CRYSTAL NUCLEATION USING SURFACE ENERGY MODIFIED GLASS SUBSTRATES

Kyle A. Nordquist, Kevin M. Schaab, Jierui Sha, and Andrew H. Bond*

DeNovX, 3440 South Dearborn Street, Lab 204 S, Chicago, IL 60616

EXPERIMENTAL SECTION

Materials & Methods. Unless specified, all H2O was initially purified by facility reverse osmosis followed by an Elga Purelab Flex 3 purification unit comprising reverse osmosis, UV organic degradation, mixed bed cation/anion exchange, and 0.2 µm filtration modules. pH was measured using a Mettler-Toledo FiveEasy pH meter with an LE409 probe calibrated with commercial standard buffers at pH = 4.0 and 7.0. Microscopic examinations used an Amscope Long Working Distance 40-400x Inverted Trinocular Zoom Microscope with digital capture, except for the microdomain cooling studies, which used an Amscope 3.5-90x Trinocular Zoom Stereomicroscope with digital capture and positioning boom.

Surface Energy Modifications. Nucleation site arrays were etched on commercially available, precleaned, 75 mm x 25 mm soda-lime glass microscope slides using Trotec Speedy 300 or Epilog Helix Model 8000 50 W CO2 laser systems. Motifs for surface energy modifications and arrays were constructed using CorelDraw® X6 or Autocad® Software, and converted into machine instructions with specified power, speed, and dots per inch (DPI) settings. Translation of vector defined drawings into bitmaps of specified DPI introduces
rounding errors, so that the actual linewidths in these studies are \(\approx 0.35-0.40\) mm. Typical laser settings for the Trotec instrument included a resolution of 1000 DPI, 70% laser power, and 30% laser head speed while operating in raster mode (i.e., pixelated pattern etching), and for the Epilog instrument a resolution of 1200 DPI, 100% laser power, and 15-30% laser head speed were used while operating in raster mode.

**Acetylsalicylic Acid Batch Crystallization.** Studies of crystallization onset times using acetylsalicylic acid (ASA) as a model system are commonly used in the scientific literature to characterize nucleation properties of substrates,\(^{25}\) and the data presented here followed a locally modified protocol. A 150 mg/mL solution of ASA in 91% (v/v) isopropyl alcohol was prepared by combining 37.5 g ASA (Sigma Aldrich A5376) with 250 mL 91% isopropyl alcohol (Aaron Industries, Inc.) and heating to 60 °C in a sealed container to facilitate dissolution. The transparent solution was allowed to cool to \(\approx 22\) °C for at least 30 min prior to use and was stable with respect to crystallization at ambient temperatures.

Both the modified nucleation surfaces and the control surfaces were cleaned prior to use by rinsing with 91% isopropyl alcohol and then soaking at 60 °C in 91% isopropyl alcohol for approximately 30 min, after which this solution was discarded. The surfaces were subsequently soaked in distilled H\(_2\)O at 60 °C for approximately 30 min, rinsed with fresh distilled H\(_2\)O, and dried upright at 60 °C in a loosely covered container to minimize dust intrusion.

Because of the stochastic nature of crystallization, replicate measurements are necessary and a statistical assessment is an important part of any conclusions drawn from such data. Further, the ubiquitous factors that can induce nucleation and affect crystal growth such as impurities, dust, and the like, require careful manipulations and careful diligence in performing control experiments. Solvent controls (i.e., no control surface or experimental nucleation surface
present) permit monitoring of solution phase variables (e.g., impurities, adventitious solids, and microscopic crystalline fragments, etc.) that can induce primary or secondary nucleation in competition with the experimental surfaces. Surface control experiments are equally important, as the unmodified regions surrounding the surface energy modifications can produce unintended nucleation. For the studies with ASA in Table 1, both the solvent controls and surface controls were randomized across different experiments to reduce the effect of random errors in any one replicate study. This approach involving careful attention to solution handling, solvent controls, surface controls, replicate experiments, and statistical analyses gives the highest probability of uncovering statistically significant and reproducible findings.

Batch crystallization onset time studies with ASA were performed in 120 mL capped vials to which ≈ 40 mL of 150 mg/mL ASA in 91% isopropyl alcohol had been added. One nucleation surface was inserted into each vial such that a single 20 mm surface energy modified nucleation site array (if present) was immersed in the solution. Each nucleation site array was oriented slightly down-facing at an angle of ≈ 60-70° so that any bulk crystallization from solution would not collect on, clog, or otherwise inactivate the nucleation site array. Vials were immediately quenched to 0 °C in an ice/tap H2O bath maintained inside a refrigerator at ≈ 3 °C. Vials were monitored for the appearance of crystalline material at 15 min intervals up to the first 180 min, and less frequently thereafter up to times of 24 h. The onset time of crystallization signaled the end point for a given experiment, and a total of six replicates were performed in the initial proof of concept studies of Table 1.

**Protein Crystallization Conditions.** Thaumatin, *Thaumatococcus daniellii* (Alfa Aesar 53850-34-3) - Protein solution: 20 mg/mL in 25 mM HEPES at pH 7.0; Precipitant solution: 0.5 M K/Na tartrate, 0.1 M sodium citrate at pH 6.3. *Bovine Pancreatic Trypsin (BPT, Alfa Aesar*
Protein solution: 20 mg/mL in 25 mM HEPES at pH 7.0, 10 mM CaCl₂, and 10 mg/mL benzamidine-hydrochloride; Precipitant solution: 0.1 M (NH₄)₂SO₄, 20% (w/v) PEG 8000. *Hen egg white lysozyme, (Amresco 0663)* - Protein solution: 20 mg/mL lysozyme in 50 mM sodium acetate at pH 4.6; Precipitant solution: 1.0 M NaCl.

**Protein Crystallization.** The protein crystallization studies used sitting drop vapor diffusion at an ambient temperature of 22(1) °C. Careful attention was paid to the consistency of reagents, age of solutions, and equipment across experiments, as well as to the control of sample evaporation and dust intrusion that can lead to adventitious nucleation and statistical aberrations. Two groups of microscope slides containing two different 20 mm x 20 mm nucleation site arrays were used:

(1) A slide containing: (a) one 20 mm x 20 mm grid array having square islands of unmodified surface with average dimensions of 1.8 mm x 1.8 mm coined G18 (for Grid 1.8 x 1.8 mm), and (b) one square grid having average island dimension of 0.6 mm x 0.6 mm (G6). See the vertical slide in Supplemental Figure 6.

(2) A second slide containing: (a) one 20 mm x 20 mm square grid array with island dimensions of 0.5 mm x 0.5 mm (G5), and (b) one series of concentric circles with a maximum outside diameter of 20 mm and progressively smaller inner circles spaced \( \approx 0.5 \text{ mm} \) apart (CC5). See the horizontal slide in Supplemental Figure 6.
Supplemental Figure 6. Schematic showing the 20 mm nucleation arrays, naming convention, and experimental layout for the proof of concept protein crystallization studies at 22(1) °C.

Crystallization studies were conducted using sitting drop vapor diffusion inside plastic Petri dishes. The slide containing the G18 and G6 arrays was placed in the bottom of the Petri dish and the G5/CC5 slide was placed on top at a 90° angle, as shown in Supplemental Figure 6, and held in place with a small amount of vacuum grease. A 2 mL volume of precipitant solution serving as the vapor diffusion reservoir was pipetted into the bottom of the Petri dish, taking care to avoid contact with the nucleation features. A 5 µL aliquot of protein solution was pipetted onto the center of each feature as well as onto the unmodified glass surface between the G5 and CC5 nucleation site arrays, and served as the surface control. A 5 µL portion of the precipitant solution was then pipetted onto each protein drop, and the two solutions were mixed via pipetting up and down a total of three times. A lid was then placed on the Petri dish and the edge wrapped with parafilm to prevent evaporation and exposure to ambient air. The dishes were monitored for crystallization using an Amscope 40-400x Inverted Trinocular Zoom Microscope after a 16 h
overnight incubation period at 22(1) °C, and then checked in alternating 8 and 16 h increments. Replicate experiments were conducted using identical protocols.

Multiplexed surface energy modified nucleation arrays were also fabricated on microscope slides, which contained four adjacent replicates of six distinct 2.5 mm arrays as shown in Figure 1:

(1) A 2x2 square grid with island sizes of 0.8 mm coined MG8 (i.e., Multiplexed Grid 0.8)

(2) A 3x3 square grid with island sizes of 0.6 mm (MG6)

(3) A pair of concentric circles with the outer circle 2.5 mm in diameter uniformly separated by 0.6 mm from an inner circle of 1.3 mm in diameter (MCC6)

(4) A pair of concentric squares spaced 0.6 mm apart at midleg with the outermost square having dimensions of 2.5 mm x 2.5 mm (MCS6)

(5) A pair of concentric equilateral triangles spaced 0.3 mm apart at midleg with each leg of the outermost triangle measuring 2.5 mm in length (MCT3)

(6) A six-point asterisk 2.0 mm across (AST)
The motifs were arranged on the slide in two groups of three rows, with each row containing four copies of each motif (Figure 2, bottom left). Motifs were spaced 3.0 mm apart in both the x and y dimensions. An intentionally unmodified 22 mm square in the middle of the slide separated the two arrays and was used for the surface control studies. Crystallization studies using these multiplexed surface energy nucleation arrays were completed in a similar manner to those of the 20 mm surface energy modifications. Specifically, a blank microscope slide was placed in the bottom of the Petri dish to elevate the multiplexed array that was placed orthogonally on top and adhered via a small dab of vacuum grease, similar to Supplemental Figure 6. Total drop sizes of 2 µL were used for these studies and comprised 1 µL of protein solution and 1 µL of precipitant solution that were mixed via pipetting three times.

**Microdomain Cooling Crystallization Experiments.** A single 20 AWG Cu wire was fashioned into a dual probe configuration where each probe was 31.8 mm in length. The dual probe configuration was attached to a TEC-12706 thermoelectric unit using Arctic Alumina Thermal Compound and sealed in place with thermally conductive Al tape. The thermoelectric unit was attached to a hollow Al heat sink through which ice cooled H2O was recirculated using a submersible electric pump. The Cu probes/thermoelectric unit/heat sink apparatus was inverted so that the Cu probes pointed downward, and the entire assembly was clamped to a ring stand (Figure 3). The unit was controlled with a programmable temperature controller (Oven Industries 5R6-900) and the temperature was monitored by thermistors (TR136-208) placed on the surface of the thermoelectric module. The reported temperatures for the crystallization experiments reflect the Cu probe temperature at 5 mm from the bottom end of the wire (i.e., a region typically immersed in solution) rather than the temperature at the thermoelectric surface; for example, a
temperature of 4 °C at the tip of the Cu probe requires a temperature at the thermoelectric surface of ≈ -4 °C due to hysteresis.

Borosilicate glass capillaries (Kimble Chase 34507) were cut to length from the open end leaving the factory sealed end to cover the tip of the Cu probe, and these unmodified capillaries served as the control surfaces for nucleation studies. Nineteen semicircular nucleation motifs of 0.35 mm line width and separated by 0.25 mm of unmodified glass surface were hemicircumferentially laser etched in a linear array onto borosilicate capillaries. (Full circumferential etching made the capillaries too fragile.) Crystallization was carried out in sealed 2 mL vials with septa containing screw-tops. The open end of the capillary was always inserted through the septum in order to prevent physical damage to the nucleation site arrays by insertion. The caps were placed on the vials and the Cu probes were carefully inserted into the capillaries. The adjacent vials containing either the control or the experimental surfaces were immersed in an H₂O heat sink at 22(1) °C, and were subjected to identical thermal treatment because the dual Cu probes shared a common thermally conductive backbone in contact with a single thermoelectric device.

Initial experiments used 150 mg/mL ASA dissolved in 91% isopropyl alcohol. For protein crystallization experiments, lysozyme was prepared at a concentration of 20 mg/mL in 50 mM sodium acetate at pH 4.6 and mixed in equal volumes with 50 mM sodium acetate at pH 4.6, 1.0 M NaCl, and 30% PEG 8000 to mimic initial conditions for vapor diffusion crystallization. A 2 mL aliquot of solution was pipetted into the vials, which were then sealed with the septum caps supporting the capillary of interest. Experiments began at 22(1) °C and the probes were cooled to 4 °C over the course of ≈ 5 min. Crystallization was monitored by time lapse microscopy using a digital camera attached to an Amscope Trinocular Zoom Stereomicroscope at 2x magnification.