are well known as highly tensioactive proteins that exhibit a tendency to self-assemble at hydrophobic–hydrophilic interfaces into amyloid fibres [12–15]. In solution, SC3 hydrophobins were shown to exist as soluble oligomers,

which were suggested to be the building blocks for both solution aggregation and interfacial assembly [15]. In this way, although the presence of small oligomers was shown to be predominant in solution, the existence of larger unstructured aggregates was also reported [17, 18]. However, the structured assembly of SC3 hydrophobins in solution and their morphological characterization has never been assessed before.

In the present study, in order to provide new insight into the SC3 hydrophobin self-assembly, the organization in solution of these amphiphilic proteins was studied. The influence of both pH and ionic strength on the protein assembly was also examined. De Vocht et al. [16] have previously shown that the SC3 hydrophobin self-assembly at the air–water interface is highly dependent on protein concentration. Indeed, at a protein concentration of 0.05 mg/mL, an overnight incubation was necessary for the organization of SC3 into rodlets, while two-times higher concentration (0.1 mg/mL) significantly increased the rate of the protein self-assembly into rodlets. Taking into account these previous results and to favour the protein self-assembly, the protein organization in solution was investigated in the present study at a protein concentration of 0.2 mg/mL.

In order to check whether SC3 hydrophobins organize in solution, their assembly process was followed directly in the protein solution by DLS experiments. For this purpose, hydrodynamic diameters of SC3 assemblies eventually formed were measured in ultrapure water (pH 5.5) for different incubation times: 5, 30, 60, 120, 180 and 240 min. Fig. 1 presents the distribution of hydrodynamic diameters of SC3 assemblies formed in solution with increasing incubation time. It comes out clearly that the size of SC3 assemblies increases with the time of incubation. Narrow size distribution was observed at each incubation time. The average diameter of 4 ± 1 nm measured after 5 min of incubation corresponds most likely to monomers and/or dimers and is in agreement with the results previously reported by

Wang et al. [15]. Larger assemblies were however formed after 30 min of incubation since the measured diameter increased up to 23 ± 6 nm. Hydrodynamic diameter of SC3 assemblies continued to increase and the size of 41 ± 8 nm was measured after 60 min of incubation. However, a threshold value of 55 ± 10 nm was reached after 120 min of incubation. DLS experiments also revealed the presence of large aggregates ($d_H > 500$ nm), which appeared already after 30 min. These aggregates correspond most likely to unstructured aggregates previously reported by Stround et al. [17]. Although qualitative, the DLS measurements carried out directly in solution revealed both that SC3 hydrophobins assemble in solution, and not only at the air–water interface, and that their assembly size increases with the incubation time.

In the next step, the morphology of SC3 assemblies formed in solution was assessed for the first time at a nanometer scale using AFM. Indeed, AFM was previously demonstrated to be an appropriate tool to study the spontaneous assembly in solution of different proteins into amyloid fibres by imaging dried protein solutions [19,20]. The AFM-based procedure developed in previous works and applied here consisted in a rapid drying of a tiny droplet of protein solution diluted 200 times onto a glass substrate prior to imaging [19]. Indeed, the abrupt dilution of the sample immediately quenches the concentration-dependent aggregation process. Moreover, deposition of a tiny droplet of this diluted bulk protein solution was followed by very fast evaporation of solvent, which additionally prevents further aggregation. Fig. 2 presents AFM phase images of dried droplets of SC3 hydrophobins solubilised in ultrapure water (pH 5.5) and incubated for 30, 60 and 120 min. The AFM images revealed for the first time that SC3 hydrophobins spontaneously self-assemble into nanorods in water. Moreover, the diameter of the nanorods, measured from the cross-section of the AFM height images, was 2 to 3 nm which corresponds to the diameter of a single SC3 hydrophobin [21]. The length of nanorods was directly measured from AFM phase images (at least 50 measurements per sample) for each incubation time (30, 60 and 120 min). After 30 min of incubation, the nanorods length was of 39 ± 6 nm. Further increase of the incubation time led to slightly longer nanorods of 66 ± 19 nm formed after 60 min of incubation. Under the assumption that all nanorods do not grow up in the solution in the same time and/or at the same rate, the larger dispersion in the measured lengths (after 1 h of incubation compared to 30 min) could be related to the larger possibility to start the protein assembly. A weak evolution in the nanorods length was observed for a prolonged incubation time (120 min) with a length value of 73 ± 20 nm. For longer incubation times, no significant evolution of the nanorods length was observed, confirming the kinetic of average hydrodynamic diameter measured by DLS (Fig. 1B).

At the air–water interface, it was established that the self-assembly of SC3 hydrophobins involves several conformational changes that lead, after several hours of incubation, to the final β -sheet-rich amyloid rodlet structure [16]. In order to get further insight into the structure of SC3 nanorods self-assembled in solution, the fluorescent probe ThT was used. Indeed, ThT greatly increases its fluorescence upon binding to stacked β -sheets such as those observed in amyloid fibres [22]. It was previously demonstrated that ThT specifically interacts with SC3 interfacial assemblies, which allowed the conclusion that SC3 hydrophobins self-assemble at interfaces in the same manner as amyloidogenic proteins, *i.e.* through β -sheet stacking [23]. The fluorescence intensity of ThT was measured directly in solution of SC3 hydrophobins at 0.2 mg/mL in a continuous manner during the protein incubation. The emission fluorescence spectra measured after 5, 30, 60 and 180 min of the incubation in ultrapure water (pH 5.5) are presented on Fig. 3. The 4-fold increase in the fluorescence intensity was measured after 5 min of incubation of SC3 hydrophobins in water in comparison to ThT alone. This increase in the fluorescence intensity comes from the β -sheets, which are involved into the nanorod formation. The fluorescence intensity increased continuously from 5 to 60 min of incubation, which suggests that more ThT interacts with longer nanorods since an

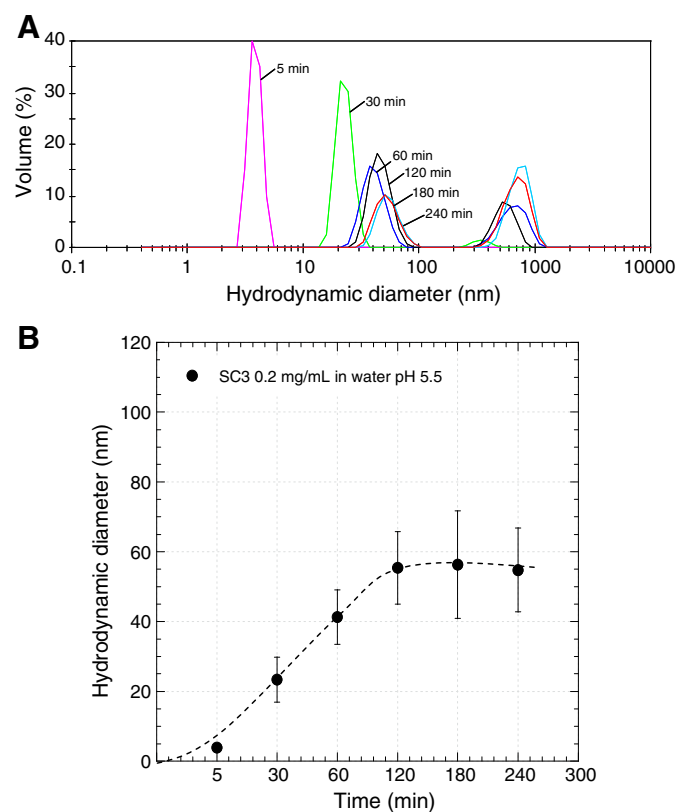


Fig. 1. (A) Distribution of hydrodynamic diameters (nm) of SC3 hydrophobin assemblies measured by DLS as a function of incubation time. (B) The evolution of average hydrodynamic diameters of SC3 hydrophobin assemblies with the incubation time (larger aggregates with the diameter above 500 nm are not taken into account). The X-axis is not linearly spaced.

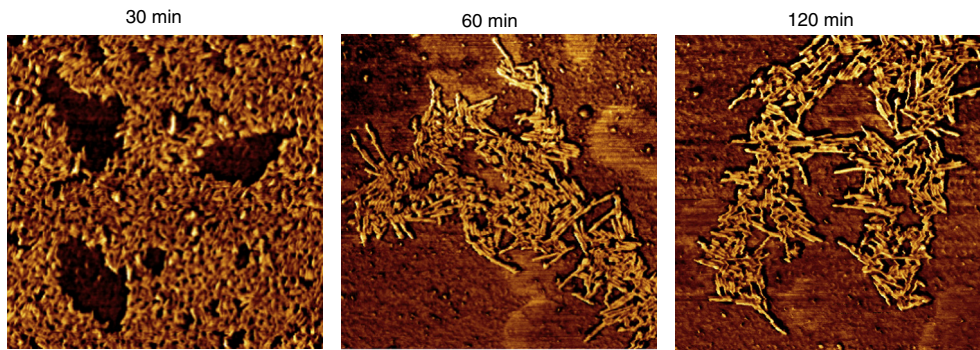


Fig. 2. AFM phase images showing the SC3 nanorods self-assembled in ultrapure water (pH 5.5) under ambient conditions at a concentration of 0.2 mg/mL after 30, 60 and 120 min of incubation. Image size $1 \times 1 \mu\text{m}$.

increase in nanorods length was evidenced by AFM after 60 min of incubation (Fig. 2). No further increase in the fluorescence intensity was measured above 60 min of incubation. Indeed, the same fluorescence intensity was recorded between 60 and 180 min of incubation (Fig. 3A). The increase in the emission fluorescence, which is due to the binding of ThT to SC3 nanorods self-assembled in solution put forward that the mechanism of nanorod formation in solution involves β -sheet stacking. This result shows that SC3 hydrophobins self-assemble into nanorods through β -sheets not only at the air-water interface, but also in solution. In Fig. 3B, the same evolution of both fluorescence maximum intensity and average hydrodynamic diameters of SC3 hydrophobin assemblies with the incubation time is evidenced.

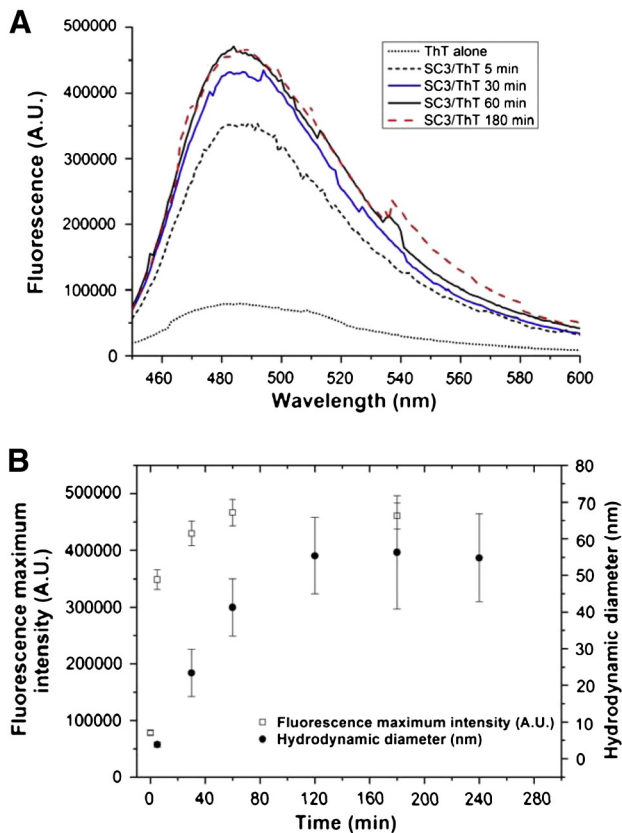


Fig. 3. (A) Fluorescence spectra of thioflavin T alone and in the presence of SC3 hydrophobins incubated in ultrapure water at pH 5.5 for 5, 30, 60 and 180 min. (B) The evolution of both fluorescence maximum intensity and average hydrodynamic diameters of SC3 hydrophobin assemblies with the incubation time. The value of the fluorescence maximum intensity at 0 min corresponds to the measurement performed in ThT alone.

3.2. Factors affecting SC3 self-assembly

At solution pH close to their isoelectric point ($pI = 4.35$) with no ionic strength (ultrapure water, pH 5.5), SC3 hydrophobins spontaneously self-assembled into nanorods through β -sheets (Figs. 2 and 3A). It was previously demonstrated that solution pH and ionic strength considerably influenced the self-assembly process of proteins by modulating electrostatic interactions between proteins [24–26]. Indeed, protein–protein interactions are highly influenced by the charges introduced in solution. However, how the ionic strength and solution pH affect the self-assembly of SC3 hydrophobins in aqueous solution remains an open question. Therefore, the influence of these parameters on the SC3 self-assembly was studied by AFM and fluorescence spectroscopy experiments.

3.2.1. Ionic strength

The effect of ionic strength on the self-assembly of SC3 hydrophobins was studied in the presence of three NaCl concentrations: 0.5 mM, 5 mM and 10 mM NaCl at pH 5.5. The incubation time was set to 60 min since the nanorod length was sufficient to allow the visualization of SC3 assemblies. Indeed, as observed by AFM, SC3 incubated for 60 min in water (pH 5.5) spontaneously self-assembled into long nanorods of 66 ± 19 nm. However, the presence of a very low salt concentration (0.5 mM NaCl) had an important effect on protein self-assembly as revealed by AFM images presented on Fig. 4. At this salt concentration, significantly longer nanorods of 134 ± 22 nm were formed in comparison to those assembled with no salt present. In contrast, the addition of higher salt amount led to a significant decrease in nanorods length. Indeed, short nanorods of 59 ± 6 nm were formed in the presence of 5 mM NaCl. Further increase in salt concentration (10 mM NaCl) led to even shorter nanorods of 40 ± 4 nm when compared to those formed in the presence of 5 mM NaCl (Fig. 4). It should be noted that increasing salt concentration not only affects the protein self-assembly into nanorods, which results in length decrease, but also promotes the aggregation of hydrophobins. Indeed, by comparing the height AFM images recorded at 5 mM and 10 mM with that obtained at 0.5 mM NaCl (Fig. 4), the undesired aggregation takes place even for a low salt concentration (5 mM).

In order to get further insight into the structure of SC3 nanorods assembled in the presence of NaCl salt, ThT probe was used. The fluorescence intensity of ThT measured in the presence of SC3 hydrophobins assembled in aqueous solutions of 0.5 mM, 5 mM and 10 mM NaCl at pH 5.5 is presented on Fig. 5. A 5.5-fold increase in fluorescence intensity of ThT was observed when the nanorods were formed in the presence of 0.5 mM NaCl, when compared to the spectrum of ThT alone (in 0.5 mM NaCl at pH 5.5). However, the presence of 5 mM and 10 mM NaCl during the protein assembly directly affected the ThT binding. Indeed, for salt concentrations of 5 mM and 10 mM NaCl, the fluorescence

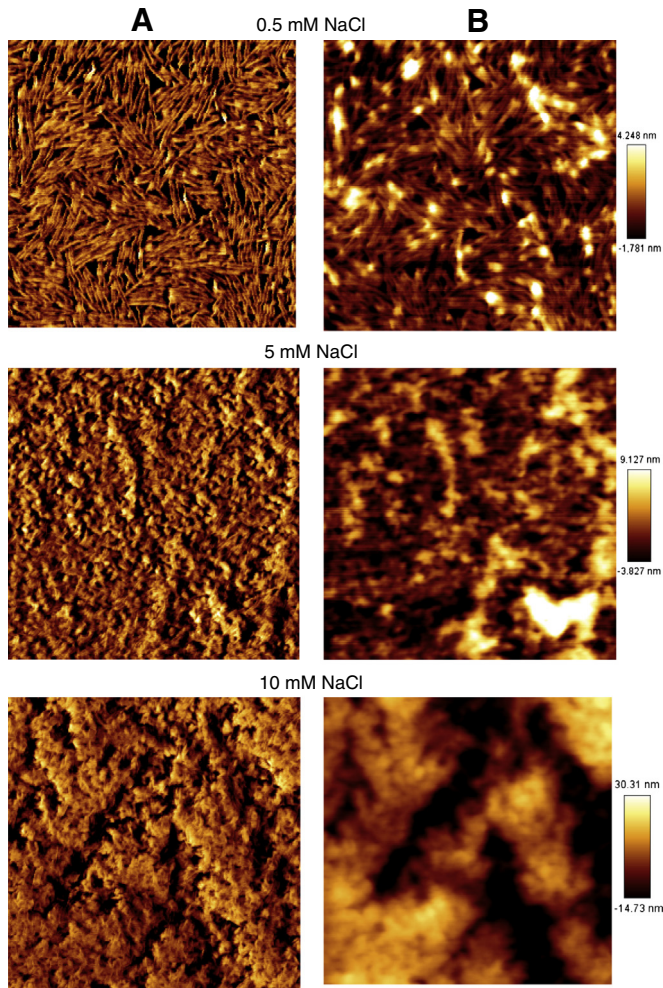


Fig. 4. AFM phase (A) and height (B) images showing the influence of varying NaCl concentrations at fixed pH on SC3 self-assembly into nanorods. SC3 hydrophobins at 0.2 mg/mL were incubated for 60 min in aqueous solutions of 0.5 mM, 5 mM and 10 mM NaCl at pH 5.5. Image size $1 \times 1 \mu\text{m}$.

intensity increased, respectively, only 4.2-fold and 3.4-fold, when compared to the spectrum of ThT alone (in 5 mM and 10 mM NaCl at pH 5.5). The increase in fluorescence intensity of ThT is directly related to the nanorod length as confirmed by AFM measurements.

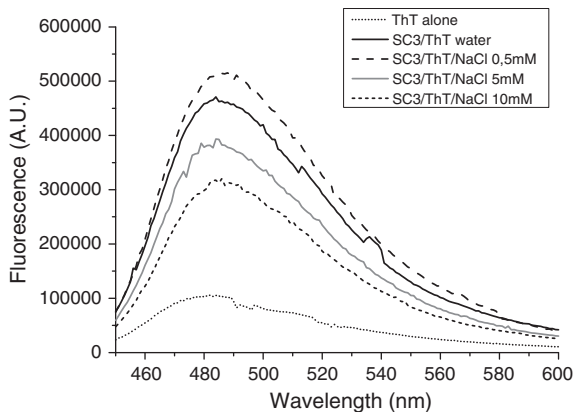


Fig. 5. Fluorescence emission spectra of thioflavin T in the presence of SC3 hydrophobins incubated in ultrapure water and in aqueous solutions of 0.5 mM, 5 mM and 10 mM NaCl at pH 5.5 for 60 min. The fluorescence intensity of ThT alone was measured in the presence of 0.5 mM, 5 mM and 10 mM NaCl at pH 5.5, and similar emission spectra were obtained.

The decrease in fluorescence intensity in solution observed with increasing salt concentration highlights that increasing salt amount inhibits the SC3 assembly through β -sheet stacks.

There are several ways by which the presence of salts in solution can modulate electrostatic and hydrophobic interactions involved in amyloid fibril formation. Indeed, salts can influence protein stability by Debye–Hückel screening of the protein charges [27] and/or by direct binding of these charges. Debye–Hückel screening with ions present in solution results from non-specific clustering around charged protein groups, causing the weakening of stabilizing charge–charge electrostatic interactions. In the same way, repulsive intermolecular interactions are screened as well, allowing the forces that govern protein–protein association to predominate. Salts can also alter the properties of the protein–solvent system by so-called Hofmeister effects [28], which contribution to the protein stability arises from the effects of ions present on the properties of water. The Hofmeister series rank ions on their ability to precipitate proteins. Kosmotrope ions, strongly hydrated and small (e.g. sodium cations), favour “salting out” (solubility decrease) of proteins, while chaotropes ions, large and weakly hydrated (e.g. chloride anions), promote “salting in” (solubility increase) of proteins. Several studies have shown that salts present in solution and solution pH have strong effects on the self-assembly of amyloidogenic proteins [20,25,26,29].

In the present study, at pH 5.5, SC3 hydrophobins are negatively charged ($pI = 4.35$). However, this net negative charge on proteins does not prevent their self-assembly into nanorods, as SC3 hydrophobins were observed to spontaneously form nanorods through β -sheet stacks in ultrapure water at pH 5.5 (Figs. 2 and 3). This can directly be related to the SC3 structure. Indeed, SC3 is only weakly charged as it contains only one basic (His) and two acidic (1 Asp and 1 Glu) amino acid residues in its sequence [30]. Therefore, hydrophobic/hydrophilic interactions constitute the main driving forces responsible for the protein assembly. This explains why even a very low salt concentration (0.5 mM NaCl) highly affects the protein assembly. It is likely that at 0.5 mM NaCl the protein assembly into longer nanorods, when compared to those formed in ultrapure water with no salt present, results from screening of protein charges by ions present in solution. However, as revealed by AFM measurements, a further increase in ionic strength (up to 10 mM NaCl) decreases the protein assembly into nanorods. This behaviour can be due to the fact that ions not only screen the protein charges in solution, but they may also bind to the protein surface [31–34]. The binding of ions would result in an increase in net charge on proteins, which disrupt the protein self-assembly into nanorods. Indeed, the mechanism of SC3 assembly through stacked β -sheets was highly affected, since an important decrease in ThT binding to nanorods was observed with increasing salt concentration (Fig. 5).

3.2.2. Solution pH

The influence of solution pH on the organization of SC3 hydrophobins was investigated for two opposite pH values, pH 3 and pH 9, at low ionic strength (0.5 mM NaCl) for the incubation time of 60 min. As observed on AFM images presented on Fig. 6, at pH 3, SC3 hydrophobins self-assembled in short nanorods of 31 ± 6 nm in length. In comparison, at pH 9, long nanorods of 113 ± 18 nm in length were formed. In addition, at pH 9, nanorods were shown to be organized in domains composed of several aligned rods, whereas isotropically dispersed nanorods were mainly observed at pH 3 (Fig. 6). Solution pH determines the protein charges and thus intermolecular electrostatic interactions between charged groups of proteins. At pH 5.5, hydrophobins are predominantly negatively charged, and they spontaneously self-assemble into nanorods through stacked β -sheets (Figs. 2 and 3). Protein–protein assembly into nanorods was additionally enhanced by the presence in solution of small amount of ions, which screen negative charges of proteins further favouring assembly (Fig. 4). At pH 9, negatively charged SC3 proteins self-assemble into long nanorods in a very similar manner to nanorods formed at pH 5.5 with 0.5 mM NaCl. Indeed, the presence of a small

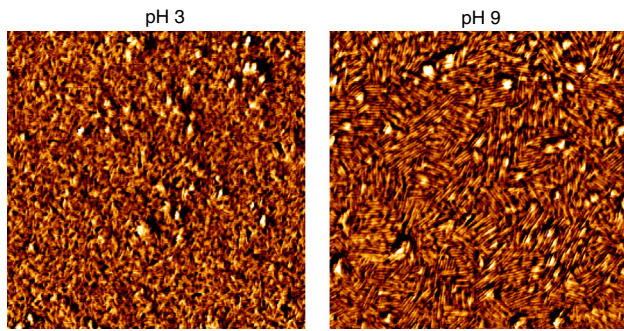


Fig. 6. AFM phase images showing the influence of pH at fixed ionic strength on SC3 self-assembly into nanorods. SC3 hydrophobins at 0.2 mg/mL were incubated for 60 min in aqueous solutions of 0.5 mM NaCl at pH 3 and pH 9. Image size $1 \times 1 \mu\text{m}$.

salt amount at pH 9 additionally enhanced the protein assembly into nanorods. In contrast, at pH 3, the net protein charge is positive. In this case, the short nanorods formed result most likely from binding of chloride anions on the protein surface, which reduces the protein assembly through repulsive negative charges. This result is in good agreement with previous works showing that proteins exhibit selective and preferential binding of anions on their surface at low ionic strength [31–34].

4. Conclusion

In the present work, the organization in aqueous solution of SC3 hydrophobins from the fungus *S. commune* was assessed. The SC3 self-assembly was followed by DLS, AFM and fluorescence spectroscopy. DLS experiments were performed directly in the protein solution in order to measure *in situ* the average size of SC3 assemblies. AFM imaging was then used to visualize the morphology of protein assemblies formed in solution and fluorescence spectroscopy with the fluorescent dye thioflavin T was carried out to characterize their structure. These complementary approaches have evidenced that SC3 hydrophobins are able to spontaneously self-assemble in ultrapure water and not only at the air–water interface. DLS experiments revealed that SC3 hydrophobins spontaneously assemble in aqueous solution and that their assembly size increases with the incubation time. AFM showed for the first time that SC3 hydrophobins are self-assembled into nanorods and confirmed that their length increased with the incubation time. The fluorescence emission of the fluorescent dye measured in solution allowed to establish that SC3 hydrophobins self-assemble into nanorods through stacked β -sheets. Then, the effects of ionic strength and solution pH on the protein self-assembly were evaluated. The presence of a very low ionic strength at pH 5.5 significantly enhanced the protein self-assembly, by screening of protein charges, since long nanorods were formed. However, a further increase of ionic strength favours binding of ions present in solution on the protein surface, which disrupt the protein self-assembly into nanorods. It appeared also that the solution pH had a significant effect on the SC3 hydrophobin self-assembly. Acidic pH was shown to considerably lower the SC3 self-assembly, which led to formation of short nanorods. In comparison, the protein assembly at pH 5.5 and pH 9 was favoured due to the presence of small salt amount, which efficiently screen the negative protein charges.

It can be concluded from this study that the self-assembly process of SC3 hydrophobins not only occurs at interfaces but also occurs in solution. The protein self-assembly in solution was shown to be tuned by solution pH and ionic strength. Therefore, the controlled assembly of SC3 hydrophobins with well-defined length of nanorods could easily be elaborated directly in aqueous solution. Elucidation of factors influencing the self-assembly of proteins and the understanding of how they affect the resulting assembly is a crucial step to efficiently control the protein organization. Such control allows opening the way

for the development of new smart self-assembled structures for targeted applications.

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Notes

The authors declare no competing financial interest.

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