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13. ABSTRACT (Maximum 200 words)  The research focus has been on the challenging issues involved in achieving two-dimensional recrystallization of protein (S-layer) monomers in large (~several hundred micron) single crystal areas. To acquire facility with the recrystallization process, we first explored the experimental conditions which influence protein crystallization - e.g., protein concentration, temperature, pH, ionic strength, concentration of various mono- and divalent cations, etc. We attempted crystallization experiments in aqueous suspension or at the air/water interface before trying to reassemble protein monomers directly on a patterned surface. We were able to obtain large (~1 $\mu$ x 2 $\mu$ ), well-ordered, recrystallized protein patches following dialysis. As with the native protein crystal, the recrystallized S-layers showed a square lattice with lattice parameter of 13 nm. We established a reproducible protocol for preparing a solution of S-layer monomers for a recrystallization experiment. We used both thin film carbon substrates on transmission electron microscope (TEM) grids as well as bulk silicon substrates for a variety of diagnostic recrystallization experiments. Protocols for these experiments were established and numerous experiments carried out, including: grid modification: float, touch, and time; glutaraldehyde crosslinking of S-layer crystal; and floatation of the formvar directly on the recrystallization solution. We attempted to extrapolate from our work with TEM grids to recrystallization experiments directly on Si wafers. Approaches included: attempts to make Si surface more hydrophilic: OTMS; lipid droplets; piranha and O <sub>2</sub> plasma cleaning; and lines of oxidation drawn with the AFM.			19991220 041	
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## Brief Outline of Research Findings

The long range goal of this AASERT research was to support our effort to use low resolution templating by means of patterned deep UV lithography of organosilane self-assembled monolayer (SAM) films to create alternating regions of intact film and hydrophilic, surface-silanol moieties. The exposed (hydrophilic) regions would then be used to nucleate the *in situ* growth of two-dimensional protein crystals and these crystals would then be used as masks for nanometer-scale pattern transfer to bulk substrates.

The research that has been performed has focussed on the challenging issues involved in achieving two-dimensional recrystallization of protein (S-layer) monomers in large (~several hundred micron) single crystal areas. In order to acquire facility with the recrystallization process, it was desirable to first explore the experimental conditions which influence protein crystallization - e.g., protein concentration, temperature, pH, ionic strength, concentration of various mono- and divalent cations, etc. We chose to do this by attempting crystallization experiments in aqueous suspension or at the air/water interface before trying to reassemble protein monomers directly on a patterned surface.

In taking this approach we grew a bacteria, the Gram-positive bacteria *Bacillus sphaericus*, possessing a protein crystal S-layer which has demonstrated remarkable recrystallization properties. We have been successful at growing the bacteria and isolating the native S-layer protein crystal. Following this, experiments were performed to attempt solubilization and recrystallization in suspension. Native *Bacillus* protein crystal aliquots were solubilized in 5 M guanidine hydrochloride in Tris hydrochloride buffer at pH 7.4 and such preps were subsequently dialyzed against 10 mM calcium chloride. TEM revealed complete solubilization of the native protein crystals with no intact monolayers or oligomeric subunit aggregates present. We were able to obtain large (~ 1 $\mu$  x 2 $\mu$ ), well-ordered, recrystallized protein patches following dialysis. As is the case for the native protein crystal, the recrystallized S-layers showed a square lattice with lattice parameter of 13 nm.

We next established a reproducible protocol for preparing a solution of S-layer monomers for a recrystallization experiment:

First, the *Bacillus sphaericus* must be grown and harvested and the S-layer protein isolated. After the S-layer protein has been isolated the protein concentration of the preparation is determined using the Pierce BCA (Bicinchoninic acid) protein assay kit. A solution of buffered guanidine hydrochloride solution (G-HCL) and S-layer protein is mixed to a final protein concentration between 1-1.5 mg/ml, 5M concentration of G-HCL, and 10mM Tris buffer pH 7.4. If the S-layer prep is found to be too dilute the tris buffer is dispensed with. For example: To make a 2ml buffered G-HCL solution containing protein we add 0.554ml of 5.51 mg/ml S-layer protein, 1.25ml of 8M G-HCL and 0.206 ml 10mM Tris.

The S-layer prep is left to solubilize in the G-HCL solution overnight at room temperature. It is then centrifuged and the supernatant is dialysed at room temperature against 3 liters of dH<sub>2</sub>O at a pH 5.0-5.6 or 10mM Tris at a pH 7.4 for 2 hours changing the solution after 1 hour. The dialysis product is centrifuged and the supernatant is used immediately for recrystallization experiments.

We found it advantageous to use both thin film carbon substrates on transmission electron microscope (TEM) grids as well as bulk silicon substrates (cut from a Si wafer with a dicing saw) for a variety of diagnostic recrystallization experiments. TEM grid experiments are stained with 50  $\mu$ l of 1% uranyl acetate (UAc) and assayed on a 100 keV TEM (JEOL 100CX). Bulk Si experiments are overcoated with 1.2 nm of titanium which oxidizes to 3.6 nm of TiO<sub>2</sub> and assayed using atomic force microscopy (AFM) using a Digital Instruments Nanoscope III. In both cases, the distinguishing signature of the recrystallized S-layers is a square lattice symmetry with a 12.8 nm lattice constant.

TEM experiments were performed on 10ul microbridge droplets whereas Si wafer experiments are performed in well plates containing between 0.8 and 1 ml of solution. The only reason for this variation is the difference in the amount of solution needed in order for the substrate (TEM grid or bulk silicon) to float on the air-water interface. (The microbridges and well plates are purchased from Hampton Research. The microbridges are small devices in the shape of a bridge to carry out sitting drop crystallization. They have a round indentation in the top surface of the bridge which holds the sample droplet during a crystallization experiment and prevents the droplet from spreading over a large area.) The actual recrystallization solution contains 50% of the dialysis supernatant and 50% of a solution made up of 200 mM CaCl<sub>2</sub> and 20mM sodium borate at pH 8.0. A number of approaches have been employed to transfer the 2-D crystal from the air-water interface:

1. ("Touch") The solution can be left undisturbed overnight allowing recrystallization to occur at the air-water interface in an area free from vibration. After this interval the substrate can be carefully touched to the liquid surface and withdrawn.
2. ("Float") The substrate can be placed on the surface immediately and left to float overnight on the air-water interface.
3. The solution can be left undisturbed for a specific time interval after which the substrate can be placed on the surface and left to float for an additional length of time.

Establishment of the basic protocol outlined above is central to the recrystallization experiments which then take a number of different approaches in regard to manipulation of substrate hydrophilicity/hydrophobicity in order to influence crystal sidedness on the substrate, site specific crystallization, etc.

While our ultimate goal is the recrystallization of S-layer on a Si wafer, substrates consisting of transmission electron microscope (TEM) grids coated with formvar and then modified in various ways are a valuable tool in exploring the recrystallization properties of *Bacillus sphaericus* S-layers. Several approaches were taken.

#### •Grid Modification

A variety of experiments were conducted on TEM grids to determine the ideal surface for recrystallization. Grids with just formvar and grids with formvar and a thin coating of carbon, were touched and floated overnight with or without first being treated by glow discharge. Crystal size averaged between 100-500 nm in diameter. Further experiments with grids used carbon-coated, glow-discharged, formvar covered grids unless otherwise stated.

#### •Float, Touch, and Time

An experiment was conducted in which the recrystallization solution was incubated at room temperature for a variety of times (0, 1, 3, 5 hrs and overnight) before adding a glow-discharged, carbon-coated formvar covered TEM grid. Some grids were then left floating for several hours. The results showed that as time left to recrystallize increased, areal coverage also increased. The protein crystals were polycrystalline and appeared as if they could fit together into a larger structure like jigsaw puzzle pieces. We believe that the process of touching the grids to the air-water interface broke a larger crystal into smaller pieces. When grids were then left to float on the surface after the initial incubation the pieces joined together in areas but remained polycrystalline. It was determined from this experiment that the time allotted for recrystallization should be at least 10 hours whether float, touch, or some combination of the methods was used.

#### •Gluteraldehyde Crosslinking of S-layer Crystal

Because the pieces of the S-layer crystals appeared as if they had broken apart from a previously larger structure due to the addition of a TEM grid to the air-water interface, a 2% gluteraldehyde solution in  $\text{dH}_2\text{O}$  was added in an attempt to crosslink and strengthen the original structure after the overnight crystallization but before the grids were added. This did not prove successful; the gluteraldehyde formed a sticky glue-like substance on the surface of the grids which was difficult to rinse away.

#### •Floating the Formvar Directly on the Recrystallization Solution

Another method employed to transfer the S-layer from the air-water interface to a substrate without dislocating it, involved floating a thin layer of formvar directly onto the recrystallization solution for 10 hours. Both grids and portions of Si wafers were then added onto the top side of the formvar before the entire complex was lifted off by rolling across it a larger piece of parafilm. In this case, the grids and wafers received no prior modification. Results were good on grids, showing large polycrystalline areas, however, no protein was found on the Si. This method was abandoned due to its requirement for copious amounts of isolated S-layer.

We attempted to extrapolate from our work with TEM grids to recrystallization experiments directly on Si wafers. Several approaches were taken.

#### •Attempts to Make Si Surface More Identical to TEM Grids

Experiments using glow-discharged, carbon-coated, formvar covered TEM grids suggested that identical treatment of Si wafers may prove beneficial to our attempts at recrystallization on Si. For reasons that are unclear to us, we were only able to obtain what appeared to be bilayer (rather than monolayer) patches and these were only ~100nm in diameter.

#### •OTMS

There is some discussion in the literature that "as a precondition for many S-layer assembly systems, the surface of the supporting layer has to be rendered hydrophobic." This led to an

experiment in which Si wafers were treated with a hydrophobic organosilane, specifically, OTMS, which may facilitate recrystallization of S-layer onto the treated wafer. Si wafers were rendered hydrophobic by treatment with OTMS after being cleaned with acetone. Small squares in the characteristic shape of recrystallized S-layer were evident yet none showed the characteristic square lattice symmetry. We believe that these were recrystallized S-layer patches which were upside-down, having the textured side facing the Si.

#### •Lipid Droplets

Drawing again from literature suggestions for recrystallization strategies, we attempted to employ the ability of lipids to alter the sidedness of a 2-D crystal at the solution-lipid interface in experiments. In these experiments, we tried to "flip" the S-layer crystal over before it was transferred to the Si wafer. A drop of dipalmitoylphosphatidylcholine (DPPC; Sigma, no. P5911) in hexane-ethanol solvent (1 mg DPPC in 1 ml of hexane-ethanol [9:1, vol/vol]) was spread on the air-water interface before or during or after adding the protein (dialysis product) to the recrystallization solution. Si wafers were touched and floated (16hrs). Analysis of the samples showed massive contamination by an unknown substance (most likely the lipid) with no S-layer lattice identified.

#### •Piranha and O<sub>2</sub> Plasma Cleaning

Having determined that hydrophilicity of the Si wafer was, in fact, an important factor in transfer of the 2-D S-layer crystal, we prepared both piranha cleaned, and piranha cleaned and O<sub>2</sub> plasma treated Si wafers which were touched and floated for the standard overnight recrystallization time. Both types of Si wafers had areas of recrystallized S-layer. The piranha cleaned wafers had only small patches of 50-300nm in diameter. The O<sub>2</sub> plasma cleaned were covered with polycrystalline S-layer made up of pieces between 100-500nm in diameter. Floated Si wafers had crystals with more rounded edges whereas on the touched wafers edges of the crystals were sharper and seemed as if they had broken apart as a result of the touching process.

#### •Lines of Oxidation Drawn with the AFM

We used the AFM to physically alter the surface of the Si wafer in only selected areas by applying a voltage across the Si wafer while the instrument was scanning. The goal was to facilitate selective recrystallization in only those areas which had been treated. Simple code written in C-like language allowed us to draw recognizable oxidation patterns in regions smaller than 10  $\mu\text{m}$ . We conducted recrystallization experiments on samples with a variety of patterns in parallel with control samples consisting of O<sub>2</sub> plasma cleaned wafers. As of this writing we have not observed different results from the selectively oxidized and the untreated areas of the patterned samples. Also, at this time we have not observed the distinctive s-layer square lattice anywhere on the lithographically treated sample, however distinctive rectangular shapes about 1-3  $\mu\text{m}$  in diameter are present suggesting that s-layer is probably present but upside-down. The O<sub>2</sub> plasma cleaned control sample showed some s-layer patches but not to the extent which was seen previously. The paucity of patches on the control samples suggests the experiments need to be repeated.