Investigation of In-Vitro Bacterial Surface Layer Formation by FBARs

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***Abstract*—We report investigations on the adsorption and *in-vitro* recrystallization kinetics of the bacterial surface layer protein of *Bacillus sphaericus* NCTC 9602 on gold surfaces by means of film bulk acoustic resonators. The acoustic resonators were operated in shear mode at about 800 MHz. From the measured changes of frequency and in dissipation, the mass and the viscoelasticity of biomolecular films formed at the top electrode of the device could be derived, respectively. The measured data revealed that protein adsorption is a fast process while the time constant for the recrystallization of the monomers into ordered two-dimensional protein crystals is typically on the order of 1h.**

I. Introduction

Bacterial surface layers (S layers) are regular two-dimensional (2D) protein crystals which form the outermost cell envelope component of many bacteria and archaea [1-3]. They exhibit different kinds of lattice symmetry (*p*2, *p*3, *p*4, and *p*6) with lattice spacings in the range of 3-30 nm. S layers are typically 5-15 nm thick and possess pores of individual but identical size and morphology with diameters in the range of 2-6 nm. They are composed of single protein or glycoprotein units with a molecular weight of 40-200 kDa. The possibility to reconstitute isolated S-layer monomers *in vitro* into 2D arrays with perfect uniformity at solid surfaces or liquid-air interfaces [4,5] makes them an almost ideal biological template for supra-molecular engineering. Taking advantage of their spatially well-defined physical and chemical surface properties, S layers have extensively been used for the template-directed chemical synthesis of regular 2D semiconducting [6,7] and metallic [8-13] nanoparticle arrays. Among others, these cluster arrays are well suited for catalytic sensor applications [14]. S layers are remarkably stable protein structures; e.g., when S layers are dried, their regular structure is preserved [13] which has recently allowed using S layers as templates for the organization of gas-phase deposited FePt nanoparticles into 2D arrays [15-17]. Moreover, the electronic structure of S layers has been investigated by photoemission und NEXAFS spectroscopy [18-22].

Here, we investigate the self-organization of the S layer of *Bacillus sphaericus* NTCT 9602 on a technical gold surface. The S layer exhibits a *p*4 symmetry with a lattice constant of 12.5 nm, and a complex pattern of pores and gaps that are 2-3 nm wide. Each unit cell of the S layer lattice is composed of 4 identical protein monomers. Each monomer consists of 1050 amino acids and has a molecular weight of 111.5 kDa. The *in-vitro* recrystallization kinetics is detected by means of a film bulk acoustic resonator (FBAR) operated in shear mode. In distinction to previous experiments [23], this allows observing the system *in-situ* without disturbing the structure formation process.

II. Materials and Methods

A. S-layer preparation

The used S layer was isolated from the bacterium *B. sphaericus* NCTC 9602. The conditions for cell cultivation and purification of the S-layer sheets are described elsewhere [11,24]. In order to disintegrate the protein sheets into monomers, 500 µl of the S-layer suspension were washed twice with distilled water and centrifuged for 45 min at 25000 g and 4°C. Then, the protein pellet was treated with 300 µl 6 M guanidinium hydrochloride for 2 h at room temperature. Thereafter, the disintegrated protein suspension was twice dialyzed against TRIS/HCl buffer, pH 9.0, for 45 min. This solution could be stored at 4°C up to 7 days. To initialize recrystallization at substrate surfaces, $MgCl_2$ was added with a final concentration of 10 mM.

B. Structural characterization of the recrystallized S layer

To study the structure of the *in-vitro* recrystallized S layers, first, monomer solutions with protein concentrations ranging from 0.05 to 10 mg/ml were recrystallized on Si substrates and carbon-coated transmission electron microscopy (TEM) grids. Atomic force microscopy was performed using a NanoScope IIIa (Digital Instruments) operated in tapping mode on air. For the TEM investigations,

the samples were stained with 2% uranylacetate and imaged using a Zeiss TEM EM 912 Omega operated at 120 keV.

C. *Film bulk acoustic resonator*

The FBAR system used in this experiment (Figure 1) was described elsewhere [5]. The assembly of the protein monomers into S layers was directly accomplished at the 100 nm thick gold top electrode. The resonator was operated in shear mode at a resonance frequency of around 800 MHz. The shear-mode operation is especially suited for biological applications in liquid environment [25]. The resonators were arranged in arrays of 200 µm wide, squared electrodes which had centre-to-centre spacings of 300 µm. The small pixel size and the direct dependence of the resonance frequency on the adsorbed mass [26] made the used FBARs highly suitable for the investigation of protein adsorption from small amounts of buffer liquids. The protein adsorption at the top electrode led to a decrease of the resonance frequency; changes of the dissipation gave information about the adsorbents' viscoelastic properties. The minimum detectable mass of the FBAR devices used was in the range of ng/cm^2.

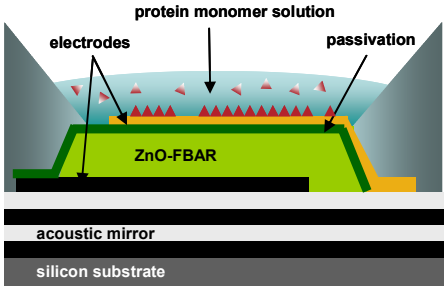

Figure 1. Schematic viewgraph of the FBAR sensor. The sensor is part of the bottom plate of a liquid reservoir to which the S-layer monomer solution is exposed. The monomers adsorb to the gold top electrode of the sensor where they - depending on the concentration of the monomer solution - assemble into single or double protein layers.

III. Results and Discussion

A. *Structure of the recrystallized S layers*

In-vitro assembly of protein layers at the substrate surface was started from monomer solutions with protein concentrations between 0.1 and 10 mg/ml by adding 10 µl of 100 mM $MgCl_2$ to a total volume of 100 µl monomer solution. Figure 2 shows an AFM image of a protein layer recrystallized from a 0.1 mg/ml monomer solution at a Si substrate. The assembled protein layer possessed regular four-fold lattice symmetry with a lattice periodicity of 12.5 nm which is in agreement with previous measurements [10]. Exactly the same behavior was found in TEM investigations on S layers which have been directly recrystallized at carbon films on copper TEM grids (Figure 3B). At low protein concentration, a single domain was formed covering the whole imaged surface area. This was different for high protein concentrations where the assembled monolayer consisted of multiple small domains (Figure 3A). Thus, the recrystallized protein monolayers were found to form single-crystalline domains which sizes depended on the protein concentrations of the monomer solutions. The higher the concentration in solution the smaller was the domain size. This behavior can be explained by the nucleation and growth of the 2D protein crystals at the surface. At high concentrations, many nucleation sites were formed. Consequently, the distance between adjacent nucleation sites, and thus, the overall size of the single-crystalline domains was small. At low concentrations, only a few nucleation sites formed from which the domains could grow large.

At concentrations ≥1 mg/ml double layers were formed (data not shown).

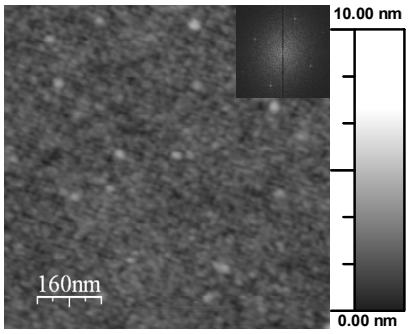

Figure 2. AFM image of a regular protein layer recrystallized from a 0.1 mg/ml monomer solution of *B. sphaericus* NCTC 9602 at a Si substrate. The inset shows the power spectrum. The latter gives clear evidence that the layer exhibits a *p*4 lattice symmetry with a lattice constant of 12.5 nm.

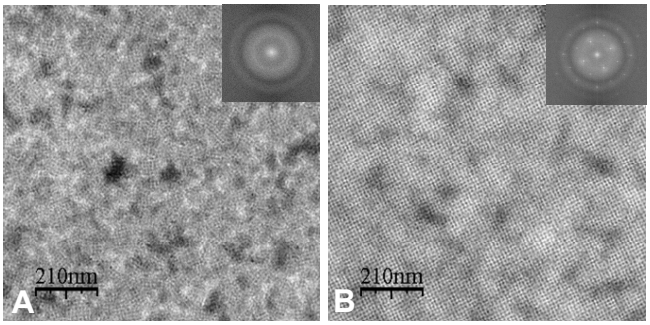

Figure 3. TEM images of S layers of *B. sphaericus* NCTC 9602, recrystallized at carbon films on copper grids and negatively stained with 2% uranylacetate. Recrystallization from 1 mg/ml (A) and 0.1 mg/ml (B) monomer solutions. In the insets the corresponding power spectra are depicted.

B. *Kinetic experiments by means of FBARs*

The recrystallization experiments described above were directly carried out at the FBAR. The experimental procedure is described elsewhere [5]. Figure 4A shows the initial negative shift of the resonance frequency plotted versus time for four different monomer concentrations in the range of 0.1-10 mg/ml. The negative frequency shift is directly proportional to the mass of the protein adsorbing at the sensor surface. The increase of adsorbed mass is proportional to the monomer concentration. A detailed analysis of the data revealed that the protein adsorption kinetics can be described by a sum of at least two exponential functions

$$\Delta f = A \cdot \left(-1 + e^{-B \cdot t}\right) + C \cdot \left(-1 + e^{-D \cdot t}\right), \qquad (1)$$

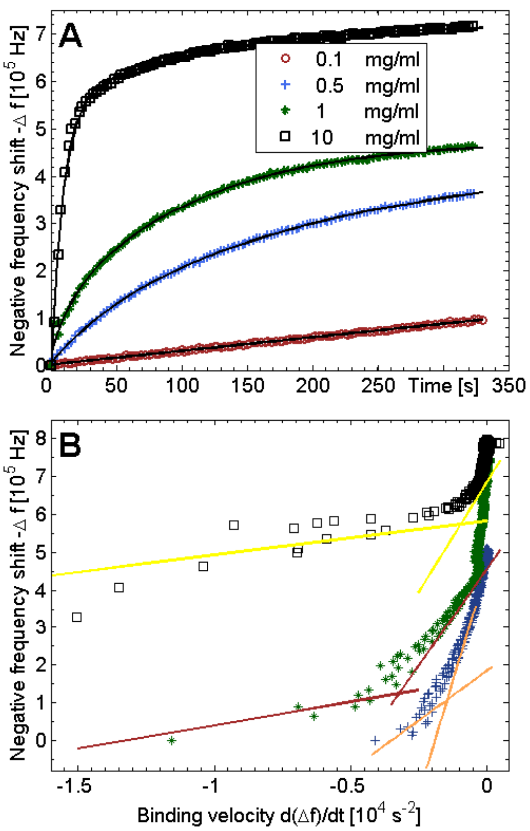

Figure 4. A: Initial negative frequency shift during the adsorption of S-layer monomers of *B. sphaericus* NCTC 9602 at the gold surface of the FBAR. The data measured for four different monomer concentrations ranging from 0.1-10 mg/ml are depicted by symbols; the solid lines represent fit curves obtained by fitting the data with the function given in Equation 1. B: Negative frequency shift versus its time derivative for the monomer concentrations 0.5, 1, and 10 mg/ml. Same color code as in (A). The straight lines represent exponential functions with 'slopes' of values B and D as derived from the fits.

where the sum of the constants A and C characterizes the mass adsorption at the surface in equilibrium for infinite time. B and D represent rate constants describing how fast the monomers adsorb. Evidence for this mathematical description is given by Figure 4B where the negative frequency shift data are plotted versus their time derivative. In addition to the data, lines are plotted representing the slopes B and D as well as the offsets A and C as derived from the fit of the data depicted in Figure 4A. It is clearly shown that the data can be represented by at least two exponential functions, proving the assumption that the initial reaction kinetics is dominated by at least two different processes of rates B and D. We suppose, the first and faster process described by Equation 1 is associated to monomer adsorption at the bare resonator surface, whereas the second process corresponds to monomer deposition to S-layer patches which have already formed at the surface. Both processes can be assigned as stochastic deposition processes which can be described by a Poisson model. The latter comprehends the monomer deposition as a stochastic process in which the deposition events at a certain substrate position occur continuously and independently of one another [27].

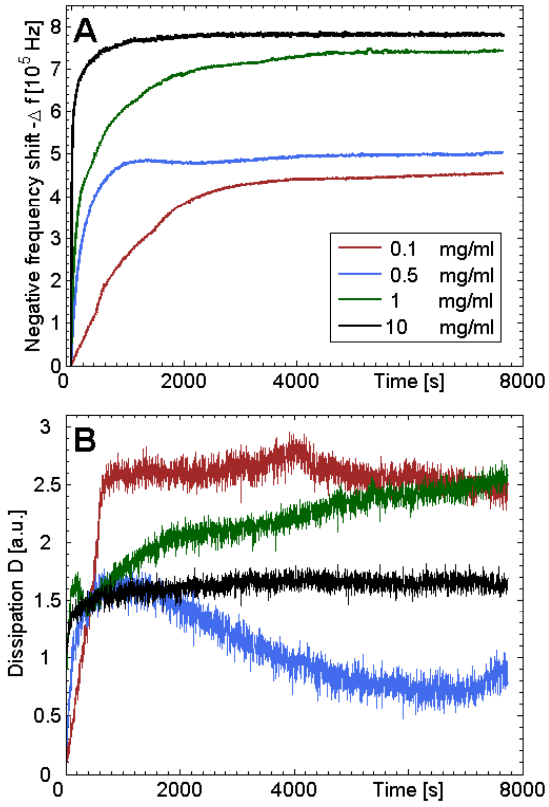

Figure 5. Negative frequency shift (A) and dissipation (B) for protein monomer concentrations of 0.1-10 mg/ml, measured over a time period of ~2.2 hours. Same color code as in Figure 4.

For times < 300 s, the development of the dissipation can be described with the same exponential functions (fit data not shown).

Figure 5 shows the long-term adsorption and dissipation behavior. The latter gives information about the viscoelastic properties of the deposited S-layer films. While the frequency shifts (Figure 5A) tend to develop into two different plateaus - a lower level at around 400 kHz for monomer concentrations ≤ 0.5 mg/ml and a higher level at 800 kHz for concentrations ≥ 1 mg/ml, the long-term dissipation values 'oscillate' with respect to protein concentration (Figure 5B). In combination with the structural investigations discussed above, the long-term behavior of the frequency shift can be interpreted as the formation of one monolayer for concentrations ≤ 0.5 mg/ml, and of two layers for higher concentrations. The dissipation behavior is more complex. As discussed above, the initial dissipation behavior is similar to the one of the initial frequency shift - the absolute values increase with increasing monomer concentration. However, for longer times the dissipation increases further or decreases depending on the monomer concentration. We suppose that this behavior can be explained by the recrystallization of the protein layers. Expecting a recrystallized S-layer to dissipate less energy than a less ordered 'amorphous' protein film, e.g., the time dependence of the 0.5-mg/ml sample can be interpreted as follows: During monomer adsorption, the dissipation increases with time. At ~1500 s reorganization of monomers into

ordered layers starts, resulting into a decrease of dissipation with time. On this basis, also the 'oscillation' of the long-term can be explained. At the lowest concentrations, the assembled films might be incomplete. Therefore, they show a high dissipation. With increasing concentration, complete films with low dissipation are formed. When the concentration is further increased, a second layer is formed. Therefore, the same, and thus, 'oscillating' behavior is observed. From here we can conclude that the characteristic time scale for the *in-vitro* recrystallization of S-layer monomers on gold surfaces is on the order of 0.5-1 h. To get more detailed information about the ongoing structure formation processes, further investigations are necessary.

IV. Conclusion

The *in-vitro* structure formation of the S layer of *B. sphaericus* NCTC 9602 was investigated by means of atomic force microscopy, electron microscopy and film bulk acoustic resonators. The high sensitivity of the FBAR sensors with a minimum detectable mass in the range of ng/cm^2 allowed studying the kinetics of S-layer protein adsorption and recrystallization at the sensor surface. From the changes in frequency shift and dissipation over time conclusions concerning the complex structure formation processes going on in thin protein layer could be derived. These investigations have generic character; they can generally be applied to structure formation processes in thin biomolecular films.

Acknowledgment

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