Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

A method to produce microseed stock for use in the crystallization of biological macromolecules

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Received 28 October 1998
Accepted 2 February 1999

A method is presented for producing a seed-stock mixture for macromolecular crystallization. A PTFE bead and microcentrifuge tube act as mortar and pestle for pulverizing seed crystals of macromolecules. Energy for the bead's motion is supplied by a vortex mixer or an ultrasonic bath. The crushed crystal is serially diluted to prepare a seed-stock mixture of the desired concentration for crystallization. Crystals produced using both hanging-drop vapor diffusion and a capillary microbatch method show expected dilution behavior. This technique of producing seed stock is compared with traditional means and advantages over the standard protocol are demonstrated.

1. Introduction

A crystallization experiment for a biological macromolecule (Fig. 1) might begin with solution conditions which are undersaturated with respect to the macromolecule; under such conditions, homogeneous spontaneous nucleation cannot occur and crystals placed in the solution will dissolve (Riès-Kautt & Ducruix, 1992). The concentrations of macromolecule and crystallizing agent might be increased until a point of saturation, located somewhere on the two-dimensional solubility curve, is reached. Here, homogeneous spontaneous nucleation cannot occur and crystals added to the solution neither dissolve nor increase in size. Supersaturation can be divided into three sequential zones. In the *metastable zone*, spontaneous homogeneous nucleation does not occur within a reasonable length of time, but crystals added to the solution will increase in size. In the *labile zone* spontaneous homogeneous nucleation can occur. In the *precipitation zone* an amorphous precipitate rapidly forms. It is widely believed that ideal solution conditions for crystal growth are not the same as ideal conditions for spontaneous homogeneous nucleation. Nucleation requires a higher level of supersaturation than crystal growth. Spontaneous homogeneous nucleation depends on the probability of aggregation of macromolecules to form a viable nucleus, which is in turn related to the level of supersaturation of the solution. These events do not occur in a reasonable length of time unless a solution reaches a labile state of supersaturation. Seeding is a method which allows growth conditions to be optimized separately from those conditions necessary to induce nucleation. Several methods of seeding are currently used. Macroseeding techniques introduce a washed single crystal to a protein solution which is in the supersaturated labile zone (Stura & Wilson, 1990). Heterogeneous epitaxic seeding techniques employ minerals (McPherson & Shlichta, 1988) or protein-binding membranes (Punzi *et al.*, 1991) which act as

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templates for protein aggregation. These methods can be effective, especially in cases where no seed crystals of the same or of a closely related protein are available. Microseeding techniques and applied methodology have been described in the literature (Stura & Wilson, 1992). In one such method, streak seeding (Stura & Wilson, 1991), microseeds are transferred to protein drops by drawing a hair across the surface of the protein crystal under study or across a closely related protein crystal (cross-seeding) to pick up nuclei. These nuclei are transferred to a protein solution by drawing the impregnated hair through a protein drop. Crystallization generally takes place along the path which the hair followed through the drop. Another method of microseeding involves the preparation of seed-stock mixtures. These seed-stock mixtures are traditionally prepared using tissue homogenizers with ground-glass surfaces. A washed seed crystal is placed into the homogenizer with a small volume of stabilizing solution. The glass surfaces, through a grinding action, pulverize the seed crystal to microscopic particles. This seed stock can then be serially diluted in stabilizing solution to 10^{-3} to 10^{-7} times its original concentration, creating a seed-stock suspension which is diluted to the point where only a few viable nuclei are transferred into a protein solution. If the seed-stock mixture is too concentrated, inoculation of a protein drop will lead to a showering of microcrystals. If the seed stock is too dilute, the transfer will not deliver any nuclei to the protein drop and will not have the intended positive effect on crystal growth. It is important to transfer the nuclei to a protein solution which is mildly supersaturated. Transfer of nuclei to an undersaturated protein solution can cause dissolution of the nuclei. Transfer of nuclei to a solution which is too highly supersaturated can result in a showering of microcrystals. Through experimentation, a properly diluted seed-stock mixture can be prepared which will lead to inoculation of the protein solution with the desired number of nuclei. The method of drop inoculation can be varied. In a variant of the streak-seeding approach, a whisker is used to transfer nuclei from the diluted seed-stock mixture to the protein solution. Alternatively, using a micropipet, a small measured aliquot of the seed-stock suspension is inoculated into the protein drop. These methods can greatly improve the likelihood of growing protein crystals in a reproducible manner.

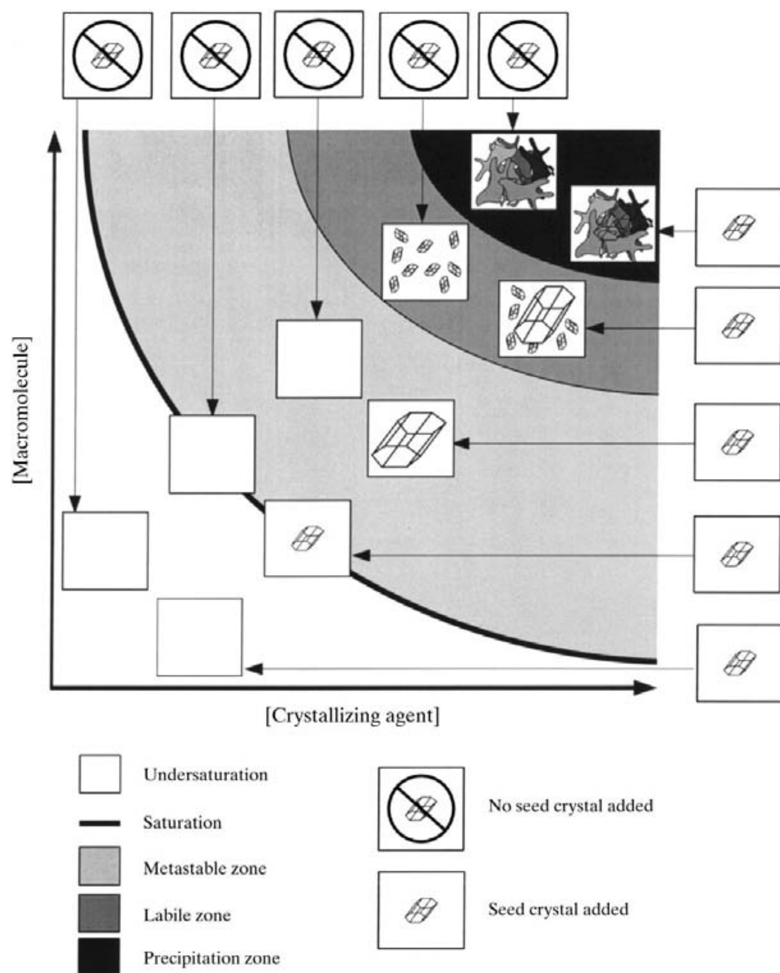

Figure 1
A two-dimensional representation of a crystallization experiment showing results with and without introduction of a macroseed. The graph is divided into three major zones: *undersaturated*, *saturated* and *supersaturated*. In the first region, represented by the area under the solubility curve, solution conditions are undersaturated with respect to the macromolecule, homogeneous spontaneous nucleation cannot occur and a crystal placed in the solution will dissolve. The next region, saturation, is measured experimentally and is represented by the two-dimensional solubility curve. Along the saturation curve, homogeneous spontaneous nucleation cannot occur and crystals added to the solution will not dissolve or increase in size. The next region of the graph, supersaturation, can be further divided into three sequential zones. The first zone, *metastable supersaturation*, is supersaturated to the point that spontaneous homogeneous nucleation does not occur in a reasonable length of time; however, crystals added to the solution will grow. The metastable zone is the region ideal for seeding a protein solution. The second region, *labile supersaturation*, represents a region where spontaneous homogeneous nucleation can occur and crystals added to the solution will grow. This region is not as well suited for seeding as the metastable region. Seed crystals added to a labile supersaturated solution can shock the solution and cause excessive nucleation. The final *precipitation* zone is an area which is many times supersaturated with respect to crystal growth and leads to the formation of amorphous precipitate.

The process of pulverizing the crystal for use in a seed-stock mixture can be somewhat difficult and is the issue we will address. Ground-glass tissue homogenizers are expensive and can be difficult to clean when PEGs and other chemical additives are required for stabilizing protein crystals. Tissue homogenizers can become less effective after several uses as the ground-glass surfaces become worn. Their relatively high cost makes it impractical to use a new clean glass tissue homogenizer for each experiment. Small crystals, which may be the only ones available for seed preparations, can sometimes fit between the ground-glass surfaces, failing to produce the desired microscopic crystalline particles. Finally, if additives which can lead to foaming are present in the protein-stabilizing solution, great care must be taken to avoid formation of foam in

the seed-stock mixture. We have developed an alternative method for pulverizing macromolecular crystals for use in seed-stock mixtures. The method uses a PTFE bead inserted into a microcentrifuge tube containing the protein crystal and a stabilizing solution. The protein crystal is pulverized using a vortex mixer or ultrasonic bath to cause the bead's motion. The PTFE bead acts as a pestle and the microcentrifuge tube as a mortar. PTFE was chosen as the bead material owing to its resistance to chemical and physical deterioration, commercial availability and low cost. We demonstrate advantages of bead pulverization which eliminates many of the difficulties encountered using the classical glass tissue-homogenizer protocol.

2. Experimental procedures

Two groups of experiments were conducted. Unless otherwise indicated, chemicals were purchased from Sigma Chemical Company (PO Box 14508, St Louis, MO 63178, USA). Hen egg-white lysozyme (HEL) was used as supplied (Sigma L2879, Lot No. 66H7050) without further purification. The HEL was shown to contain 5.87% chloride ion by weight by IC analysis (Censullo, 1996). Dissolved in acetate buffer at a pH of 4.5, HEL is calculated to have ten chloride counterions. If the remaining chloride counterion is in the form of NaCl, 1 mg of HEL dissolved in 1 ml total solution volume will contain 1.02 m*M* NaCl. This assumption gives a calculated total mass of 0.993 mg for a 1.000 mg sample. Any other impurities which may have been contained in the HEL preparation were not taken into account during calculations or sample preparations. Water used in the preparation of solutions was distilled and deionized using a Barnstead NANOpure II with a resistance >17 MΩ cm^{-1}. Protein solutions were sterile-filtered using 0.22 µm cellulose acetate centrifuge filters (Fisher No. CFA0215SM). All other solutions were filtered using disposable sterile syringe filters (25 mm, 0.20 µm, cellulose acetate membrane; Corning, 21052-25). Filter preservatives were removed by washing with distilled deionized water followed by a small volume of stock solution prior to sample collection. The results of the experiments were digitally captured using a Cambridge SZ-6 stereozoom photomicroscope with a Hitachi Model VK-C150 color video camera and a Snappy video-capture device (Play, Inc., 2890 Kilgore Road, Rancho Cordova, CA 95670-6133, USA) and processed using a Pentium Pro 200 PC. JPEG images were optimized and arranged using Adobe Photoshop 4.0 and Pagemaker 6.5 (Adobe Systems Inc., 345 Park Ave, San Jose, CA 95110-2704, USA).

2.1. HEL (I). Hanging-drop vapor-diffusion crystallization of HEL

A stabilizing solution, used as both a protein dropping solution and for dilution of seed-stock mixtures, contained 5.1 mg ml^{-1} HEL, 690.7 m*M* NaCl and 68.5 m*M* sodium acetate buffer pH 4.6. Using available solubility data (Broutin *et al.*, 1995), the solution was calculated to be 1.75 times saturated at 291 K and gave no indication of spontaneous homogeneous nucleation. Protein concentrations were determined using a Shimadzu 1201S UV–VIS spectrophotometer to measure the optical density at 280 nm, using an absorbance of 2.64 for a 1 mg ml^{-1} HEL solution. Seed stocks were prepared using three different methods to pulverize the seed crystals of HEL.

2.1.1. Ultrasonicated bead seed stock. A single crystal of HEL (0.7 mm edge) was transferred from a stabilizing-solution wash to a microcentrifuge tube (Fisher No. 05-406-16) containing 50 µl of stabilizing solution. Volumes of stabilizing solution which were 50 µl or less were most effective for crushing the crystal. A paper wick (Hampton Research No. HR4-211) was used to transfer the crystal to ensure a minimum carryover of liquid. A 3 mm diameter PTFE bead (Fisher No. 09-191-30A) was placed into the microcentrifuge tube to act as a pestle. The microcentrifuge tube was placed in an ultrasonic cleaner (Fisher No. FS-30) for two 1 min intervals (Fig. 2). After the crystal was pulverized, 450 µl of stabilizing solution was added to the suspension. The shape of the bottom of the microcentrifuge tube was found to be critical. The microcentrifuge tube used must have a bottom conical section which is 'matched' to the shape of the bead to be effective.

2.1.2. Vortexed bead seed stock. The protocol in §2.1.1. was followed, except that a vortex Genie II mixer (Fisher No. 12-812) set at speed 3 (midpoint) for 2 min was used to pulverize the crystal. It was imperative to use a high enough vortexer setting to cause the bead to randomly 'bounce' in the microcentrifuge tube. Too low a setting on the vortex Genie caused the bead to rotate in a circular path above the seed crystal, leaving it intact. Volumes of stabilizing solution which were 50 µl or less were again found to be most effective. Once pulverized, 450 µl of stabilizing solution was added to the suspension.

2.1.3. Glass tissue-homogenizer seed stock. A single crystal of HEL (0.7 mm edge) was transferred from a stabilizing-solution wash to a glass tissue homogenizer (Fisher No. 08-416C) containing 50 µl of stabilizing solution. The seed crystal

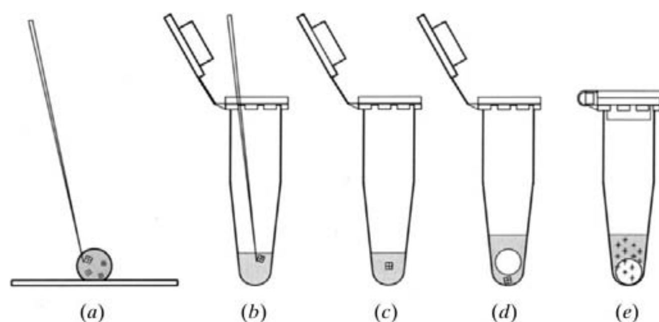

Figure 2
A paper wick is used to transfer a seed crystal with a minimum carryover of mother liquor to a microcentrifuge tube containing stabilizing solution (*a*), (*b*). After the transfer is complete (*c*), a 3 mm diameter PTFE bead is added to the microcentrifuge tube containing the crystal and stabilizing solution (*d*). Finally, the crystal is pulverized, moving the bead either by placement in an ultrasonic bath, or vortex mixing, to produce a homogeneous seed stock (*e*).

was pulverized with 50 strokes of the pestle. Again, 450 µl of stabilizing solution was added to the suspension.

The crystallization experiment was set up as hanging-drop vapor diffusion using *HANGMAN* (Luft & DeTitta, 1992), with Manco crystal-clear label tape (Hampton Research No. HR4-510) and a VDX plate (Hampton Research No. HR3-140). Reservoir solutions were 1 ml of 1.050 *M* NaCl, 105.0 m*M* sodium acetate buffer pH 4.6. After vapor equilibration, and assuming none of the dissolved protein undergoes a phase change in the drop, the protein concentrations would be nine times saturated (Broutin *et al.*, 1995).

Serial dilutions of the seed-stock suspensions obtained by all these methods were prepared *via* 1:10 dilutions of successively diluted seed stocks with stabilizing solution, beginning with the most concentrated seed stock and continuing to a 10^{-4} diluted solution. Stabilizing solution without seeds was used as infinitely diluted seed stock. The drops were initially 10 µl in volume, set up in quadruplicate with each of the six dilutions of seed stock in the protein dropping solution. Three VDX plates were set up, one for each of the methods of pulverizing the seed crystals described above. The experiments were left to equilibrate for a one week period at 291 K.

2.2. HEL (II). Crystallization of HEL with three concentrations of ultrasonicated bead seed stock

Solutions prepared included: *A*, 8%(*w*/*v*) NaCl in 0.1 *M* sodium acetate buffer pH 4.6; *B*, 40 mg ml^{-1} HEL in 0.1 *M* sodium acetate buffer pH 4.6; *C*, 0.1 *M* sodium acetate buffer pH 4.6. All solutions were sterile filtered as described. Using solubility data (Ewing *et al.*, 1994), a saturated solution of HEL (s = 1) at 291 K, 4%(*w*/*v*) NaCl, 0.1 *M* sodium acetate buffer pH 4.6 will contain 2.15 mg ml^{-1} HEL. From this data, integral values of supersaturation from two to nine times saturated (s = 2–9) were calculated and 300 µl aliquots of s = 2–9 were prepared by combination of solutions *A*, *B* and *C* in microcentrifuge tubes. To prepare the two times saturated (s = 2) crystallizing solutions, a single 0.5 mm edge seed crystal of HEL was washed in s = 2 solution. The seed crystal, 50 µl of s = 2 solution and a PTFE bead were placed in a microcentrifuge tube. The sample was ultrasonicated for two 1 min intervals to pulverize the crystal. This is s = 2, d = 1 (two times saturated, undiluted seed stock) crystallizing solution. An aliquot of s = 2, d = 1 solution was diluted 100 times with s = 2 solution to make s = 2, d = 3 (two times saturated, 100 times diluted seed stock) crystallizing solution. Finally, s = 2 solution, without seeds, was used as is to represent s = 2, d = 5 (two times saturated, infinitely diluted seed stock) crystallizing solution. The same protocol was followed for s = 3, 4, 5, 6, 7, 8 and 9. After being prepared, all of the crystallizing solutions were set up in duplicate (*r*1, *r*2) to crystallize using a capillary microbatch technique (Fig. 3; Luft *et al.*, 1999). The capillary microbatch protocol was set up by placing a 10 µl aliquot of the crystallizing solution into a microcentrifuge tube. A capillary (Fisher No. 02-668-68) was placed into the solution and the microcentrifuge tube inverted over the capillary to fill the tube. The ends of the capillaries were sealed using Hemato-Seal sealing compound (Fisher No. 02-678). After all the capillaries were prepared, the experiments were left to equilibrate for a one week period at 291 K. The results of the experiment were digitally captured.

3. Results

HEL (I) results compare seed stock from crystals crushed using an ultrasonicated bead, a vortexed bead and a glass tissue homogenizer (Fig. 4). The protein solution would be nine times saturated after complete vapor-diffusion equilibration between the drop and reservoir if no protein underwent a phase change. Results for the inoculated drops are very similar, regardless of the method used to pulverize the seed crystal. HEL (I) shows the effect of serial dilution of the seed-stock mixtures. The general trend shows a decrease in the number of crystals with decreasing concentration of the seed stock. Drops containing seed stock at high dilution produced more variation in results than drops containing seed stock at low to moderate dilution. It is possible that the seed stock had reached a dilution which was too high to consistently transfer seeds to the protein solution. Another likely explanation is the presence of dust in some of the drops, despite careful filtration of all solutions prior to use. A Zerostat anti-static instrument (Aldrich Z10 881-2) was found to be very useful in eliminating static problems. The instrument was extremely useful when setting up hanging drops, which would spread out if static was not neutralized on the tape prior to the placement of the drops. Static neutralization helped limit dust particles from being attracted to surfaces during all stages of the experimental setup.

HEL (II) used a microbatch approach to examine the relationship between the level of saturation and the seed-stock dilution on crystallization outcomes. HEL (II) is a batch experiment and levels of supersaturation are not intended to

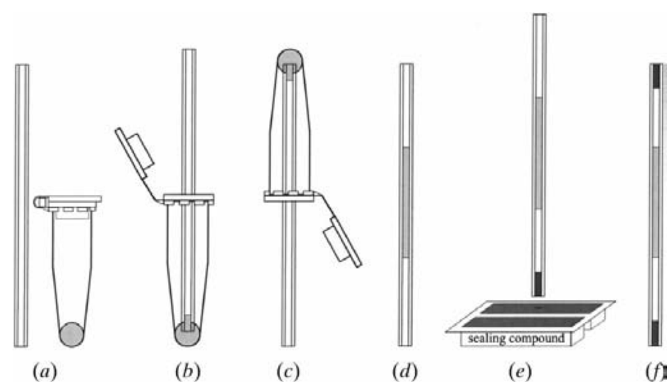

Figure 3
Setting up a capillary microbatch experiment. (*a*) An aliquot of crystallizing solution is placed in a microcentrifuge tube; (*b*) a capillary is placed into the microcentrifuge tube and inserted into the crystallizing solution; (*c*) the entire unit is inverted to allow the liquid to completely load into the capillary; (*d*) the capillary is removed from the microcentrifuge tube and the liquid plug allowed to move slightly away from the end of the capillary; (*e*) one end of the capillary is pressed into Hemato-Seal sealing compound to form a plug seal in the capillary; (*f*) the sealing process is repeated for the other end of the capillary, leaving a plug of sealant in both ends of the capillary.

be identical to the levels of supersaturation used in HEL (I), which were based upon a vapor-diffusion protocol. Results (Fig. 5) are consistent with expected trends. The microbatch experiments examined the growth of crystals at varying levels of supersaturation (s = 2–9). Crystallization occurred at lower levels of supersaturation for the seeded microbatch experiments than for unseeded experiments. The $d1$ seed stock (most concentrated) produced crystals at s = 2. The $d3$ seed stock (100 times dilution of $d1$) produced crystals at s = 5. The $d5$ seed stock (infinite dilution, no seeds added) produced crystals at s = 7. There were fewer crystals present and there was an increase in overall single-crystal dimensions when the concentration of seed stock was decreased. The consistency of results for the seeded experiments was better than the unseeded (infinite dilution) experiment.

4. Concluding remarks

All three methods of pulverizing crystals for use in microseeding were shown to be equally effective under 'ideal solution' circumstances. Visual examination of the mixtures immediately after pulverizing the seed crystals showed them to be optically identical. The freshly pulverized seed mixtures appeared to scintillate during observation under low-power magnification with transmitted light. All three methods produced predicted experimental results for a successful seeding experiment. Results were consistent for seeding experiments. The onset of crystallization was observed at lower levels of supersaturation, more numerous smaller crystals were observed with increasing concentration of seed stock and fewer larger crystals with decreasing concentration of seed stock.

In summarizing the benefits and drawbacks of the three methods, we note the following.

(i) Glass tissue homogenizers are the standard method used to produce microseed stock. They perform effectively and are easy to use. The drawbacks of the method are the expense of the homogenizers, difficulty in cleaning the ground-glass inner surfaces and a relatively short lifetime. If additives which foam are present, great care must be taken to avoid the formation of foam in the crystal-stabilizing solution.

(ii) The vortexed bead method produced suitable microseed stock. The method is inexpensive, reproducible and, because it always uses a new microcentrifuge tube and PTFE bead, eliminates any chance of contamination from past experiments. It would not be a good choice if detergents and/or other chemical additives which may foam are present in the stabi-

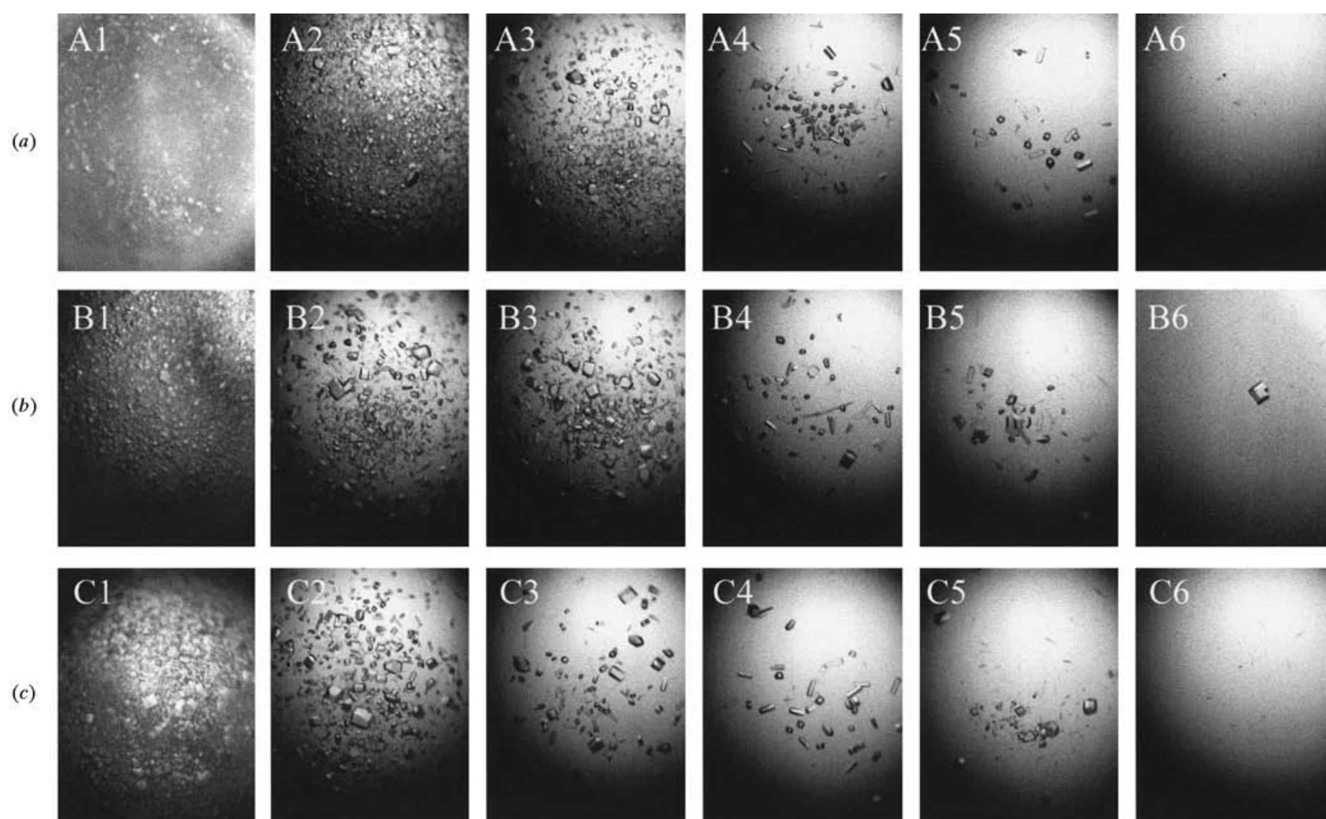

Figure 4
Results from a hanging-drop vapor-diffusion crystallization experiment using HEL solutions starting at s = 1.75. The experiments all initially contained 10 µl drops of 5.1 mg ml^{-1} HEL, 690.7 mM NaCl, 68.5 mM sodium acetate buffer pH 4.6 and were equilibrated against 1 ml reservoirs of 1.050 M NaCl, 105.0 mM sodium acetate at 291 K. Drops in column 1 contain undiluted seed stock. Drops in column 2 contain seed stock diluted 10 times, in column 3 diluted 100 times, in column 4 diluted 1000 times and in column 5 diluted 10000 times. Drops in column six are at infinite dilution of the seed stock. Seed stocks were prepared using (a) glass tissue homogenizer, (b) ultrasonicated bead, (c) vortexed bead. Results show a relative decrease in the number of crystals with decreasing concentration of seed stock.

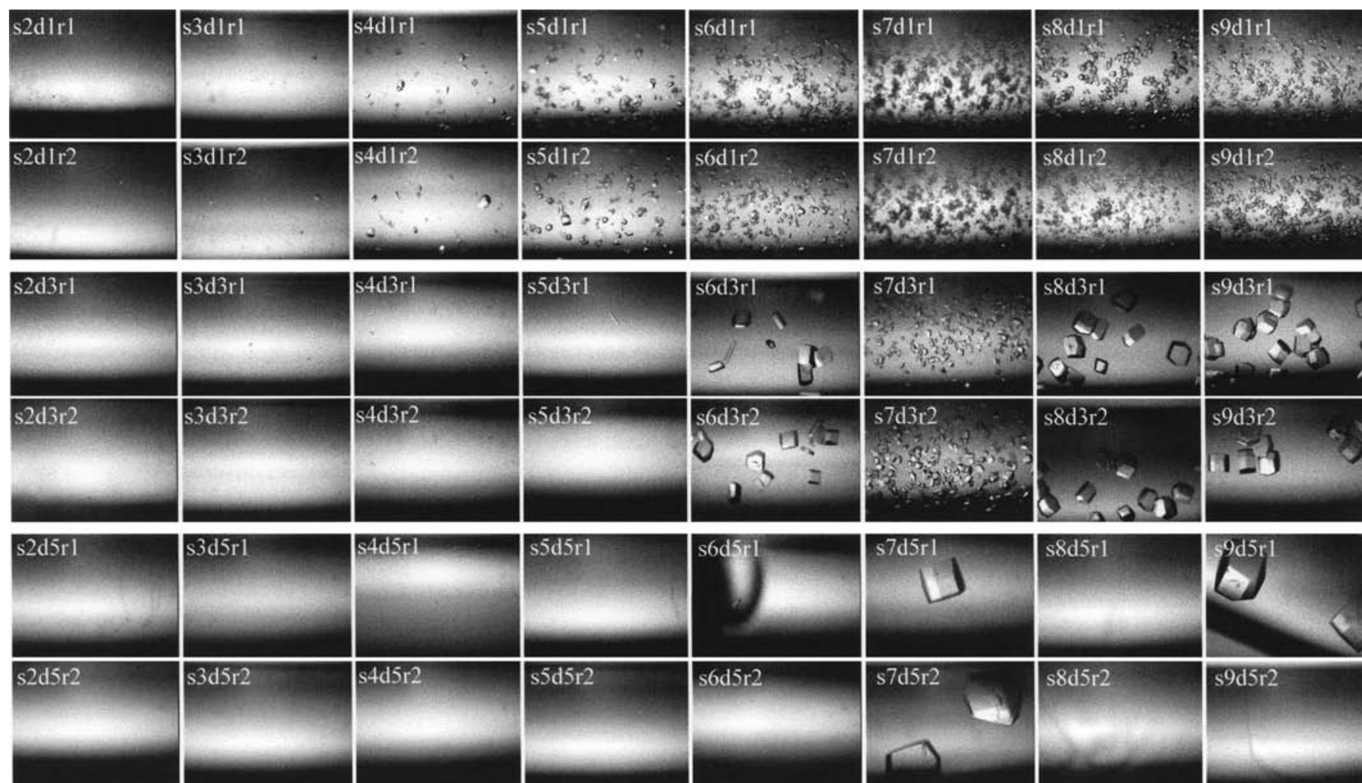

Figure 5
Results of a capillary microbatch crystallization experiment of HEL. Experiments were set up at varying integral levels of supersaturation from 2–9 times saturated (s = 2–9). All seed stock was prepared using an ultrasonicated bead. Three different concentrations of seed stock were used for the experiment, *d*1 (undiluted seed stock), *d*3 (100 times diluted seed stock) and *d*5 (infinitely diluted seed stock). The experiments were set up in duplicate (*r*1, *r*2). Photographs are grouped according to the level of supersaturation. The lowest levels of supersaturation to produce crystals for each concentration of seed stock were consistent with predicted results. The *d*1 seed stock produced crystals at s = 2. The *d*3 seed stock produced crystals at s = 5. The *d*5 seed stock produced crystals at s = 7.

lizing solution. It will not heat the sample, as ultrasonication can do, which could solubilize the seed crystals.

(iii) The ultrasonicated bead produced suitable microseed stock. The method is inexpensive, reproducible and, because it always uses a new microcentrifuge tube and PTFE bead, eliminates any chance of contamination from past experiments. The main drawback of the technique is that the sample will warm up unless short bursts are used during sonication. The method is a good choice if detergents and/or other chemical additives which may foam are present in the stabilizing solution. The method can be used with very small volumes of stabilizing solution and small seed crystals which may be difficult to grind using traditional methods.

This work was supported by NAG8-1152 from NASA.

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