Chapter 2

Bacterial membrane formation monitored with atomic force microscopy and quartz crystal microbalance

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Referencing this chapter

1. Introduction

Proteins are the machinery of life (Alberts, Bray, Johnson, Lewis, Raff, Roberts et al., 1998). They have a prominent role in food, pharmaceutical, or cosmetic technology (Yampolskaya & Platikanov, 2006; McPherson, 2003). Protein structure relates to function. Thus, to be able to perform their biological function, proteins fold into a specific spatial conformation. However, a general problem that arises in the laboratory is that protein adsorption on surfaces can denature (Gray, 2004). Therefore protein adsorption (and protein kinetics) are useful experiments to gain insight over protein adsorption and functionality.

S-layers are composed of single (glycol)proteins and constitute the most common outermost envelope component in prokaryotic organisms. They show oblique, square and hexagonal lattice symmetry with pore size between 2-8 nm and a thickness of 5-25 nm (Sleytr, Messner, Pum & Sára, 1999). A very important property of isolated S-proteins in solution, which few proteins exhibit, is their ability to form regular protein crystals on different (soft) interfaces (i.e. silicon oxide, mica, polyelectrolytes, self-assembly monolayers, or lipid films (Delcea, Krastev, Gutberlet, Pum, Sleytr & Toca-Herrera, 2008; Delcea, Krastev, Gutberlet, Pum, Sleytr, Toca-Herrera et al., 2007; Toca-Herrera, Moreno-Flores, Friedmann, Pum & Sleytr, 2004; Györvary, Stein, Pum & Sleytr, 2003; Martín-Molina, Moreno-Flores, Perez, Pum, Sleytr & Toca-Herrera, 2006; Moreno-Flores, Kasry, Butt, Vavilala, Schmittel, Pum et al., 2008; Eleta-Lopez, Pum, Sleytr & Toca-Herrera, 2011). In the last years, the production of fusion proteins based on Sproteins have constituted an advance in sensor and nano technologies (Sleytr, Huber, Ilk, Pum Schuster & Egelseer, 2007; Kainz, Steiner, Sleytr, Pum & Toca-Herrera, 2010). In this work, we report on the adsorption and S-layer formation of the S-protein SbpA on silicon oxides substrates and at the air/water interface. Quartz Crystal Microbalance with Dissipation (QCM-D) monitoring was used to follow protein adsorption in real time, and estimate the adsorbed protein mass deposited per unit area. The nanostructure of the protein surface layers was investigated with atomic force microscopy measurements. Finally, the SbpA activity at the air water interface was determined with surface tension measurements.

2. Materials and methods

2.1. Materials

Silicon oxide coated quartz crystals (Q-sense, Gothenburg, Sweden) were used as substrates. SbpA (Mw=120 kDa), were isolated from Lysinibacillus sphaericus CCM 2177 according to a reported procedure (31). Protein recrystallization buffer was prepared with 5mM Trizma (Sigma) base and 10mM $CaCl_2$ (98%, Sigma) and adjusted to pH=9 by titration.

2% sodium dodecyl sulphate, SDS, (99%, Fluka) and Hellmanex II (2%, Hellma) were used as cleaning solutions. Aqueous solution of 100mM NaCl (Sigma) was used as media in AFM experiments.

Silicon oxide treatment. Silicon substrates with native silicon oxide layers were cleaned in 2% sodium SDS for 30 minutes, rinsed with ultrapure water (Barnstead) and dried under a stream of nitrogen. Afterwards the substrates were treated with ultraviolet radiation (Bioforce Nanoscieces) for other 30 minutes before silane modification or protein self-assembling.

S-protein adsorption. The S-protein solution was isolated as explained in reference (Sleytr, Sara, Küpcü & Messner, 1986). Due to the ability of S-proteins to self-assemble in solution, the protein extract solution (1mg/ml) was centrifuged at 5 rpm for 5 minutes to separate S-protein monomers from self-assembly products. The protein concentrate was determined with a Nanodrop spectrometer (Thermo Scientific, Wilmintong, USA). Before starting the experiments, the supernatant was diluted using the appropriate amount of recrystallizing buffer. On one hand, in-situ QCM-D experiments were carried out by protein solution injection into the experimental set up, once the substrates were stabilized in tris-buffer. On the other hand, ex-situ experiments were done using substrates where S-protein had been previously recrystallized. Small volume humidity chambers were used for that purpose preventing water evaporation. The protein was incubated between one and three hours at room temperature and afterwards the substrates were rinsed with recrystallizing buffer in order to remove excess of protein.

2.2. Apparatus

2.2.1. Quartz Crystal Microbalance with Dissipation monitoring (QCM-D)

The QCM-D set up consists of a thin AT-cut piezoelectric quartz crystal sandwiched between two electrodes. The crystal oscillates in shear modus when AC voltage is applied. The interesting feature is that the resonant frequency of the sensor depends on the total mass, which includes the entrapped water. For many cases, the measured change in frequency is proportional to the adsorbed mass (more details are given in the results section). This technique also permits to evaluate the dissipated frictional energy delivering information about the viscoelastic properties of the absorbed molecules on the quartz crystal (more details are given in the results section). In our investigation, QCM-D (Q-sense E4, Gothenburg, Sweden) was used to carry out real time/in situ experiments.

2.2.2. Atomic Force Microscopy (AFM)

The sensor of the AFM consists on a sharp tip mounted on a soft cantilever of a specific spring constant. In particular, due to the interaction forces between the tip and the sample it is possible to obtain topographical information at the nanoscale and quantify interaction forces down to dozens of pN. The optical detection system of the AFM consists of a photodiode that follows the cantilever deflection (through the reflection of the laser beam at its back side) while scanning the sample. The photodiode electrical signal is later processed by a computer that keeps the force on the tip constant by generating a feedback signal for the piezoscanner.

The original images presented in this manuscript were recorded in contact (at different scan rates) in 100mM NaCl aqueous solution, at room temperature using a Nanoscope V controlled Multimode AFM (Bruker, Santa Barbara, USA). Back side gold coated silicon nitride (Si3N4, NP-S, Bruker) cantilevers with nominal spring constant of 0.06 N/m were used.

3. Results and discussion

SbpA adsorption was monitored in real time by QCM-D. Figure 1 illustrates the variation of the frequency (a) and dissipation (b) as a function of time for three protein concentrations. When the protein solution is injected in the experimental chamber, the frequency decreases due to protein deposition, while the dissipation increases. Once no further changes in frequency and dissipation are observed, the excess of protein is removed from the chamber by rinsing with fresh buffer solution. This process hardly affects frequency and dissipation meaning that the freshly formed protein layer does remain on the silicon dioxide surface.
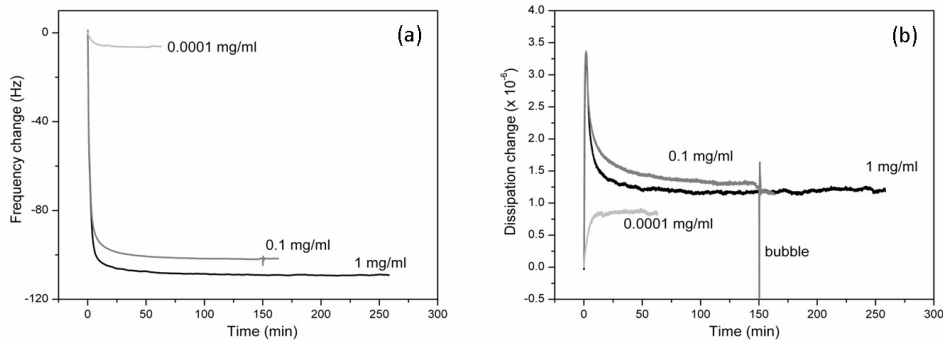
Figure 1. QCM-D measurement. (a) frequency change for SbpA adsorption on silicon dioxide as a function of time. A frequency shift of about 100 Hz for the two largest concentration is observed. Note the low frequency value obtained for 0.0001 mg/ml. (b) dissipation change for three SbpA concentrations as a function of time. The largest concentrations have a similar final value after protein layer is formation. (The straight line refers to an air bubble that appeared when rinsing with buffer)

In order to quantify the incorporated mass one should make certain assumptions: i) the protein layer is homogeneously distributed and rigid enough to avoid any oscillatory deformation, and ii) the added protein mass is lower than the quartz crystal itself.

Furthermore, if the overall dissipation-frequency shift ratio is smaller than $0.2 \times 10^{-6} Hz^{-1}$, (Gläsmäster, Larsson, Höök & Kasemo, 2002) the Sauerbrey equation can be used to determine adsorbed mass once the frequency change is known:

$$\Delta m = - \frac{C \cdot \Delta F}{n}$$

here Δm is the mass surface density (ng/cm^2), C is a constant that depends on the intrinsic properties of the sensor ($-17.7 \, ng/(Hz \cdot cm^2)$), ΔF is the frequency change and n is the overtone number. In this way the mass per unit area calculated using the 5th overtone (ΔF), is $1930 \, ng/cm^2$, $1800 \, ng/cm^2$, and $108 \, ng/cm^2$ for 1 mg/ml, 0.1 mg/ml and 0.0001 mg/ml respectively. (Note that the smallest concentration did not lead to a compact protein layer, as the force-distance curves will show later, and therefore the values reported are just illustrative.).

Figure 2 shows the variation of the adsorbed mass with time for SbpA adsorption on silicon dioxide.
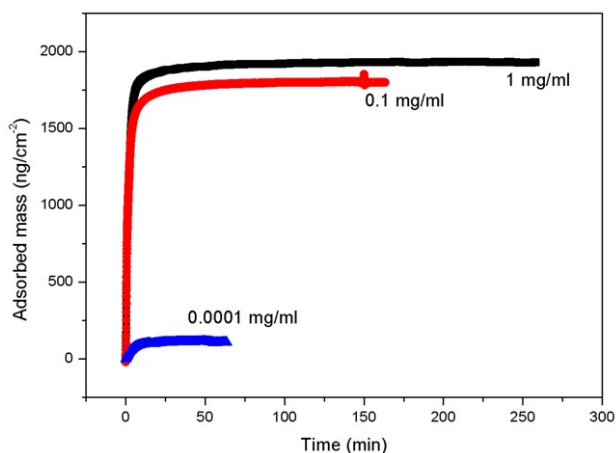

Figure 2. SbpA mass adsorption on silicon dioxide as a function of time for three concentration. The adsorption is constant after ca. 60 minutes. The difference in mass for the two largest concentrations is about 100 ng/cm^2

A quick analysis of the exponential growing shows that the adsorption for 1 mg/ml is the fastest one. Similar values for the adsorbed mass (and also frequency and dissipation) were obtained for SbpA adsorption studies on (poly(sodium 4-styrenesulfonate)) and self-assembled monolayers (Delcea et al., 2008; Eleta-Lopez et al., 2011).

The (surface) structure of the adsorbed protein on silicon dioxide QCM-D sensors was determined with atomic force microscopy. Figure 3 shows AFM deflection images of the three studied protein concentrations. On the left, it can be seen that the smallest protein concentration (0.0001 mg/ml) does no form a crystalline layer. The image of the middle corresponds to a protein concentration of 0.1 mg/ml. Although the image shows bumpy S-layer areas, the characteristic P4 pattern of the Slayer is to be recognised. On the right, the highest protein concentration (1 mg/ml) leads to a more homogeneous protein layer of clear P4 structure similar to the crystalline structure found in bacteria (Sleytr, Sára, Pum, & Schuster, 2001).
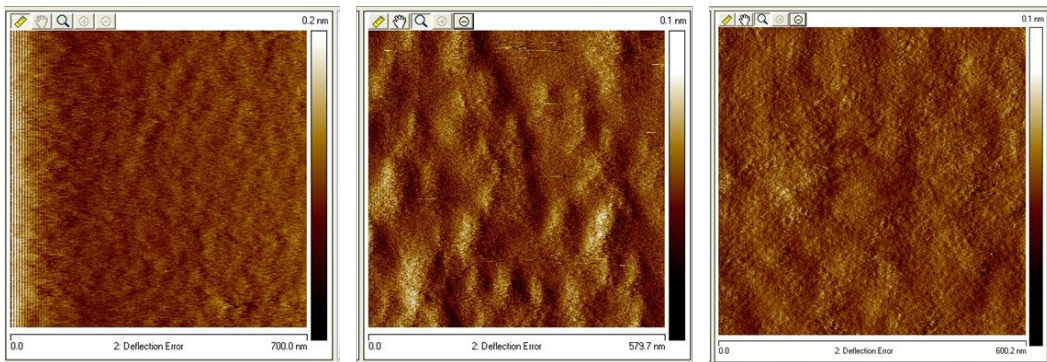
Figure 3. AFM deflection images measured in contact mode of adsorbed SbpA on silicon dioxide. All the measurements were performed in 100 mM NaCl aqueous solution. The result of the lowest concentration (0.0001 mg/ml), left image, is an indication that the adsorbed proteins on the QCM-D crystal do not form a crystalline protein layer. On the contrary, S-layer formation took place for higher concentrations (0.1 mg/ml and 1 mg/ml), middle and right images respectively

Recrystallization processes normally occur in two stages, nucleation and growth (McPherson, Kuznetsov, Malkin & Plomp, 2003). While the substrate-protein interaction is crucial to the formation of the nucleation points, the protein-protein interaction might be more important for the incorporation of new proteins and coalescence of 8 crystal domains leading to the final compacted protein layer. QCM-D frequency-dissipation curves is an easy and qualitative way to link the layer formation and the mechanical properties of the layer. Figure 4 illustrates the variation of the dissipation as function of the frequency change (adsorption of mass). Few proteins adsorbed for a concentration of 0.0001 mg/ml leading to a small increase in the dissipation. More interesting is the tendency for highest concentrations. The dissipation starts to rise at the same moment that proteins adsorb on the substrate in a very similar way until reaching a maximum value of about 3.5×10^{-6}. In this first part, protein random adsorption with high degrees of freedom of molecular movement and a hydrodynamic process (depending on the protein concentration) could be the reason for the observed effect. After the maximum of the curve, the dissipation decreases while more protein is being adsorbed on the substrate (the frequency becomes more negative). This can be interpret as the start of formation of a "rigid" structure (of less molecular motion freedom). Finally, at frequency values of about 90 and 100 Hz the dissipation reaches its lower value, which means that the S-layer is formed (as shown by AFM measurements).
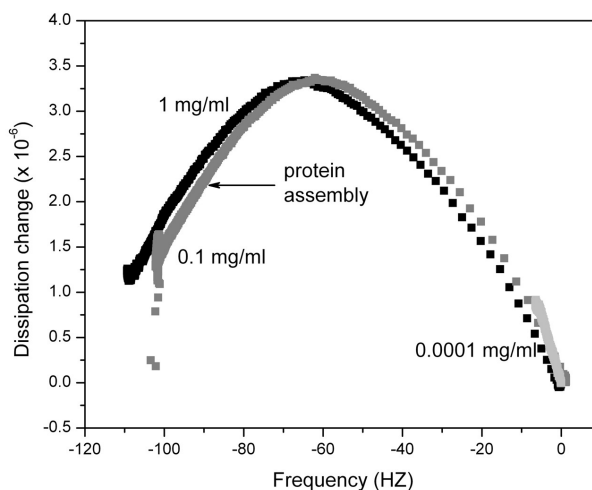
Figure 4. Dissipation change versus frequency shift for SbpA adsorption on silicon dioxide. The dissipation shows a maximum for a frequency value of about 60 Hz, showing the possible moment at which protein self-assembly takes place. Note that a dissipation value of zero would mean that the protein layer has similar mechanical properties as the QCM-D substrate

In order to get more insight about the mechanical properties of the adsorbed protein layer, AFM force distances curves were carried out (see Figure 5). Force-distance curves at a loading rate of 1000 nm/s showed that the protein layer is stable for the two highest proteins while soft and elastic protein domains were detected for protein concentration of 0.0001 mg/ml. On one hand, the left image of figure 5 confirms that the AFM tip senses a hard wall (in this case the S-layer) and that practically no energy is lost in the process (the approaching and the retracting curves overlap each other, blue and red respectively). On the other hand, on the right image, the red curve (when the AFM tip leaves the surface) shows a saw-tooth pattern typical for polymer stretching (as well as adhesion energy) with forces of about 100 pN and extension lengths of tenths of nm. This curve also shows a larger curvature before the contact zone between the AFM tip and the substrate indicating a higher repulsive force (probably of electrostatic and entropic nature) and a different "protein layer" of softer texture.
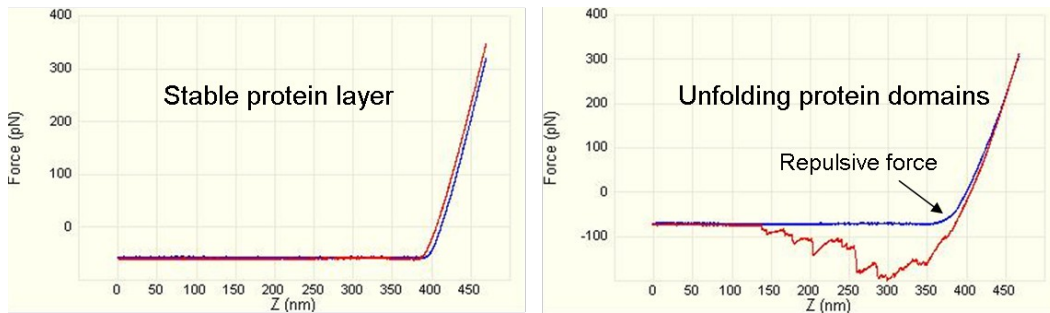
Figure 5. On the left, a force-distance curve showing the robustness of a protein crystal (S-layer). The approaching curve (blue) and the retracting curve (red) practically overlap each other showing no hysteresis or elastic domains. On the right, it can be seen that AFM tip could not easily be removed from the surface. I this case the is pulling proteins out of the substrate after applying a load of 300 pN. Note that the adhesion/unfolding forces are about 100 pN

4. Conclusions

S-protein adsorption on silicon dioxide and at the air-water interface has been studied with atomic force microscopy and quartz crystal microbalance and tensiometry. A first analysis of the results indicate that protein adsorption is primary governed by diffusion (until saturation is reached at the highest concentration). Low SbpA protein concentrations (0.0001 mg/ml) do not form a crystalline protein layer but a soft one of elastic nature as AFM image and force-curves show. On the contrary, protein concentrations of 0.1 mg/ml and 1 mg/ml form a "rigid" crystalline layer corresponding to a surface mass density of about 1800 ng/cm^2 and 1930 ng/cm^2 respectively. Finally, it has been confirmed that SbpA adsorbs at the air-water interface reducing in about 20 mN/m the surface tension of water for the highest protein concentration. New experiments would be needed to refine the protein adsorption on silicon surfaces and to map a molecular model of protein adsorption at such interfaces.

Acknowledgments

References

Alberts, B., Bray, D., Johnson, A., Lewis J., Raff, M., Roberts, K., et al. (1998). *Essential Cell Biology: an introduction to the Molecular Biology to the Cell.* Garland Publishing Inc New York & London. 133-181.

Delcea, M., Krastev, R., Gutberlet, T., Pum, D., Sleytr, U.B., & Toca-Herrera, J.L. (2008). Thermal stability, mechanical properties and water content of bacterial protein layers recrystallized on polyelectrolyte multilayers. *Soft Matter, 4,* 1414-1421. http://dx.doi.org/10.1039/b719408k

Delcea, M., Krastev, R., Gutberlet, T., Pum, D., Sleytr, U.B., Toca-Herrera, J.L., et al. (2007). Mapping bacterial surface layers affinity to polyelectrolytes through the building of hybrid macromolecular structures. *Nanotechnol., 7,* 4260-4266.

Eleta-Lopez, A., Pum, D., Sleytr, U.B., & Toca-Herrera, J.L. (2011). Influence of surface chemistry and protein concentration on adsorption rate and S-layer crystal formation. *Phys. Chem. Chem. Phys., 13,* 11905-11913. http://dx.doi.org/10.1039/c1cp00052g

Gläsmäster, K., Larsson, Ch., Höök, F., & Kasemo, B. (2002). Protein adsorption on supported phospholipid bilayers. *J. Colloid Interface Sci., 246,* 40-47. http://dx.doi.org/10.1006/jcis.2001.8060

Gray, J.J. (2004).The interaction of proteins with solid interfaces. *Curr. Opin. Struct. Biol., 14,* 100-115. http://dx.doi.org/10.1016/j.sbi.2003.12.001

Györvary, E.S., Stein, O., Pum, D., & Sleytr, U.B. (2003). Self-assembly and recrystallization of bacterial S-layer proteins at silicon supports imaged in real time by atomic force microscopy. *J. Microsc., 212,* 300-306. http://dx.doi.org/10.1111/j.1365-2818.2003.01270.x

Kainz, B., Steiner, K., Sleytr, U.B., Pum, D., & Toca-Herrera, J.L. (2010). Fluorescent S-layer protein colloids. *Soft Matter, 6,* 3809-3814. http://dx.doi.org/10.1039/c0sm00008f

Martín-Molina, A., Moreno-Flores, S., Perez, E., Pum, D., Sleytr, U.B., & Toca-Herrera, J.L., (2006). Structure, surface interactions and compressibility of bacterial S-layers through surface forces techniques. *Biophys. J., 90,* 1821-1829. http://dx.doi.org/10.1529/biophysj.105.067041

McPherson, A. (2003). Macromolecular crystallization in the structural genomics era. *J. Struct Biol., 142*, 1-2. http://dx.doi.org/10.1016/S1047-8477(03)00033-9

McPherson, A., Kuznetsov, Y.G., Malkin, A., & Plomp, M. (2003). Macromolecular crystal growth as revealed by atomic force microscopy. *J. Struct. Biol., 142*, 32-46.
http://dx.doi.org/10.1016/S1047-8477(03)00036-4

Moreno-Flores, S., Kasry, A., Butt, H.J., Vavilala, C., Schmittel, M., Pum, D., et al. (2008). From native to non-native two-dimensional protein lattices via underlying hydrophilic/hydrophobic nanoprotrusions. *Angew. Chem. Int. Ed., 47,* 4707-1717. http://dx.doi.org/10.1002/anie.200800151

Sleytr, U.B., Huber, C., Ilk, N., Pum, D., Schuster, B., & Egelseer, E.M. (2007). S-layers as a tool kit for nanobiotechnological applications. *FEMS Microbiol. Let., 267,* 131-144.
http://dx.doi.org/10.1111/j.1574-6968.2006.00573.x

Sleytr, U.B., Messner, P., Pum, D., & Sára, M. (1999). Crystalline Bacterial Cell Surface Layers (S Layers): From Supramolecular Cell Structure to Biomimetics and Nanotechnology. *Angew. Chem. Int. Ed., 38,* 1034-1054. http://dx.doi.org/10.1002/(SICI)1521-3773(19990419)38:8<1034::AID-ANIE1034>3.0.CO;2-#

Sleytr, U.B., Sara, M., Küpcü, S., & Messner, P. (1986). Structural and chemical characterization of S-layers of selected strains of Bacillus stearothermophilus and Desulfotomaculum nigrificans. *Arch. Microbiol., 146,* 19-24. http://dx.doi.org/10.1007/BF00690152

Sleytr, U.B., Sára, M., Pum, D., & Schuster, B. (2001). Characterization and use of crystalline bacterial cell surface layers. *Prog. Surf. Sci., 68,* 231-278. http://dx.doi.org/10.1016/S0079-6816(01)00008-9

Toca-Herrera, J.L., Moreno-Flores, S., Friedmann, J., Pum, D., & Sleytr, U.B. (2004). Chemical and Thermal Denaturation of Crystalline Bacterial S-Layer Proteins. *Microsc. Res. Techniq., 65,* 226-234.

Yampolskaya, G., & Platikanov, D. (2006). Proteins at Fluid Interfaces: Adsorption Layers and Thin Liquid Films. *Adv. Colloid Interf. Sci, 128-130,* 159-183. http://dx.doi.org/10.1016/j.cis.2006.11.018