Attachment of Calcium Oxalate Monohydrate Crystals on Patterned Surfaces of Proteins and Lipid Bilayers

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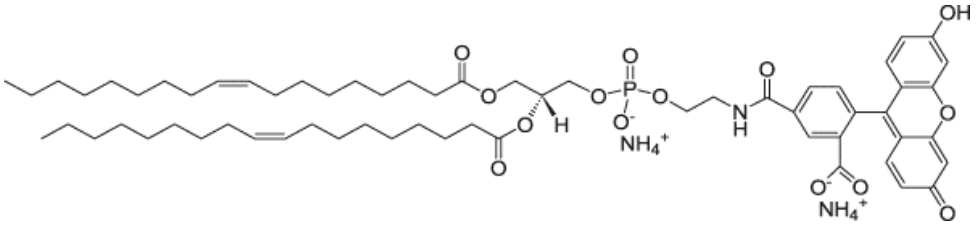
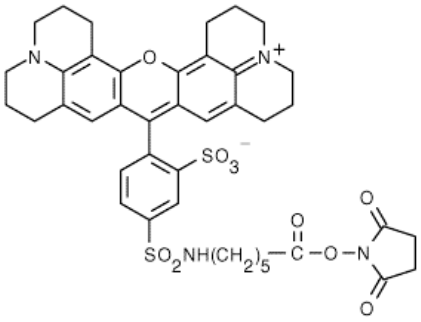
Materials. Bovine Osteopontin (OPN) was kindly provided by Dr. Sorenson at Aarhus University, Denmark. Texas red was purchased from Invitrogen. Sodium chloride, sodium oxalate, calcium chloride and human serum albumin (HSA) were purchased from Sigma. 1,2-Dipalmitoyl-sn-Glycero-3-Phospho-L-Serine (sodium salt) (DPPS) and 1,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine-N-Carboxyfluorescein (ammonium salt) (DOPE-CF) were purchased from Avanti Polar Lipids. Sylgard 184 silicone elastomer kit was obtained from Dow Corning. Deionized (DI) water with a resistivity greater than 18 MΩ was obtained from a Millipore system. All chemicals were used as obtained without further purification.

Fluorescence labeling and solution preparation of proteins. OPN and HSA were labeled with Texas red according to the manufacturer's recommendations. Texas Red fluoresces at roughly 615 nm, resulting in little spectral overlap with carboxyfluorescein, which fluoresces at roughly 517 nm. Briefly, the protein-Texas Red conjugate was synthesized by adding 100 μL of a solution of Texas Red-X succinimidyl ester in dimethylsulfoxide (10 mg/mL) to 1 mL of OPN or HSA in 0.2 M sodium bicarbonate buffer (10 mg/mL, pH 8.3), followed by incubation for 1 hr at room temperature. The reaction was then stopped by adding 100 μL of fresh prepared 1.5 M hydroxylamine (pH 8.5). Unconjugated dye was removed by dialysis for 12 hours using 2 kDa dialysis tubing (Spectra/Por, Spectrum Labotatories). The conjugated protein (TR-OPN or TR-HSA) was then dissolved in phosphate buffered saline solution to 250 μg/mL.



Carboxyfluorescein-labeled DPPE



Texas Red -X succinimidyl ester

Preparation of small unilamellar vesicles (SUVs). SUVs comprised of DPPS and 2% DOPE-CF were prepared by extrusion method described as the following. First, the mixed lipid was dissolved in a solvent (chloroform: methanol =1:1). The solvent was evaporated at 30°C by rotary evaporation yielding a thin lipid film on the sides of a round bottom flask. The lipid film is thoroughly dried to remove residual organic solvent by placing the flask on a vacuum pump overnight. Lipid solution (1 mg/mL in 10mM Tris-HCl buffer, pH 7.4) was obtained by hydration of the dry lipid film for 1 h at 60°C. The extrusion was performed on a mini-extruder through 100 nm filters (Avanti Polar Lipids). SUVs with 100 nm diameters were obtained.

Preparation of lipid/protein-patterned surface. Silicon wafers patterned with photoresist were fabricated using standard photolithographic techniques.[1] Briefly, a layer of photoresist (SU-8) was spin coated onto the silicon wafers. UV light with 365 nm wavelengths shine through a plastic printing mask with the desired features to transfer the features onto the photoresist (SU-8). Following the removal of the non-polymerized photoresist, resulting in surfaces that have 3 cm × 3 cm active areas containing desired futures with a depth of 5 μm. Polydimethylsiloxane (PDMS) stamps were prepared with these silicon surfaces as master molds. Briefly, PDMS was first thoroughly mixed with the curing agent (at a 10:1 ratio) and allowed to remove air bubbles under vacuum for 30 min at room temperature. Then the mixed elastomer was poured into the silicon master mold and cured in a vacuum oven for 1 h at 100°C.

Lipid/protein patterned surface were prepared by microcontact printing technique on silicon wafers or glass slides.[2,3] PDMS stamps were treated with air plasma for 30s in plasma cleaner and immediately incubated with 100 μL 250 μg/mL OPN or HSA solution for 30 min. The stamp was dried with nitrogen gas, removing excess protein solution from the stamp. The stamp was then placed in contact with a clean silicon surface under 20 g weight for 2 min. The protein patterned silicon wafer was then incubated with 100 μL 1 mg/mL lipid SUVs suspension to form supported membranes on the exposed regions, and finally the surfaces was rinsed extensively in water to remove excess vesicles and weakly bound protein.

Attachment of COM crystals on patterned surfaces. The growth solution was prepared by adding 2 mL 10 mM $CaCl_2$ stock solution (150 mM NaCl) into 16 mL 150 mM NaCl solution in a glass vial and stirred for 5 min, 2 mL 10 mM Na_2Ox solution (150 mM NaCl) was added drop wise. The silicon wafer patterned with OPN/DPPS and HSA/DPPS vesicles were placed into the vials filled with growth solution perpendicularly and kept in an oven for 24 hours at 60 °C. The silicon wafer was cooled down for 30 minutes at room temperature. Then, it was rinsed with water for 3 times, and dried with the nitrogen stream. Other substrate including silicon wafer, silicon wafer coated merely with OPN or DPPS were also used for comparison experiments with the same procedure as described above. Crystal's attachments on OPN/DPPS patterned substrates were carried out at several different time periods in the same method as described above.

Characterizations. A Leitz optical microscope with a CCD camera was used to image the samples. A Lieca DM IRE 2 fluorescence microscope with CCD camera was used to capture fluorescence images. Images were acquired with two filter sets (one set for protein labeled with Texas Red, with excitation/emission wavelength at 567/615 nm and one set for lipid labeled with carboxyfluorescein, with excitation/emission wavelength excitation/emission wavelength at 492/517 nm). The two data channels for the different filter sets were combined, following automatic false color assignment to aid visualization of the fluorescence color, using the Lieca FW 4000 software module.

Atomic Force Microscopy (AFM) images were obtained using Asylum MFP-3D-SA (Asylum Research) in a tapping-mode. Scanning election microscope (SEM) was used to characterize the samples. A thin film of gold (~2 nm) was sputtered onto the surface. A voltage of 5 kV was used during sample examination on a Hitachi 3500 SEM (Hitachi Ltd., Japan). Circular dichroism (CD) spectra were recorded by an Aviv 202SF CD spectrometer (Aviv Biomedical, Inc.) equipped with a thermostated five compartments cell holder over a range of 185-260 nm. All measurements in solution were recorded in a quartz cell with a 1 mm path length.

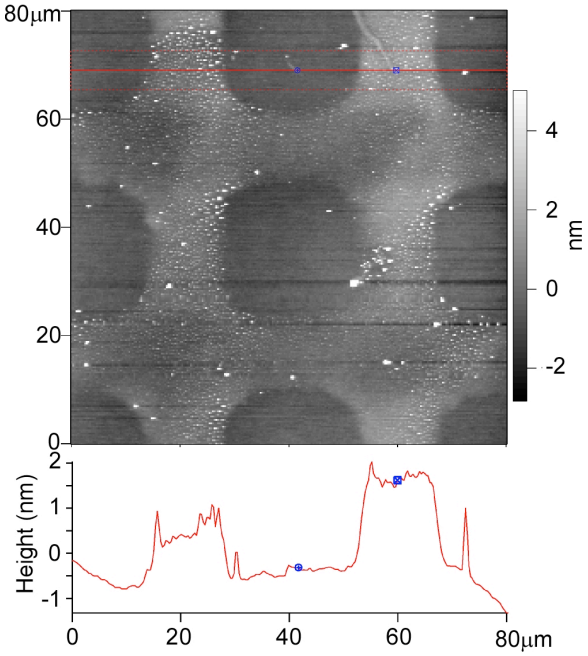


Figure S1. AFM tapping-mode image and analysis of OPN patterns on silicon surfaces revealed that 15 μm wide OPN grids have a thickness of ~2 nm.

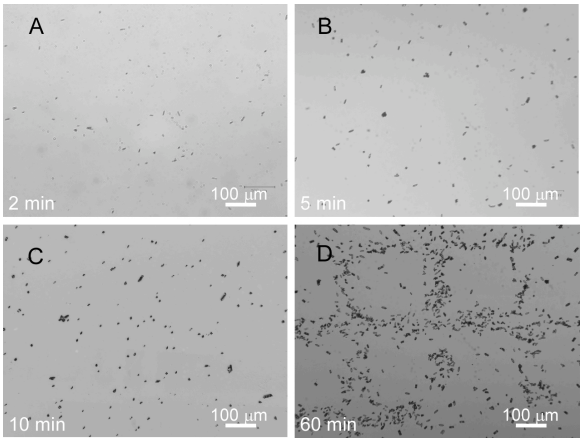


Figure S2. Optical microscopy images of the crystals attached to the DPPS/OPN patterned surface after being kept in the solution at 60°C for (A) 2min, (B) 5min (C) 10min and (D) 60min.

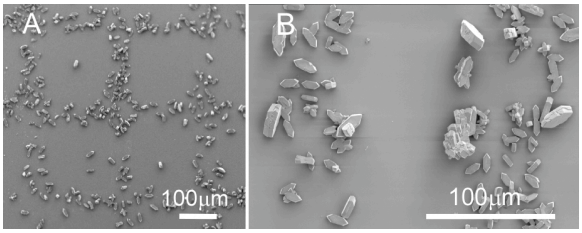


Figure S3. SEM images of COM crystals attached to OPN regions on DPPS-OPN complex surface after 24 hours at 60°C.

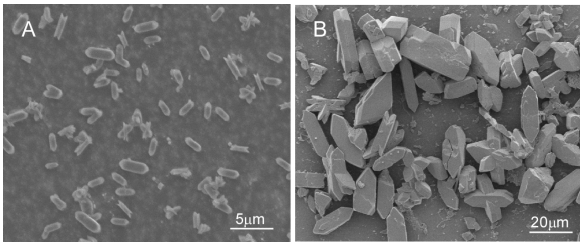


Figure S4. SEM images of crystals collected from (A) the fresh prepared crystal growth solution and (B) the same crystal growth solution being kept at 60 °C after 24 hours.

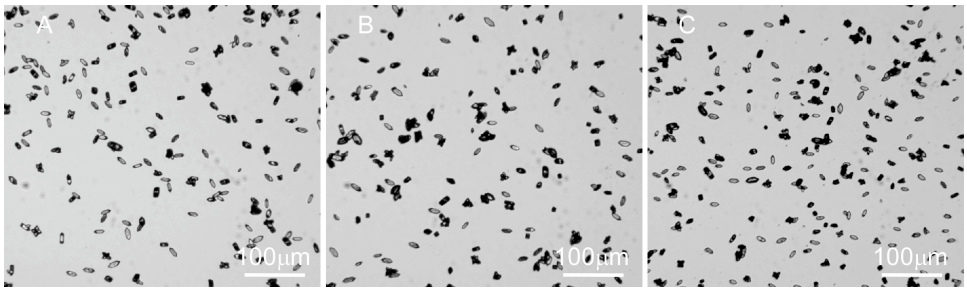


Figure S5. Optical microscopy images of the crystals attached to (A) bare silica wafer, (B) OPN coated surfaces and (C) DPPS membrane coated surface.

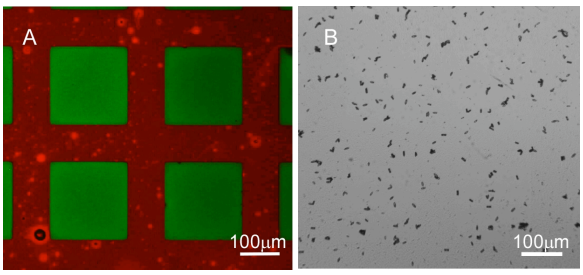


Figure S6. (A) Epifluorescence image of the hybrids surface of HSA and DPPS bilayers. HSA is labeled by Texas red dye (red color) and DPPS membrane regions are identified by their 2% DOPE-CF content (green color). (B) Optical microscopy image of the crystals grown on DPPS-HSA patterned surface.

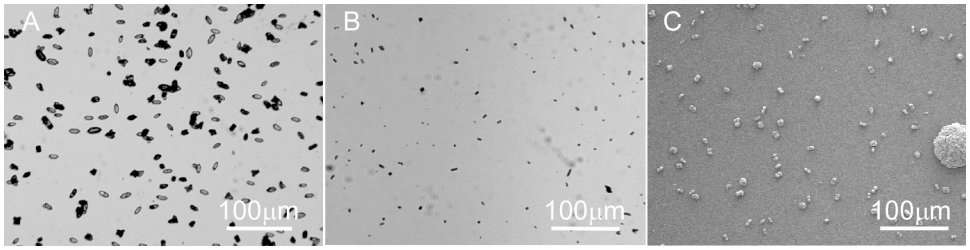


Figure S7. Optical microscopy and SEM images of COM crystals attached to a DPPS membrane-coated silicon surface immersed in the CaOx growth medium (A) after 24 hours without OPN, (B) after 24 hours in the presence of OPN in solution (5 μg/mL) , and (C) after 24 hours in a solution prepared by adding $CaCl_2$ (1 mM) to a solution of OPN (5 μg/mL) and Na_2Ox (1mM).

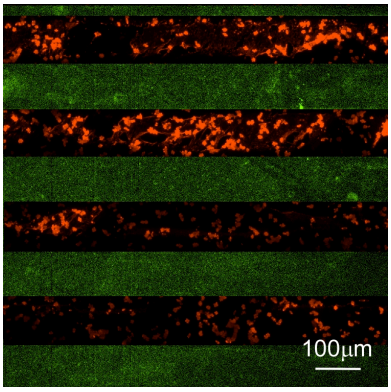


Figure S8. Epifluorescence images of the patterned surface of OPN and DPPS bilayers incubated for 24 hours in 1mM CaOx solution at 60 °C.

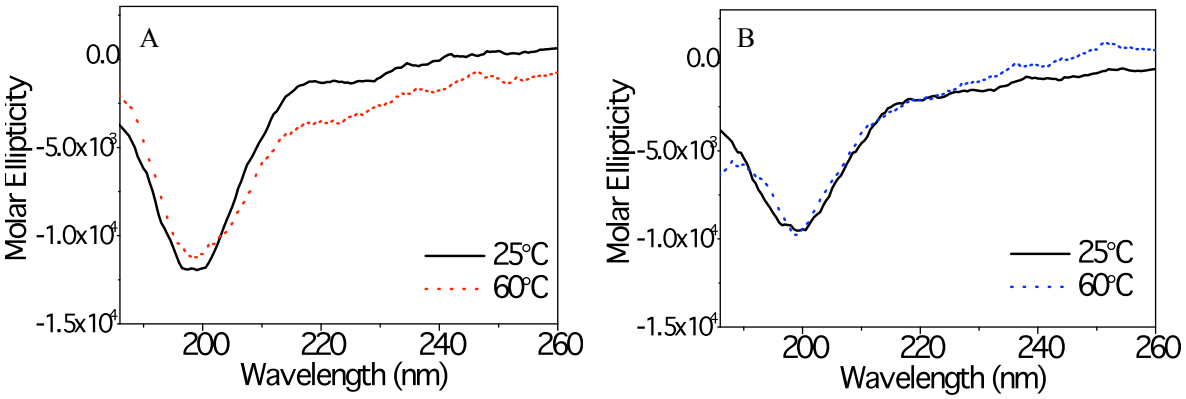


Figure S9. CD spectra of OPN at 50 μg/ml in (A) water and (B) 10mM Tris-HCl buffer solution, pH 7.4, at 25°C and 60°C. The CD spectra display a minimum at 198nm, which can be attributed to a random coil conformation. Increasing the temperature from 25 to 60°C had little effect on the OPN spectra.

References

[1] Wilbur, J. L.; Kim, E.; Xin, Y. N.; Whitesides G. M. *Adv. Mater.* **1995**, 7, 649.

[2] Groves, J. T.; Ulman, N.; Boxer, S. G. *Science* **1997**, 275, 651.

[3] McDonald, J. C.; Duffy, D. C.; Anderson J. R.; Chiu, D. T.; Wu, H. K.; Schueller, O. J. A.; Whitesides, G. M. *Electrophoresis* **2000**, 21, 27.