Chemical and Thermal Denaturation of Crystalline Bacterial S-Layer Proteins: An Atomic Force Microscopy Study

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ABSTRACT Crystalline monomolecular cell surface layers, S-layers, are one of the most common outermost cell envelope components of the prokaryotic organisms (bacteria and archaeda) that protects them from competitive habitats. Since isolated S-protein subunits are able to re-assemble into crystalline arrays on lipid films and solid supports making biomimetic surfaces, S-layer technology is currently used in nanobiotechnology. An important aspect of the biomimetic surfaces built with S-layers is their stability under extreme solvent conditions or temperature. Chemical (pH, alcohol) and physical (thermal) denaturant conditions were employed to test the stability of S-layers. Recrystallized bacterial surface layers from *Bacillus sphaericus* (SbpA) on hydrophilic silicon wafers loses the crystalline structure at 80% ethanol/water mixtures, the change in structure being reversible after treating the surface with buffer solution. SbpA on silicon supports denatures at pH 3 and at 70°C, and the process is irreversible. Cross-linking of SbpA enhances the stability for high ethanol and acidic conditions, but it does not improve thermal stability. Recrystallized SbpA on secondary cell wall polymer (SCWP), a natural environment for the protein layer, is more resistant to ethanol and pH exposure than recrystallized SbpA on hydrophilic silicon supports. Atomic force microscopy (AFM) was used to monitor the loss of stability and the changes in protein layer conformation. *Microsc. Res. Tech. 65:226–234, 2004.*

INTRODUCTION

Crystalline bacterial cell surface layers (S-layers) are one of the most common outermost cell envelope components of the prokaryotic organisms. They represent the simplest biological membranes developed during evolution (Sleytr et al., 1999). They are composed of a single protein or glycoprotein, and have the ability to assemble into a closed lattice during all stages of cell growth and cell division. They exhibit oblique, square, or hexagonal lattice symmetry. On the one hand, S-layers must be structurally robust to protect bacteria from adverse conditions in competitive habitats, reflecting the structure of the outermost layer-specific adaptations to environmental conditions. Factors that break the S-layer structure among others are extreme variations of pH, mechanical stress, high temperatures, changes of osmotic pressure, and foreign organisms (Engelhardt and Peters, 1998). On the other hand, S-layers should allow cell division, cell growth, or the passage of vesicles through the membrane.

The interest of S-layer stability and robustness is not only biological but technological. S-layers are currently used in biotechnology due to the ability of their subunits to reassemble into monomolecular arrays at the air water-interface (Pum and Sleytr, 1995), and on lipid films (Gufler et al., 2004), liposomes (Mader et al., 2000), solid supports (Gyorvary et al., 2003), and hollow polelectrolyte capsules (Toca-Herrera et al., 2004). They also have been used to make fusion proteins with functional biomolecules (Moll et al., 2002; Völlenkle et al. 2004).

Atomic force microscopy (AFM) (Binnig et al., 1986), has been widely used to study biological molecules due to its high topographical resolution (Müller et al., 1995) and to the possibility of measuring interaction forces (Capella and Dietler, 1999) or investigating elastic properties of polymers (Rief et al., 1997). Chemical denaturation of proteins is a basic experimental procedure to investigate the so-called protein-folding problem that deals with the prediction of the three-dimensional structure of a protein from its amino acid sequence. (Ferhst, 1999).

In this work, we study with AFM the loss of recystallized S-layers on hydrophilic silicon supports via chemical and physical denaturation. The denaturation procedure took place in three different ways: making hydrophobic residues soluble with different water/ethanol mixtures, breaking weak electrostatic interactions by changing the pH, and lowering the energy barrier of

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intermolecular interactions by increasing the temperature. We will show that only after denaturation with water/ethanol mixtures, is it possible to recover the crystalline structure. Chemical cross-linking with glutaraldehyde preserves the S-layer structure for the water/ethanol mixtures and pH variation but fails when the temperature is increased. We will also show that S-layer recrystallization on secondary cell wall polymer increases the stability of the protein layer in the presence of chemical denaturants (low pH, water/ethanol mixtures).

MATERIALS AND METHODS

Materials

The bacterial cell surface layer proteins SbpA used in this study were isolated from Bacillus sphaericus CCM2177. Growth in continuous culture, cell wall preparation, and extraction of SbpA with guanidine chloride, dialyzation, and further centrifugation were done according to (Sleytr et al., 1986). S-layer protein self-assembly products formed during dialysis were sedimented for 15 minutes at 4°C. The concentration of the clear supernatant containing the disassembled S-layer subunits was adjusted with Millipore water to 1 mg mL^{-1}.

Millipore water (18.2 MΩ cm, pH 5.5) and ultra pure ethanol (Sigma, St. Louis, MO) were used to make four water/ethanol mixtures: 80/20, 60/40, 40/60, and 20/80 in v/v. Buffer solutions were prepared with Millipore water and citric acid-monohydrate (Merck) for pH 3 and Tris-HCl (Gerbu) for pH 7 and 9. To enhance the stability of the S-layer by cross-linking, 0.5% glutaraldehyde (GA, Merck) in 100 mM cacodylic acid buffer (Fluka) was used.

Sample Preparation, Protein Recrystallization, and Measurement Procedure

Silicon wafers (IMEC, Belgium) with a native silicon oxide layer were cut into pieces 1×1 cm^2 in size. The wafers were O_2 plasma-treated to make them hydrophilic (Gala Instruments, Germany). The cleaning procedure was done as follows: 20 seconds at 0.01 mbar; power density, 70%; and high purity grade O_2 (Linde). The advancing contact angle (Kruess contact angle measurement system G1, Hamburg, Germany) of water for a sample set of 40 silicon wafers was $45 \pm 1°$. The advancing contact angle of water on the plasma-treated silicon supports was $10 \pm 2°$. The hydrophilic silicon supports were used for protein recrystallization immediately after plasma treatment.

Treated silicon wafers and gold glass slices covered with thiol-secondary cell wall polymer (thiol-SWCP) (Sleytr et al., 2001) were immersed in a recrystallizing solution containing 0.1 mg mL SbpA in 0.5 mM Tris-HCl buffer, pH 9, 10 mM $CaCl_2$, and kept there overnight. Samples with recrystallized protein were washed with Millipore water before starting the AFM experiments. The samples were scanned in aqueous solutions containing 100 mM NaCl to avoid electrostatic repulsion between tip and sample and, hence, optimise the lateral and vertical resolution of the images. Once the crystalline S-layer pattern was resolved, the sample was exposed to the denaturant solution. After 30 minutes, the denaturant solution was exchanged by aqueous solutions containing 100 mM NaCl and the effect of the denaturant on the sample was investigated. The samples that presented a partial or total loss of structure were introduced overnight in recrystaillizing buffer without protein. These treated samples were scanned again to test if the loss of the S-layern pattern was irreversible or not. The thermal experiments were carried out as follows: (1) exposure of the protein surface of the sample to water at the desired temperature for 10 minutes, (2) replacement of the warm water by aqueous solution with 100 mM NaCl, and (3) measurement of the sample at room temperature after thermal reequilibration. Crosslinking of S-layers was performed by exposing the sample to glutaraldehyde in 100 mM cacodylic acid buffer for 15 minutes.

At least five samples were investigated for every denaturant condition and three sample regions were studied. This procedure was carried out with three independent set of samples.

Apparatus

Scanning force microscopy images were obtained using a Nanoscope III multimode (Veeco Instruments Inc. Santa Barbara, CA) in contact mode. Oxide-sharpened tip silicon nitride (Si_3N_4) cantilevers (Nanoprobe, NP-S) with a nominal spring constant of about 0.1 N/m were used. The surfaces were scanned at 4–5 Hz on areas ranging from 1000 nm down to 250 nm at constant loads ranging from 300 pN to 1 nN. The low loads prevent damage of the surface by the scanning tip and optimise the quality of the image. Force-distance curves were measured by approaching and separating the AFM tip to and from the surface while monitoring the deflection of the cantilever spring to which the AFM tip is attached. In this way, it is possible to obtain the so-called approach and retract curves and investigate the tip-surface interactions. The thermal treatment was carried out with a programmable oven, model 47900 (Bamstead-Thermolyne).

RESULTS AND DISCUSSION

Chemical and Temperature Denaturation

The first denaturation experiment on S-layers recrystallized on hydrophilic silicon supports was carried out treating the surface with different water/ethanol mixtures. Figure 1a shows the typical S-layer pattern, with lattice parameters a = 14.9 nm, b = 13.5 nm, $\gamma = 90°$. The rms roughness (mean root square, Rq) of such a surface is typically 0.1–0.2 nm. Figure 1a also shows a force-distance curve where no adhesion is observed. This is a common feature for recystrallized S-layer, which behaves as a solid, non-adherent surface. Figure 1b shows the topography of the S-layer after exposure to 80% ethanol. It can be seen that at this ethanol concentration, the periodicity of the S-layer lattice is lost. Another kind of structure with distance between sub-units of about 30 nm in which one can distinguish square-like aggregates (presumably four sub-unit complexes) appears, although the lateral order is partially lost. Additionally the retract part of the force-distance curve shows unfolding peaks, with forces below 1 nN, that might correspond to arbitrary events of the S-layer protein. The force-distance measurement also shows hysteresis between the approach and retract curves, meaning that the sample has become

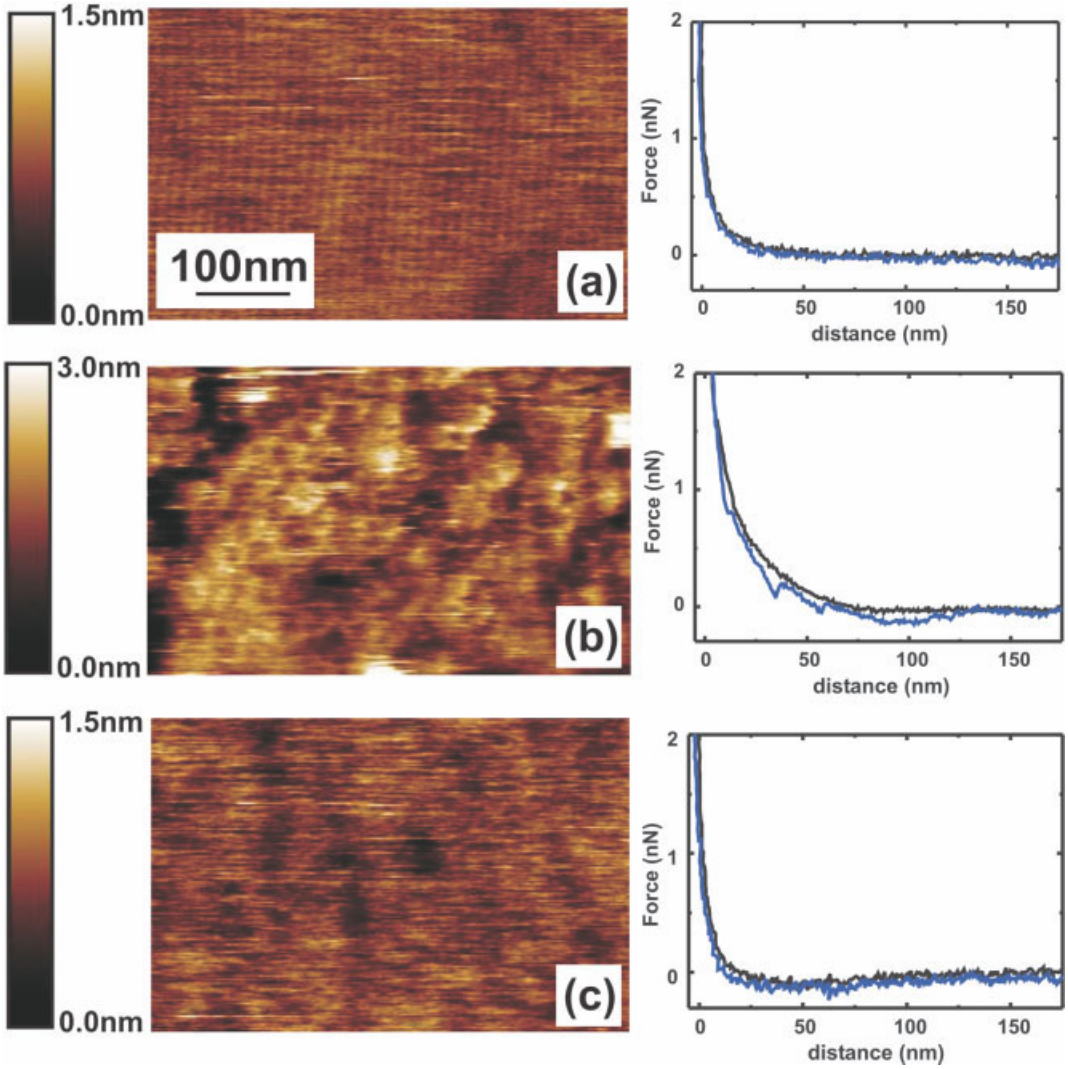

Fig. 1. AFM height images measured in contact mode of S-layers recrystallized on hydrophilic Si wafer. All the measurements were performed in 100 mM NaCl aqueous solution. **a:** S-layer just as it was formed; **b:** S-layer treated with EtOH/H_2O 80/20 (v/v); **c:** S-layer treated with recrystallizing buffer that follows the treatment described in b. On the right-hand side, force-distance curves show the AFM tip-S-layer interactions in each case. In the force-distance curves, the black line indicates that the tip aproaches the surfaces and the blue line that the tip leaves the surface.

"softer" with an augmentation of the sample rugosity (Rq = 0.544 nm). This fact also shows that the protein has not been desorbed from the surface. An interesting feature is shown in Figure 1c, where after treatment of the same sample with pH 9 buffer, the structure of the denaturated sample is recovered. The force distance-curve shows that no protein unfolding events happen, and the image shows the typical S-layer structure.

The second set of experiments consisted of exposing the S-layer to different temperatures and studying its thermal stability. Figure 2 shows some of the results from this study. It was found that the S-layer structure resisted 10-minute thermal treatment at different temperatures, ranging from 30 up to 60°C. At 70°C, the structure is considerably changed with respect to the typical lattice shown in Figure 2a. The force-distance curve that corresponds to Figure 2a shows again no adhesion and no deformation regions, although occasionally the approach and retract curves do not show a monotonous repulsion, like in the case shown. This may be due to the experimental dispersion when using different AFM tips (Butt, 1991). However, an extensive study has not been done in this regard. Figure 2b shows a region within the surface after heating at 70°C for 10 minutes. It should be said that, taking into account images done on different positions within the surface, the sample is heterogeneous. Distorted, square lattice-like regions of periodicity ranging from 20–30 nm as well as regions showing no structure at all can be detected on the same sample. Occasionally holes can be seen, indicating that loss of material may also eventually occur when the temperature is increased. The rms roughness, Rq, is larger (0.6) than that of the non-distorted S-layers, which is typically in the range

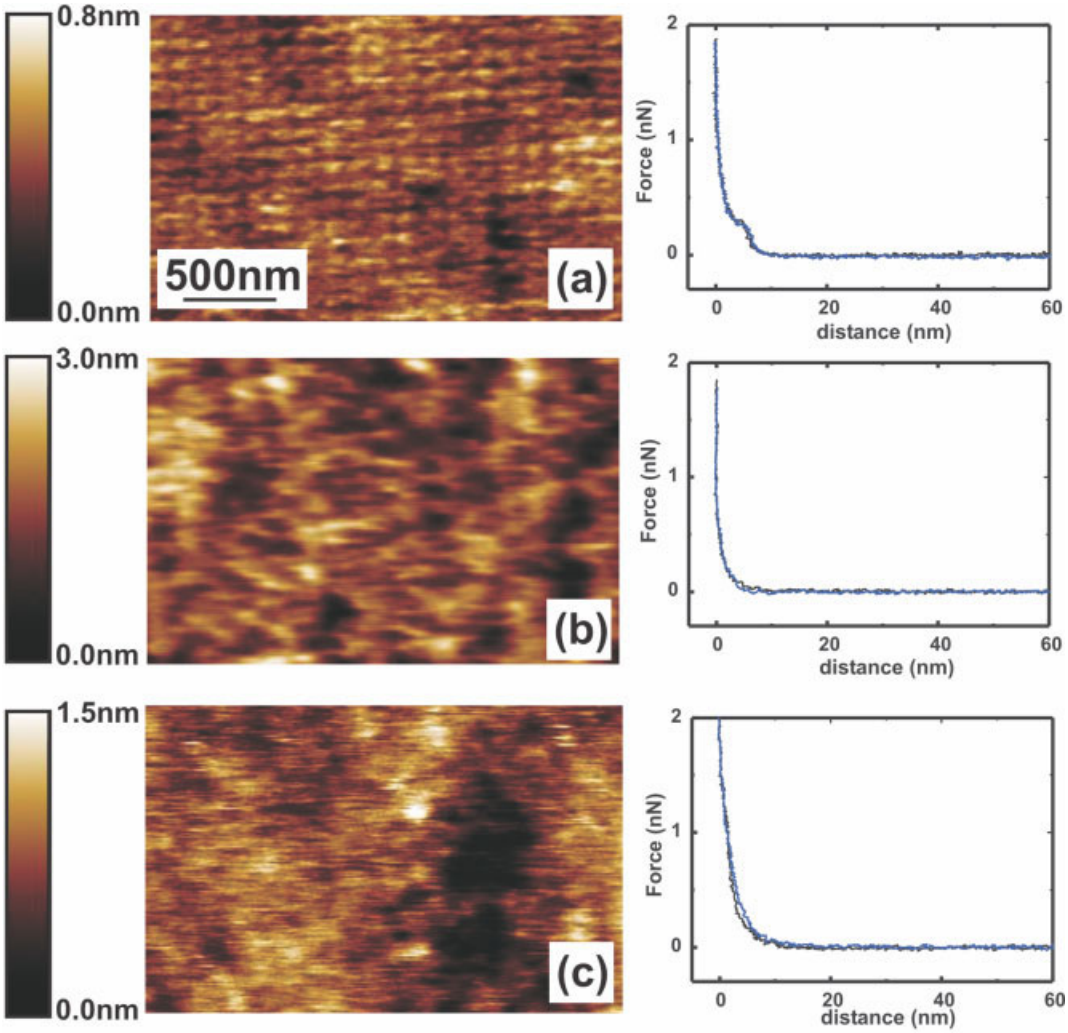

Fig. 2. AFM height images measured in contact mode of S-layers recrystallized on hydrophilic Si wafer. All the measurements were performed in 100 mM NaCl aqueous solution. **a:** S-layer just as it was formed; **b:** S-layer heated at 70°C for 10 minutes; **c:** S-layer treated with recrystallizing buffer follows the treatment described in b. On the right-hand side, force-distance curves show the AFM tip-S-layer interactions in each case. Note that the black line indicates the tip is aproaching and the blue line that the tip is retracting. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com]

of 0.1–0.2 nm when Si wafer is used as substrate. In this case, the force-distance curve does not show either elastic domains or hysteresis when the load is below 2 nN. This indicates that no softer regions were detected in the sample within this load range. Figure 2c shows the sample after buffer treatment. Ocassionally, periodical structures spaced by less than 20 nm due to a distorted square lattice can be seen together with some aggregates. However, no recovery of the S-layer structure just as it is seen in Figures 1a and 2a was possible.

The results of the denaturation with pH are shown in Figure 3. The S-layer structure was stable at pH values of 9, 7, and 5. At pH 3, the structure of the layer was lost. This result is similar to former results found in bacteria (Sleytr, 1975). Figure 3a shows the usual control experiment with the typical S-layer pattern showing no adhesion between the AFM tip and surface. Figure 3b shows the effect of pH on the same surface. The image reveals large features with spacing of about 40 nm. The former S-layer lattice is deformed and the typical structure has disappeared. Additionally, the rms roughness of the surface is one of the highest encountered in these investigations (Rq = 1.7 nm), which may indicate an extensive degree of denaturation of the structure. The force-distance curves show protein unfolding peaks of about 500 pN, indicating the existence of elastic domains involving one or more protein subunits, which corroborates the loss of crystal structure and the fact that the S-layer proteins are denatured and loosely adsorbed on the silicon wafer. The buffer treatment does not help to recover the structure of the S-layer, possibly due to the big loss of material and the extent of denaturation. This is shown in Figure 3c. This can be seen from the scanning micrograph where there is

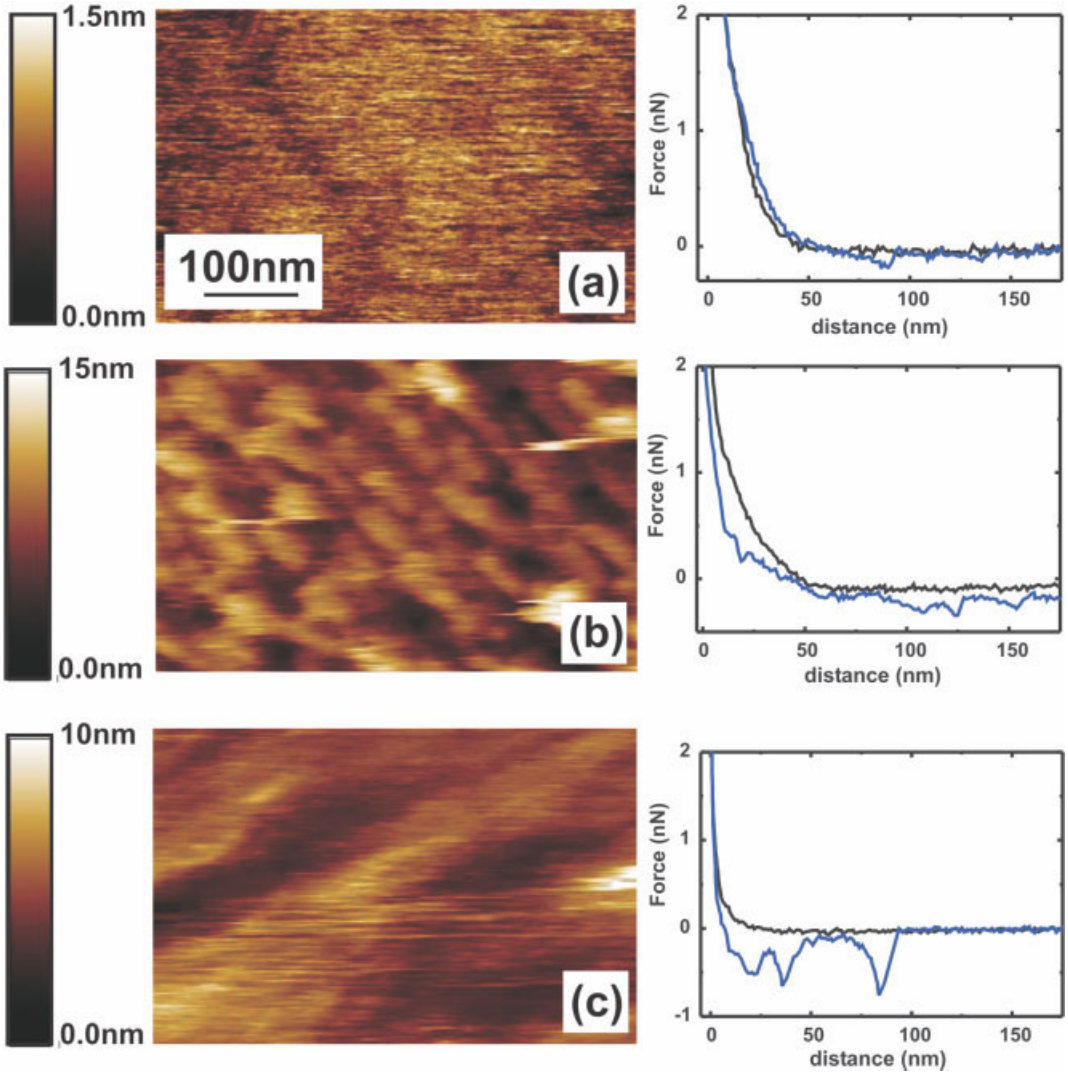

Fig. 3. AFM height images measured in contact mode of S-layers recrystallized on hydrophilic Si wafer. All the measurements were performed in 100 mM NaCl aqueous solution. **a:** S-layer just as it was formed (pH = 5.5); **b:** S-layer treated with pH = 3 buffer; **c:** S-layer treated with recrystallizing buffer (pH = 9) that follows the treatment described in b. On the right-hand side, force-distance curves show the AFM tip-S-layer interactions in each case. Note that the black line indicates the tip is aproaching and the blue line that the tip is retracting. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com]

no crystalline structure. In addition, the force-distance still shows elastic domains.

Stability of Cross Linked S-Layers

The same denaturation experiments were carried out with samples treated with glutaraldehyde, which was used to enhance the S-layer stability. Figure 4 shows the results. Glutaraldehyde is able to keep the S-layer structure after being treated with ethanol (Fig. 4a) and pH = 3 buffer (Fig. 4b), since the lattice parameters are the same as those measured for the controls. However, the cross-linking process is unable to keep the structure when the sample is exposed at 70°C (Fig. 4c).

Stability of Biomimetic S-Layer

The last sample to be investigated consisted of S-layers (SbpA) recrystallized on thiol-secondary cell wall polymer. Secondary cell wall polymer is composed of teichoid acids, teichuronic acids, lipoteichoic acids, or lipoglycans (Navarre and Schneewind, 1999) and is part of the cell envelope layer of gram-positive bacteria. This polymer interacts specifically with the so-called SLH-motifs of the S-layer protein SbpA (Sleytr et al., 2001), making our system more biomimetic than those investigated above. Figure 5 shows the results obtained for this system. This system has been seen to have a higher roughness; therefore, the AFM images shown are deflection images for a better appreciation of the image features.

Figure 5a shows an image on the sample prior to the denaturant process. Again, a typical S-layer lattice can be seen. However, the roughness (Rq = 0.9 nm) is larger than on silicon wafers, due to the polymer cushion. Figure 5b shows that the S-layer structure is partially lost after treatment with 80% ethanol (top of

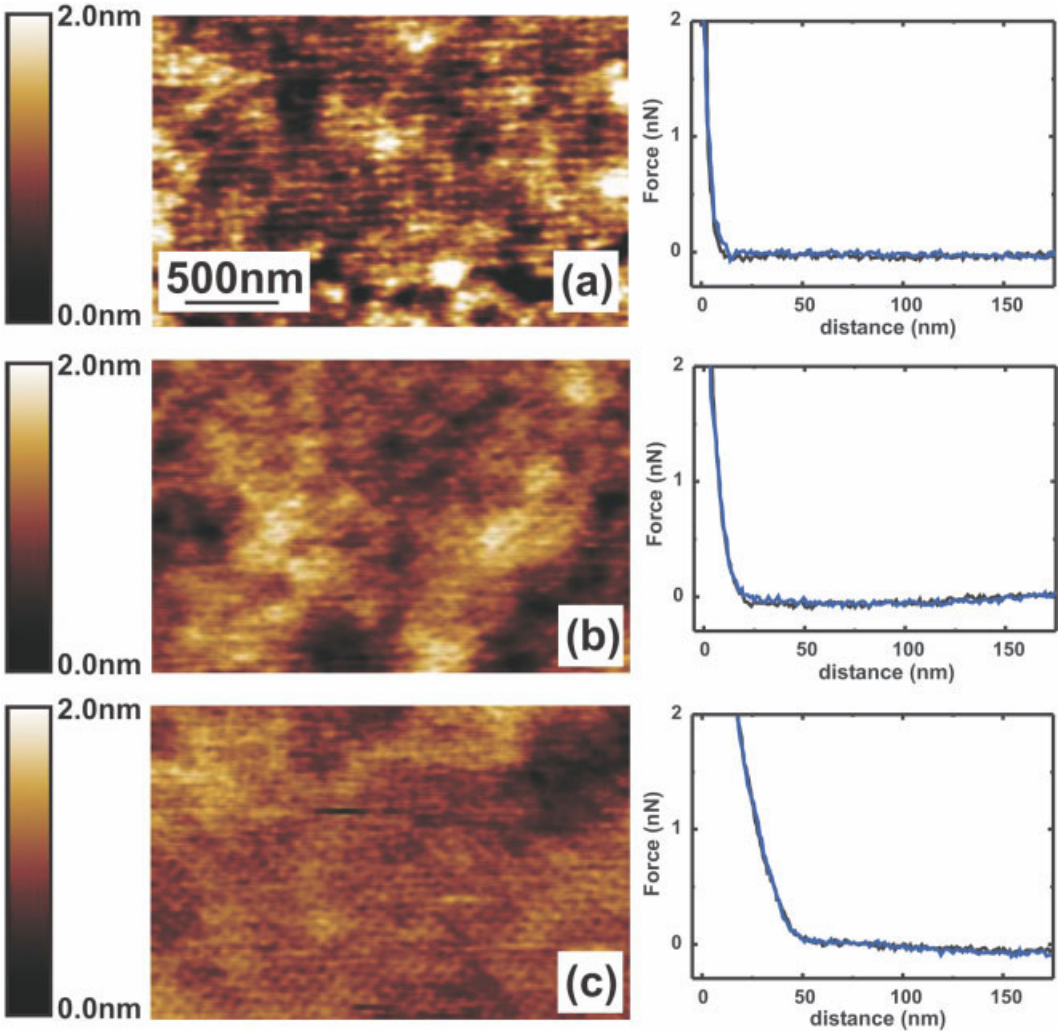

Fig. 4. AFM height images measured in contact mode of S-layers recrystallized on hydrophilic Si wafer and treated with glutaraldehyde solution. All the measurements were performed in 100 mM NaCl aqueous solution. **a:** Cross-linked S-layer just as it was formed (pH = 5.5); **b:** Cross-linked S-layer treated with pure EtOH; **c:** Cross-linked S-layer treated with pH = 3 buffer. On the right-hand side, force-distance curves show the AFM tip-S-layer interactions in each case. Note that the black line indicates the tip is aproaching and the blue line that the tip is retracting. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com]

micrograph), but that there are regions in which the lattice structure is preserved (bottom of micrograph), unlike what was observed in S-layers on Si wafers. On the other hand, the roughness that exhibits the surface is not appreciably different from that prior to the treatment (Rq = 0.8 nm). This may indicate that roughness is due to the polymer cushion and that the latter is not modified during the treatment. Figure 5c shows the state of the S-layer after buffer treatment. It can be observed that the structure has been preserved. Figure 5d shows the S-layer structure after exposing the sample at pH 3. Although an increase in roughness is detected (Rq = 1.7 nm), the S-layer pattern is visible. After treating the sample with buffer solution, the roughness decreases to 0.9 nm (Fig. 5d), returning to the original value and making the S-layer structure more evident. It is likely that pH may modify both the polymer cushion (i.e., a polyacid complex) and the S-layer to a greater extent than the alcohol. Like the previous systems, the lattice structure does not resist thermal treatment (10 min, 70°C). Buffer treatment did not help to recover the S-layer structure (results not shown here).

DISCUSSION

Although the experimental conditions for the crystallisation of bacterial cell surface layers SbpA on hydrophilic silicon wafers are known (Gyorvary et al., 2003), no comparative studies about the stability through chemical and thermal denaturation have been carried out. Table 1 summarizes the experimental observations concerning the conditions where the structure of the S-layer is lost and whether this change is reversible. Table 2 depicts the topological changes undergone on the different S-layers studied before and after the denaturant treatment. The results show that

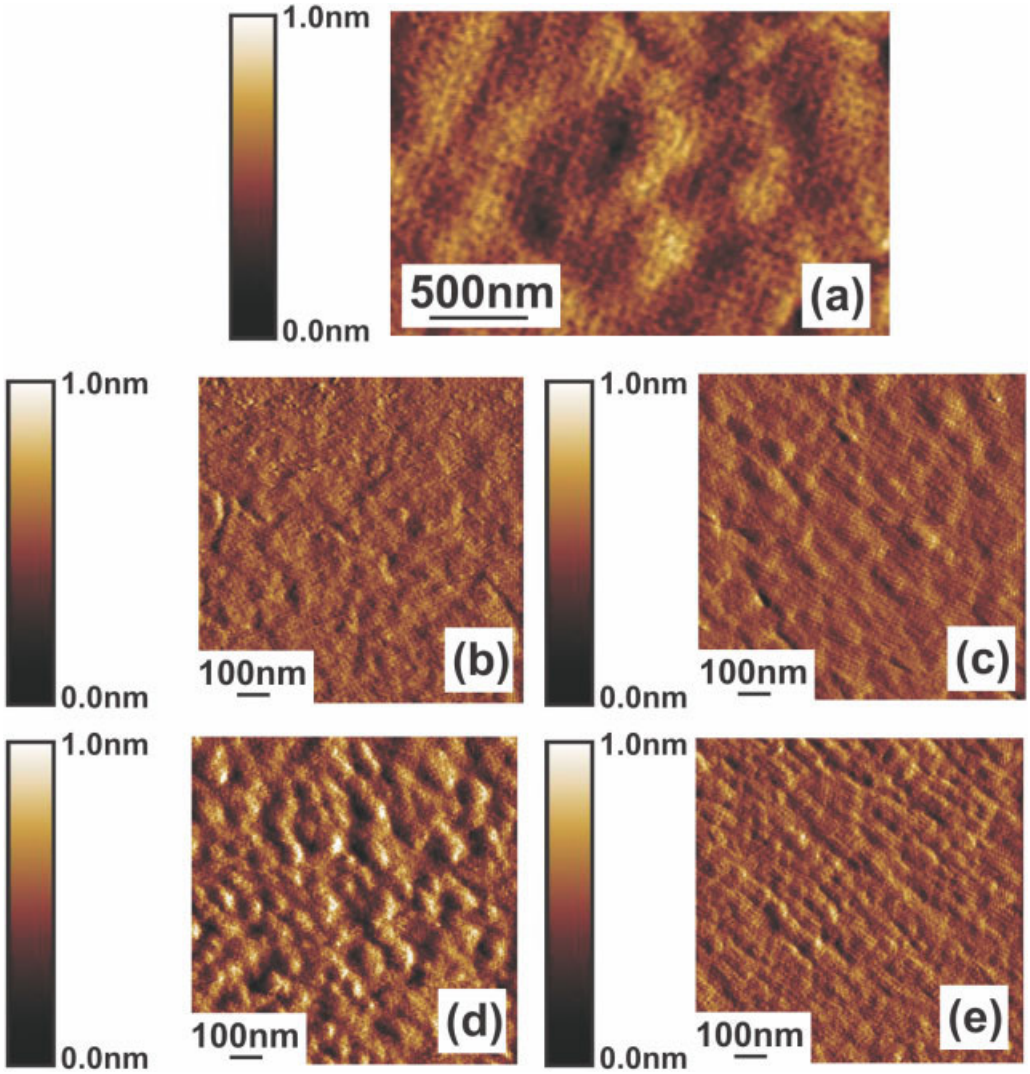

Fig. 5. AFM deflection images of S-layers recrystallized on self-assembled thiol-modified SCWP. Gold-coated Si wafers were used as substrate. All the measurements were performed in 100 mM NaCl aqueous solution. **a:** S-layer as it was formed; **b:** S-layer treated with EtOH/H_{20} 80/20 (v/v) mixture; **c:** S-layer following b and recrystallizing buffer treatments; **d:** S-layer treated with pH = 3 buffer; **e:** S-layer after recrystallizing buffer treatments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com]

TABLE 1. Summary of the experimental findings on the chemical and thermal stability of S-layers on Si wafer, a biomimetic support (SCWP), and crosslinked with glutaraldehyde (GA)

	Alcohol presence		pH		Temperature	
System	Stability	Reversibility	Stability	Reversibility	Stability	Reversibility
S-layer on Si wafer	Stable in <80% EtOH	Reversible at 80%EtOH	Stable at pH > 3	Irreversible at pH = 3	Loss of structure at 70°C (10 min)	Irreversible at 70°C
S-layer + GA on Si wafer	Stable in 100% EtOH	—	Stable at pH = 3	—	Loss of structure at 70°C (10 min)	Irreversible at 70°C
S-layer on thiolated-SCWP	Partial loss of structure in 80%EtOH	Reversible at 80% EtOH	Stable at pH = 3, rougher	Rugosity decreases	Loss of structure at 70°C (10 min)	Irreversible at 70°C

TABLE 2. Summary of structural parameters of S-layers prior to and after the denaturant treatment

	Before alcohol treatment (nm)		After alcohol treatment (nm)	
System	Lattice parameters	Roughness (Rq)	Lattice parameters	Roughness (Rq)
S-layer on Si wafer	a = b = 14–15	0.1–0.2	No lattice: square features 20-nm big	0.5
S-layer + GA on Si wafer	a = b = 13–16	0.2–0.4	a = b = 14–15	0.3
S-layer on thiolated-SCWP	a = b = 14–15	0.9	Partial loss a = b = 14–16	0.7–0.9
	Before pH treatment (nm)		After pH treatment (nm)	
System	Lattice parameters	Roughness (Rq)	Lattice parameters	Roughness (Rq)
S-layer on Si wafer	a = b = 14–15	0.1–0.2	No lattice: features sizing 20–40 nm	1.7
S-layer + GA on Si wafer	a = b = 13–16	0.2–0.4	a = b = 14–15	0.3
S-layer on thiolated-SCWP	a = b = 14–15	0.9	Rough surface, lattice present but not clear	1.7
	Before thermal treatment (nm)		After thermal treatment (nm)	
System	Lattice parameters	Roughness (Rq)	Lattice parameters	Roughness (Rq)
S-layer on Si wafer	a = b = 14–15	0.1–0.2	Loss or distorted lattice a = b = 20–30	0.6
S-layer + GA on Si wafer	a = b = 13–16	0.2–0.4	Loss of structure	1.2
S-layer on thiolated-SCWP	a = b = 14–15	0.9	Loss of structure	—

the recrystallized S-layers on hydrophilic silicon supports are resistant to high concentrations of ethanol (60–80%) and temperature (60–70°C) and that irreversible changes occur via rearrangement of the S-layer structure, which generally involves distortion of the lattice via subunit swelling and subsequent desorption of subunits. In all cases, complete loss of periodic structure occurs when roughness reaches a certain value (Rq = 1.7 nm).

It is shown that acidic conditions below pH 3 lead to a loss of crystalline structure. The low pH induces the protonation of carboxylic and amine groups in the hydrophilic shell and, therefore, a new distribution of charges that induces conformational changes. On the other hand, it is known that S-layers have a negative zeta potential (Toca-Herrera et al., 2004) and the distribution of charges on their surface is preferentially negative. Such a scaffold is maintained via divalent cations (Ca, Mg) (Gyorvary et al., 2003, Toca-Herrera et al., 2004) that keep the protein subunits bound to each other as well as to negatively charged surfaces, such as Si wafer. The protonation at low pH makes the protein subunits less negative, and thus induces loss of affinity to the cations and subsequent protein desorption. The protein-unfolding events show that this actually happens. Also in this case, a loss of material is detected, making it impossible to recuperate the initial bilayer S-layer structure (Gyorvary et al., 2003). This is shown when the denaturant is removed and replaced with buffer and the structure cannot be recovered. The structure of SbpA could only be recuperated in the case of ethanol denaturation. The ethanol can distort the protein structure in two ways: it can replace solvating water molecules that stabilize the hydrophilic shell as well as solubilize the hydrophobic core due to its aliphatic character. The AFM images show that there is evidence of a swollen structure and force-distance curves show relatively low desorption. Temperature denaturation increases the thermal energy of the S-layer protein leading to distortion of the S-layer structure possibly induced by conformational changes in the protein subunits. This is observed at temperatures around 70°C, prior to lattice collapse and desorption of subunits from the surface. S-layer cross-linking with glutaraldehyde proved to stabilise the protein layer structure at low pH and high ethanol concentrations, but failed to keep the structure at high temperatures. Since cross-linking is an artificial way to enhance stability of protein (See http://www.emdbiosciences.com/SharedImages/TechnicalLiterature/1_CB0539_protmod.pdf), this was a good test to check the weakness of natural S-layer samples (not cross-linked).

The S-layers recrystallized on secondary cell wall polymer most resemble the behaviour of the S-layer in its natural environment. In this case, the protein layer is bound specifically to the rough polymer cushion forming a monolayer (Sleytr et al., 2001) and has proved to be more resistant than S-layers on silicon supports. The experiments reveal that the specific interactions driven by the presence of SCWP as support play an active role in the stabilization and robustness of S-layers.

CONCLUSIONS AND OUTLOOK

In this work, we have studied the chemical and thermal denaturation of (1) recrystallized S-layers (SbpA) on hydrophilic silicon wafers, (2) recrystallized S-layers (SbpA) on secondary cell wall polymers/gold substrates, and (3) cross-linked S-layers on hydrophilic silicon wafers. Results show that after ethanol denaturation, the protein layer is able to recuperate its structure after exposing the denatured sample to a recrystallization buffer in all cases. Thermal denaturation induces the loss of crystalline structure at 70°C for every sample, the process being irreversible. S-layer denaturation at acidic pH 3 was only irreversible for sample (1). Samples (2) and (3) proved to be structurally resistant, although samples (2) proved to be

rougher. These results show that chemical cross-linking makes the S-layer more stable, but it is still a question whether this surface has the same biomimetic properties as the non-cross-linked ones.

The present work opens the way to the understanding of the denaturing processes taking place on S-layers. Chemical and physical denaturants induce changes on their structure that steadily lead to denaturation, which may be reversible or irreversible. This work has helped to characterize the reversibility from a phenomenological point of view. The turning point to define reversibility is hinted at in this work as a transition state of a distorted lattice of swollen sub-units, characterized by a certain roughness. However, dynamic scenery of how the changes take place and induce irreversibly is missing and this can be further investigated.

In addition, it has been found that the resistance to denaturation is enhanced when S-layers sit on a biomimetic surface, such as SCWP. Apart from the biomimetical advantages of using SCWP, this biopolymer is difficult to purify. This could be solved in the future by using synthetic polyelectrolytes with similar physical chemical properties as cushions for S-layers.

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