Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Enhancing drop stability in protein crystallization by chemical patterning

Viatcheslav Berejnov* and Robert E. Thorne

Cornell University, Ithaca, NY 14853, USA

Correspondence e-mail: vb54@cornell.edu

Received 21 June 2005
Accepted 12 September 2005

The motion of protein drops on crystallization media during routine handling is a major factor affecting the reproducibility of crystallization conditions. Drop stability can be enhanced by chemical patterning to more effectively pin the drop's contact line. As an example, a hydrophilic area is patterned on an initially flat hydrophobic glass slide. The drop remains confined to the hydrophilic area and the maximum drop size that remains stable when the slide is rotated to the vertical position increases. This simple method is readily scalable and has the potential to significantly improve the outcomes of hanging-drop and sitting-drop crystallization.

1. Introduction

Sitting-drop and hanging-drop vapor-diffusion methods are among the most popular techniques for protein crystallization (Hampton Research, 2003; McPherson, 1999). In the sitting-drop technique, protein crystals can sediment onto the glass or plastic surface supporting the drop, frequently adhering so strongly that they are damaged during retrieval. Adhered crystals are much more likely to crack during growth because of stresses associated with contact with the support.

The hanging-drop method largely eliminates these problems. Crystals sediment away from the supporting surface toward the liquid–air interface (although some may still nucleate and grow on the supporting surface). Freely suspended crystals often have unperturbed facets, show less cracking and have smaller mosaicities and (in the absence of protein 'skins') are easily retrieved for diffraction studies. Consequently, hanging drops are preferred for final crystallization trials to obtain the largest, highest quality crystals for data collection.

However, obtaining well formed hanging drops with well defined surface-to-volume ratios can be difficult. Depending on the mother-liquor composition, the drop can move relatively freely over the hydrophobic siliconized glass cover slides used to minimize drop spreading. While the cover slide is being inverted, larger drops can slide off entirely and smaller drops can become distended, changing their shape and rate of equilibration with the well solution. Similar problems occur for drops dispensed onto polymer (*e.g.* Teflon) films.

We have demonstrated a simple method to fix the drop shape and position. Chemical patterning of a circular area increases the strength of pinning of the drop's contact line and thereby defines the drop shape. Drops can be flipped without sliding or permanently distorting and the drop area and curvature for given drop volume can be customized. Furthermore, the drop size and shape for a given drop volume

(which determines the equilibration time with the well) is now much more reproducible. This eliminates an important variable factor affecting nucleation rates and subsequent growth in both hanging-drop and sitting-drop vapor-diffusion growth.

2. Model for drop behavior on an inclined surface

The boundary of a liquid drop on the surface of a flat substrate is a line separating liquid, solid (substrate) and vapor (air) and is called the contact line. The angle between the tangent to the vapor–liquid interface at the contact line and the substrate gives the contact angle θ (Fig. 1). On a horizontal perfectly uniform substrate, the contact angle would have a unique value determined by the liquid–vapor, liquid–substrate and vapor–substrate surface energies. If such a substrate were tilted from the horizontal, the drop would immediately slide off.

On a real substrate, the contact line is pinned by interaction with disorder at the substrate surface, so that the contact angle is no longer unique but depends on the drop's history (*e.g.* how it was dispensed and how it has been tilted). The strength of the contact-line pinning is reflected in the range of contact angles that can be supported before the contact line and thus the drop de-pin and move.

When a substrate supporting a drop is rotated from the horizontal, the drop initially distorts while its contact line remains pinned and fixed, as shown in Fig. 1. As the substrate is rotated toward the vertical, the net of the gravitational and surface-tension forces may locally exceed the pinning force. The contact line may then become locally unstable and distort from its initially circular form, producing a 'downhill' spreading of the drop. Upon further rotation, the gravitational force may exceed the maximum pinning force on the drop as a whole. The entire drop then becomes unstable and slides off the substrate.

To understand this process more formally, consider a simplified essentially two-dimensional model shown in Fig. 1(*b*) (MacDougall & Ockent, 1942; Frenkel, 1948), which can be extended to three dimensions (Furmidge, 1962; Dussan & Chow, 1983). We assume the drop's contact line is pinned by some specific but isotropic interaction with the substrate. In this simplified geometry there are only two contact angles, θ_a and θ_r, defining the angle of the tangent to the air–liquid interface at the advancing (downhill) and receding (uphill) edges of the contact line. For a true three-dimensional drop, as in Fig. 1(*a*), the contact angle θ varies along the (initially circular) contact line between extreme values at its uphill and downhill edges (Brown *et al.*, 1980; Rotenberg *et al.*, 1984; El Sherbini & Jacobi, 2004).

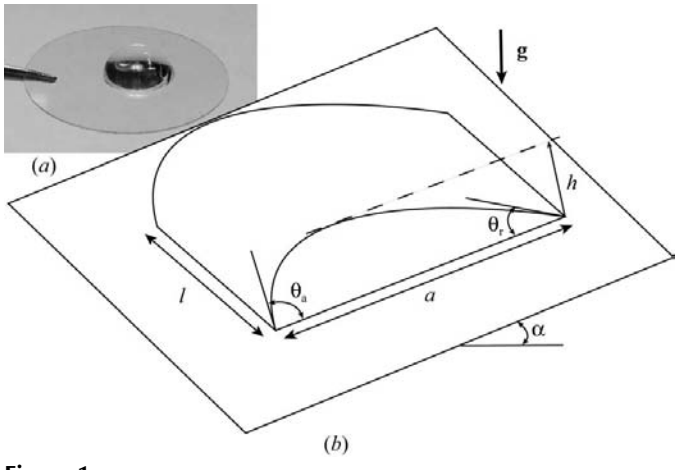

Figure 1
(*a*) Photograph of a protein-containing drop on a siliconized glass slide. (*b*) Simplified two-dimensional model for a pinned drop on a flat inclined substrate.

When the substrate is horizontal, $\theta_a = \theta_r$. When the substrate is tilted from the horizontal by an angle α, the contact line remains pinned, the advancing contact angle θ_a increases and the receding contact angle θ_r decreases. For some critical tilt $\alpha = \alpha^*$ (corresponding to values of $\theta_a = \theta_a^*$ and $\theta_r = \theta_r^*$), a sufficiently large drop will depin and slide down the substrate. Balancing the downhill component $F_{g,\|}$ of the force arising from gravity $\mathbf{F_g}$ with the uphill force arising from capillarity $\mathbf{F_c}$ provides a boundary condition that must be satisfied by a stationary pinned drop (Frenkel, 1948). The drop remains stationary and pinned if for a given inclination, drop mass and contact conditions we are able to find a drop shape consistent with this boundary condition (Frenkel, 1948; Lawal & Brown, 1982). Beyond a critical inclination angle α_c no solution can be found and $F_{g,\|} > F_c$.

The net capillary force is very roughly given by $F_c \simeq l\sigma\Delta(\cos\theta)$, where l is the characteristic width of the drop, σ is the vapor–liquid surface tension and $\Delta(\cos\theta) = \cos\theta_r - \cos\theta_a$. Analogous to a static friction force, $\Delta(\cos\theta)$ increases as the tilt angle increases so as to keep the drop stationary. The maximum value $\Delta(\cos\theta)_{max} = (\cos\theta_r - \cos\theta_a)_{max}$ that can be sustained by the contact-line pinning is called the contact-angle hysteresis (De Gennes, 1985). For $\alpha > \alpha^*$, the drop slides and $\Delta(\cos\theta)$ depends upon the flow conditions (Dussan & Chow, 1983).

The downhill component of the drop's weight $\mathbf{F_g}$ is $F_{g,\|} = \rho g V \sin\alpha$, where ρ is the density of the liquid, g is the acceleration due to gravity and V is the drop volume. For the two-dimensional drop of Fig. 1(*b*), $V \simeq alh$, where $a \simeq l$ is the drop's diameter and h is its characteristic height. Combining the formulas for F_c and $F_{g,\|}$ yields the condition for drop stability $F_{g,\|} = F_c$ on a surface inclined at angle α,

$$\rho g a^2 h \sin\alpha = l\sigma(\cos\theta_r - \cos\theta_a). \qquad (1)$$

For any angle α between 0 and α^* the drop is able to adjust its surface shape by varying the contact-angle values θ_a and θ_r and thus $\Delta(\cos\theta)$. The last contact angles θ_a^* and θ_r^* the drop reaches at α^* are determined by the contact-angle hysteresis $\Delta(\cos\theta)_{max}$. At that critical point, $(\cos\theta_r - \cos\theta_a)$ corresponds to a surface-energy difference that is an invariant of the drop shape.

For hanging-drop crystallization, we are particularly interested in the case $\alpha = 90^\circ$ when the drop is oriented vertically. For such a drop to be stable against sliding,

$$V_c(\sin\alpha = 1) = \text{const} \simeq \frac{l\sigma}{\rho g}(\Delta\cos\theta). \quad (2)$$

A drop satisfying this criterion is absolutely stable. It may still move, however, if accelerations during flipping or other handling, which produce forces on the drop that add to the gravitational force, are large enough. The stability criterion depends on the properties of the drop, which are affected by the presence of protein. Proteins have hydrophobic and hydrophilic parts and thus can behave as surfactants (Mobius & Miller, 1998). Protein present at the vapor–liquid interface decreases the solution's surface tension. In addition, protein adsorption on the substrate surface can change the contact angles, surface-pinning properties and contact-angle hysteresis.

In protein crystallization, we often want to minimize the drop surface-to-volume ratio to produce slow equilibration with well solutions favourable for nucleation. For hanging-drop crystallization, we would also like to maximize the stability of the drop, equivalent to obtaining the largest possible drop volume that is stable at $\alpha = 90^\circ$. Increasing the drop volume will decrease the drop surface-to-volume ratio until drop flattening by gravity becomes important. Increasing the contact-angle hysteresis (*i.e.* the strength of contact-line pinning) will increase the maximum size of an absolutely stable drop.

By chemically patterning a substrate to produce hydrophobic and hydrophilic areas so as to increase the strength of contact-line pinning and $\Delta(\cos\theta)_{\max}$, we show in the following that the maximum absolutely stable drop volume can be increased. Strong pinning of the contact line at the hydrophobic/hydrophilic boundary also yields much more accurate control over drop shape and surface-to-volume ratio.

3. Materials and methods

Six times recrystallized and lyophilized hen egg-white lysozyme from Seikagaku America (Falmouth, MA, USA) was dissolved in a sodium acetate (NaAc) aqueous buffer prepared by adding concentrated acetic acid to a 50 m*M* sodium acetate solution to adjust the pH to 5.0. Protein concentration was measured using a Spectronic Genesys TM 5 spectrophotometer (Spectronic Instruments, NY, USA).

Solution surface tension was measured as a function of protein concentration using the pendant drop-counting method. A pipette tip is filled with protein solution and then held vertically and the number of drops that fall from the tip in a given time and the total mass of these drops is measured. Using these values, the drop radius as it emerges from the tip and the liquid density, the vapor–liquid surface tension can be calculated. For the protein solutions studied, the drop radius was measured to be roughly 11% larger than the external tip diameter.

Siliconized and initially flat glass slides HR3-231 with diameter 22 mm were purchased from Hampton Research (Laguna Niguel, CA, USA). On a freshly unpackaged slide, a 40 µl buffer drop formed a reproducible contact angle of 90–92°.

To increase contact-angle hysteresis and thus drop stability under inclination, a hydrophilic circular region was fabricated on some of these hydrophobic siliconized glass slides. A drop of 1 *M* NaOH was placed on the surface at room temperature and the slide was then heated on a hotplate in air to 373 K until all liquid evaporated. The base removes the silanol layer and reacts with the glass, producing water-soluble silicates. These were washed away using a jet of distilled deionized water and the slide was then dried using pure N_2 gas. NaOH drop volumes of 25, 50, 75 and 100 µl were used to produce etched areas with diameters of 5.5 ± 0.5, 7.0 ± 0.5, 8.0 ± 0.5 and 9.5 ± 0.5 mm.

Following this treatment, the etched areas were highly hydrophilic, with contact angles for pure water of less than 1°. When a drop was dispensed onto the etched region, the large difference in contact angle between the etched and unetched regions strongly pinned the drop's contact line at the boundary between these regions. This strong pinning produced a contact-angle hysteresis for pure water of $90 \pm 1^\circ$, compared with a value of $12 \pm 1^\circ$ for a uniform siliconized glass slide.

The patterned and unpatterned slide surfaces were analysed by contact-mode AFM (DI MultiMode III, Santa Barbara, CA, USA) using NSC 1215 tips from MikroMasch. A typical surface profile in Fig. 2 shows that the treated area is etched to a depth of about 1 µm.

To determine drop stability on a given unpatterned or patterned slide, a drop was dispensed onto a horizontal slide using a 100 µl micropipette (Pipetman Co., France). The slide was then slowly rotated in 2–4° steps, allowing roughly 1 min after each rotation to allow transient relaxations of the drop to die out. The contact angles of the drop were measured using a vertical goniometer and its shape and position recorded using a digital camera. Dispensed drop volumes were accurate to

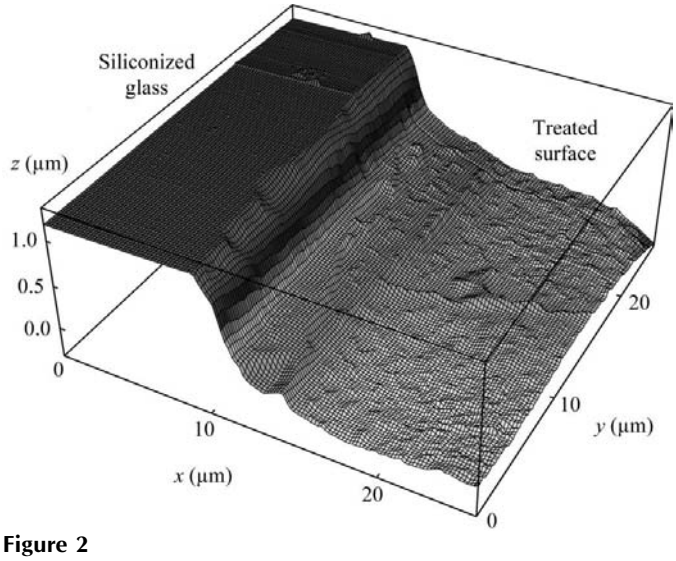

Figure 2
AFM image of the boundary between untreated and NaOH-treated parts of the slide. The average etched depth of the NaOH-treated hydrophilic area is about 1 µm.

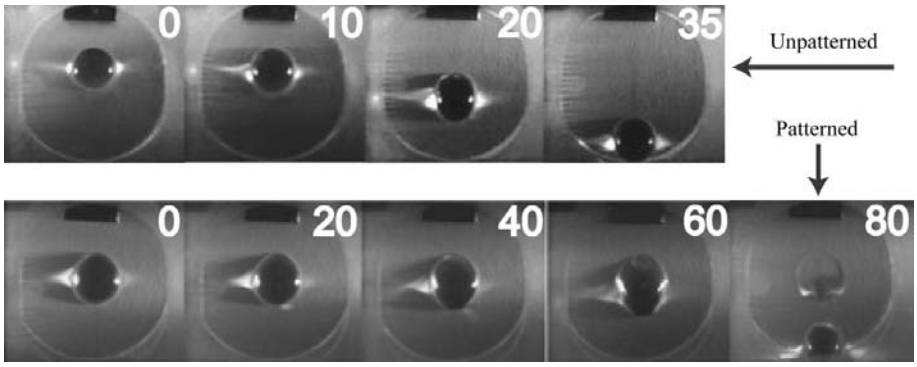

Figure 3
Effect of substrate patterning on stability of protein-free buffer drops. The numbers in each frame indicate the tilt angle α in degrees. Substrates are hydrophobic siliconized glass slides. Patterned slides have circular hydrophilic areas with diameters of 8 mm.

1% and tilt and contact-angle measurements were accurate to 1–2°. Each measurement on an 'unpatterned slide' used a fresh slide and each measurement on a 'patterned slide' used a new fabricated slide to avoid surface-contamination problems between measurements.

4. Results and discussion

Drop stability on patterned and unpatterned slides was investigated as a function of drop-orientation angle, drop volume, drop area and protein concentration. As the inclination angle was incremented upward, the contact angles and contact-line position varied around the drop circumference, in part through local instabilities (similar to avalanches) that caused abrupt jumps in contact-line position. For larger drops, the drop became absolutely unstable at a critical angle $\alpha^* \leq 90^\circ$ and slid off the slide, as shown in Figs. 3 and 4. To construct a drop-stability phase diagram, the patterned area (radius) and protein concentration were fixed and the critical angle measured as a function of drop volume. Repeating these measurements with different protein concentrations and patterned areas provided a complete characterization of drop stability.

Fig. 4 shows a typical drop-stability diagram for patterned and unpatterned slides with fixed patterned radius and fixed protein concentration. For a given inclination $\sin\alpha$, a drop remains stable (even though its contact line may undergo local displacements around the drop's circumference) for volumes up to a critical volume $V_c(\sin\alpha)$. Drops smaller than the critical volume $V_c(\sin\alpha = 1) = \text{const}$ will be absolutely stable; they will not slide off even at $\alpha = 90^\circ$. Drops larger than V_c become unstable and slide off at a critical inclination $\sin\alpha_c \propto 1/V$ as follows from (1). For the conditions in Fig. 4, substrate patterning increases the volume range of absolute stability by ∼15%.

For hanging-drop crystallization, we want to maximize the range of volumes for which a drop is absolutely stable and thus the critical volume $V_c(\sin\alpha = 1)$. V_c depends on the protein concentration and on the area of the patterned region, which determines the drop's area.

Fig. 5 shows how the critical volume for absolute stability $V_c(\sin\alpha = 1)$ varies with protein concentration, for a fixed drop diameter. Drop volumes below each curve are absolutely stable. For both unpatterned and patterned slides, the critical volume decreases beyond a protein concentration of roughly 1 mg ml^{-1}. This behavior is consistent with the measured

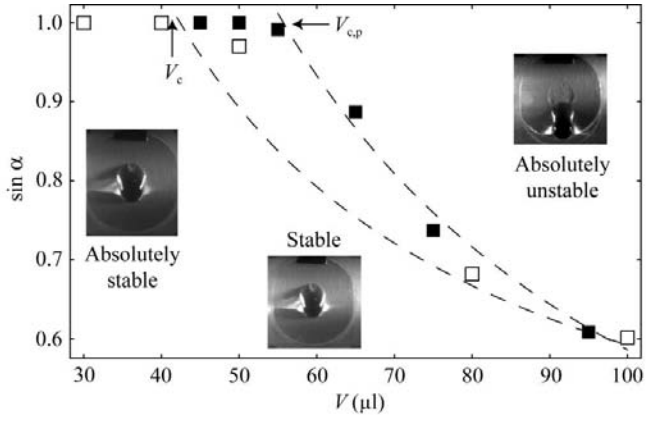

Figure 4
Stability diagram for drops on unpatterned siliconized slides (open squares) and siliconized slides patterned with an 8 mm diameter hydrophobic region (solid squares) for drops containing 1 mg ml^{-1} lysozyme in 50 m*M* NaAc buffer pH 5. The critical volumes beyond which drops are unstable at $\alpha = 90^\circ$ are $V_c = 47 \pm 2$ and $V_{c,p} = 55 \pm 2$ ml for the unpatterned and patterned slides, respectively. Drops with smaller volumes are absolutely stable. Dashed lines represent the function $\sin\alpha \propto 1/V$.

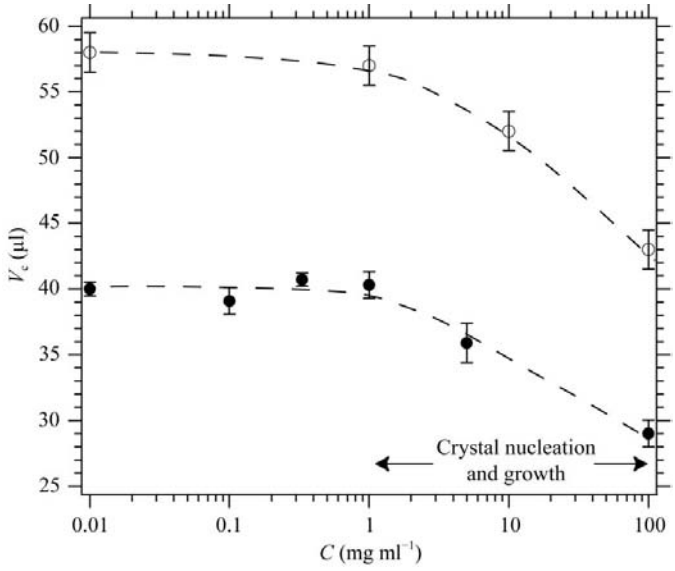

Figure 5
Critical drop volume $V_c(\sin\alpha = 1)$ for stability on vertically oriented substrates *versus* protein concentration. The open and solid circles show data for unpatterned siliconized slides and siliconized slides patterned with a 9.5 mm diameter hydrophobic region, respectively. The approximate concentration range over which crystal growth can be induced by addition of NaCl precipitant is indicated.

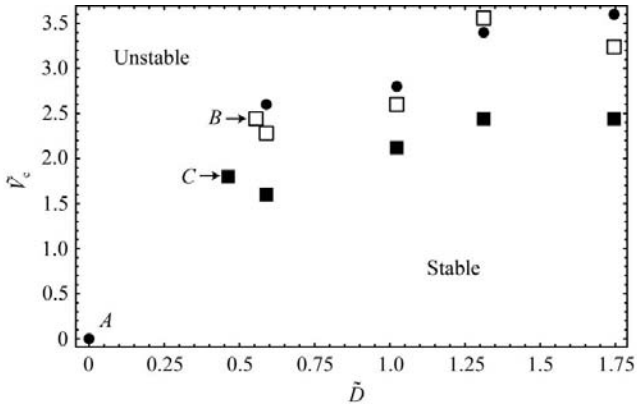

Figure 6
Enhancement of drop critical volume V_c by patterning a circular hydrophilic region of diameter D. The points A, B and C correspond to drops on unpatterned slides. The raw data is scaled by the value of the point A (V_{c0} = 12.5 µl and D_0 = 3.5 mm) as described in the text. Solid circles correspond to protein-free buffer solution and open circles and squares correspond to protein concentrations of 1 and 100 mg ml^{-1}, respectively.

increase in drop surface tension with increasing protein concentration (Mobius & Miller, 1998).

For pure protein-free buffer on an unpatterned slide, the critical volume V_c is 12.5 µl, a factor of 3.2 smaller than for the smallest protein concentration (0.01 mg ml^{-1}) shown by solid circles in Fig. 5. Thus, variation of V_c with protein concentration on unpatterned slides is non-monotonic. This complex behavior is likely to result because protein adsorbs to the slide surface, modifying the contact angle and contact-angle hysteresis. Even very small solution concentrations of protein are sufficient to modify these properties. On the other hand, the critical volume V_c for the patterned slides (open circles) is constant between 0 and 0.01 mg ml^{-1}, indicating that contact-line pinning dominates the drop–substrate interaction.

Over the whole concentration range, patterning provides large increases in the critical volume for absolute stability, from 45% at C = 0.01 mg ml^{-1} to 48% at 100 mg ml^{-1}. At concentrations that yield the largest protein crystals (near 2 mg ml^{-1}), the increase is 38%.

Fig. 6 shows how the critical volume $V_c(\sin\alpha = 1)$ for absolute stability varies with the drop diameter D (measured when the slide is horizontal) for pure buffer and for solutions with different protein concentrations. To simplify representation of the data, Fig. 6 uses the dimensionless variables $\tilde{V}_c$ and $\tilde{D}$ defined by the formulas $\tilde{V}_c = (V_c - V_{c0})/V_{c0}$ and $\tilde{D} = (D - D_0)/D_0$, respectively. Here, V_{c0} = 12.5 µl and D_0 = 3.5 mm are the critical volume and corresponding wetting diameter of the drop measured for protein-free buffer solution on an unpatterned hydrophobic siliconized slide, indicated by point A.

Points B and C are also for unpatterned slides, but with protein concentrations of 1 and 100 mg ml^{-1}, respectively. At 1 mg ml^{-1} (point B), the critical volume is 41.4 µl, 3.3 times larger than for protein-free drops. Increasing the protein concentration to 100 mg ml^{-1} (point C) decreases the critical volume to 32.5 µl, 2.6 times larger than for protein-free drops.

On patterned slides, the wetted drop diameter is equal to the patterned diameter and can be made much larger than on unpatterned slides. For protein-free buffer (solid circles in Fig. 6), increasing the wetted diameter from 3.5 to 9.5 mm increases the critical volume by a factor of 4.6. For 1 mg ml^{-1} and 100 mg ml^{-1} protein, increasing the diameter from 6.1 and 5.1 mm, respectively, to 9.5 mm increases the critical volume by roughly 20–25%. This increase comes at the cost of a factor of 0.94 decrease in drop surface-to-volume ratio.

5. Conclusions

We have demonstrated that the overall stability of drops on an inclined flat substrate can be significantly increased by patterning the substrate surface so as to strongly pin the drop's contact line. This allows larger drops to be used in hanging-drop crystallization. More important, patterning precisely defines the drop shape and thus its surface-to-volume ratio, independent of how the drop is dispensed and flipped. Patterned substrates should thus provide more repeatable drop equilibration rates with well solutions and thus more reproducible crystal nucleation and growth.

The largest increases in drop stability and volume occur for protein-free solutions. This suggests that surface patterning can be used to define 'well' drops on the same substrate as the protein drop and with volumes 10–100 times the protein drop volume. Consequently, with surface patterning wells can be eliminated, yielding a crystallization platform that can be used in any orientation.

This work was funded by NASA (NAG8-1831) and by the NIH (R01 GM65981-01). We thank Eugene Kalinin for assistance with the AFM measurements.

References

Brown, R. A., Orr, F. M. & Scriven, L.E. (1980). *J. Colloid Interface Sci.* **73**, 76–87.
De Gennes, P. G. (1985). *Rev. Mod. Phys.* **57**, 827–863.
Dussan, V. E. B. & Chow, R. T.-P. (1983). *J. Fluid Mech.* **137**, 1–29.
El Sherbini, A. I. & Jacobi, A. M. (2004). *J. Colloid Interface Sci.* **273**, 556–565.
Frenkel, Y. I. (1948). *J. Exp. Theor. Phys. (USSR)*, **18**, 659–667 (In Russian). English translation available at http://arxiv.org/abs/physics/0503051.
Furmidge, C. G. L. (1962). *J. Colloid Sci.* **17**, 309–324.
Hampton Research (2003). *Crystallization Research Tools*, Vol. 13, No. 1. Laguna Niguel, CA, USA: Hampton Research.
Lawal, A. & Brown, R. A. (1982). *J. Colloid Interface Sci.* **89**, 346–352.
MacDougall, G. & Ockent, C. (1942). *Proc. R. Soc. London Ser. A*, **180**, 151–173.
McPherson, A. (1999). *Crystallization of Biological Macromolecules.* New York: Cold Spring Harbor Laboratory Press.
Mobius, D. & Miller, R. (1998). Editors. *Proteins at Liquid Interfaces.* Amsterdam: Elsevier.
Rotenberg, Y., Boruvka, L. & Neumann, A. W. (1984). *J. Colloid Interface Sci.* **102**, 424–434.