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Random Microseeding: A Theoretical and Practical Exploration of Seed Stability and Seeding Techniques for Successful Protein Crystallization

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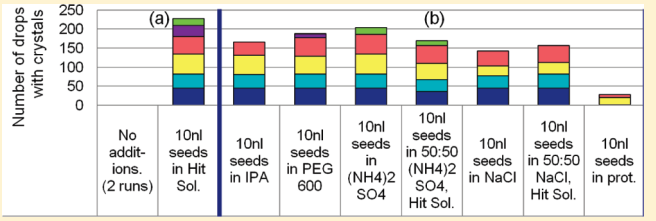
Supporting Information

ABSTRACT: Microseed matrix-screening combined with random screens (rMMS) is a significant recent breakthrough in protein crystallization. In this study, a very reproducible assay for crystal seeds was set up that allowed the following recommendations to be made: (1) the suitability of a solution for suspending seed crystals can be predicted by incubating (uncrushed) crystals in it for one day and observing crystal stability. (2) For routine rMMS, seed crystals should be suspended in the crystallization cocktail that gave the original crystals. (3) Seed crystals can be suspended in PEG or NaCl solutions to reduce the prevalence of salt crystals. (4) Protein complexes can be seeded with seed crystals suspended in PEG. If necessary, seed crystals can also be suspended in the original crystallization cocktail with any individual ingredients that destabilize the complex removed. (5) "Preseeding" of the protein stock should not be used if rMMS is available, because it is less effective. (6) Seed crystals can be harvested from microfluidic devices. (7) Heterogeneous nucleants and cross-seeding are less effective than rMMS, but they can be used if seed crystals cannot be obtained. A theoretical case and practical suggestions are also put forward for producing crystals with different space groups.



INTRODUCTION

Traditionally, microseeding has been used as an optimization step, where seed crystals are transferred into conditions that are similar to previously known crystallization conditions.[1,2] Ireton and Stoddard[3] introduced a novel, more systematic approach, referred to as microseed matrix-screening. This method was automated and further improved by D'Arcy et al.,[4] who were the first to report the use of seeding with random screening kits. Experience has confirmed that "random MMS" (rMMS) not only produces extra hits[4,5] but also generates better-diffracting crystals.[6]

As shown in Figure 1, seeding in screening experiments picks up viable crystallization conditions in the metastable zone that without seeds would be overlooked as clear drops. The metastable zone may be much larger than the labile zone, so many additional hits may be found. Indeed, the labile zone may appear not to exist, although the metastable zone does; see the work of Ireton and Stoddard[3] for an example.

In spite of its effectiveness, several aspects of rMMS are poorly understood. We started this project with the objective of using seeding and other nucleation methods to generate better-diffracting crystals. We had eight specific questions in mind: (1) how can we carry out rMMS in a way that will give the maximum number of crystal hits? (2) How can we produce crystals with different space groups? (3) How can we reduce the number of salt crystals that arise in rMMS experiments? (4) How can we use microseeding for crystallizing protein complexes? (5) How can we stabilize seed stocks? (6) Is it helpful to "preseed" the protein stock? (7) Can we harvest seed crystals from microfluidic devices and capillaries? And, (8) can we encourage crystal nucleation if we have not yet obtained our first crystals?

One of the innovations in D'Arcy's 2007 paper[4] was to suspend seed crystals in the solution that was in the reservoir of the well that the seed crystals were taken from, saving protein and simplifying the procedure. We will refer to this solution as the "Hit Solution". We wanted to investigate the stability of the seed crystals in the Hit Solution and in other precipitants because the seed stock generally contains very little protein. In a typical experiment, the contents of a well with crystals (with a volume of say 0.6 μL) are suspended in 50 μL of Hit Solution. This means that the protein concentration in the seed stock is about 1.2% of the concentration that gave crystals in the original hit, say 0.06 mg/mL. This is likely to be near the point labeled "SHS" in

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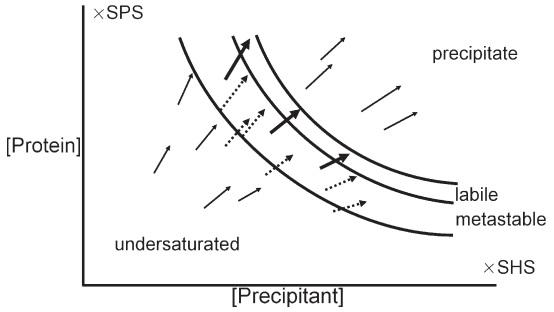

Figure 1. A screening experiment can be thought of as a set of points that land randomly on the phase diagram of a protein. (These "points" are shown as arrows because a vapor diffusion setup is assumed.) This schematic phase diagram[7] has four main areas: an unsaturated zone where drops remain clear and no crystals can grow (labeled "undersaturated"); a zone where precipitation takes place ("precipitate"); a zone where crystal nuclei form and grow into visible crystals ("labile"); finally, there is a zone just below the labile zone, often called the "metastable" zone. Here, crystals do not form spontaneously, but if you take a crystal, for example from the labile zone, and put it into this zone, it will grow. The line that divides the metastable zone from the undersaturated zone is the solubility curve of the protein. In a normal screening experiment, crystals will only appear in wells where the arrows end up in or pass though the labile zone (thick arrows). If, however, you add seed crystals to the screen, you will obtain a set of additional hits where conditions end up in the metastable zone (dashed arrows). SHS and SPS indicate the rough positions of seed stocks made up in Hit Solution and protein stock, respectively (see text).

Figure 1, that is, well below the metastable zone; therefore, the crystals are likely to be thermodynamically unstable and to dissolve. To put it another way, if a protein molecule happens to fall off a microcrystal in the seed stock, it is unlikely that this molecule or any other protein molecule will subsequently attach itself to replace the loss. However, it is common experience that (macro-) crystals that have been harvested into the reservoir solution (containing no protein) may appear to be stable for limited periods. This implies that, as long as the precipitant concentration is relatively high, the rate of dissolution may be so low that it is not normally noticed. A second objective of the project was therefore to find out whether, in the conditions typically used, instability of the seed stock was an important consideration. This was of interest because some groups have reported that the rMMS method does not work in their hands, and we wondered if they were handling their seed stocks inappropriately. Moreover, other groups have reported that seed stocks that are left on the bench for a few hours may become inactive.

Another area of concern was the tendency for rMMS experiments to generate salt crystals, which may waste investigators' time and energy. This is a particular problem when the main precipitant in the Hit Solution is a salt. For example, if a seed stock containing ammonium sulfate is added to all conditions in a typical screen, one would expect to find several wells containing crystals of calcium sulfate (gypsum), because Ca^{2+} is common in crystallization screens. Moreover, Mg, Ca, Zn, and Cd frequently form phosphate crystals. We therefore investigated methods of reducing the number of salt crystals in rMMS experiments.

Radaev and Sun[8] showed that crystallization conditions for protein complexes heavily favor (71% versus 27%) polyethylene glycols rather than ammonium sulfate or other high-salt crystallization conditions. Seed stocks obtained from high-salt conditions may introduce harmful concentrations of salts that can interfere with the crystallization of protein−protein, protein−peptide, and protein−small molecule complexes and with the formation of heave atom derivatives. A method that replaces high-salt precipitants with for example PEG would therefore be helpful.

An obvious disadvantage of the rMMS method is that it can only be used when one has found at least one crystal, in order to make the first seed stock. We had several ideas for tackling this problem: first, we conjectured that seed crystals could be harvested from microfluidic devices. Second, we noted the approach of Habel & Hung,[9] who collected precipitated protein from the wells of unsuccessful screening experiments and added it to random screens. It is likely that, even when crystals are not visible, some of the precipitates obtained are nevertheless crystalline (the crystals may be smaller than the wavelength of light, therefore undetectable with optical microscopes). Third, we were interested in cross-seeding with crystals of unrelated proteins (see Results and Discussion). Fourth, we noted the investigations of Chayen & Saridakis[10] of diverse nonprotein materials for protein nucleation. A nanoporous biocompatible glass ("bioglass") was the most successful of these.[11] This material has cavities that are comparable in size to protein molecules, and it has previously been shown to induce crystallization of at least 14 proteins, several of which could not be crystallized without bioglass.[12] Sugahara et al.[13] proposed the use of synthetic zeolite to induce nucleation. By including these heterogeneous materials in our study, we planned to compare directly their effectiveness with regular microseeding.

We emphasize that we were not seeking to prove here that rMMS microseeding is an effective way to crystallize proteins. This has already been clearly demonstrated by several publications[4−6] and a multitude of PBD entries (e.g., 3CL0, 3CKZ, 3CL2, 3I0M from the National Institute for Medical Research, UK). Instead, we investigated the *relative* effectiveness of different methods of inducing nucleation, with enough data to give good statistics, in order to improve nucleation techniques.

EXPERIMENTAL SECTION

Choice of Test Proteins. Table 1 shows the proteins used in this study. We selected proteins that could be obtained in large quantities and which did not typically crystallize in a large number of conditions in a screen. Other proteins, including lysozyme, concanavalin A, and ferritin, were not used because reliable Receptive Conditions could not be found (see below).

Reagents and Screens Used. The commercial crystallization screens used were Molecular Dimensions' Structure Screen 1 and JCSG+, Jena Bioscience's JBScreen Membrane 3, and Qiagen's PEG Suite. In the experiments of Figures 4 and 8 (Supporting Information) and Table 4, we looked at the effect on crystallization of suspending seed crystals in the following solutions: Hit Sol.; 100% isopropanol ("IPA"); 100% polyethylene glycol, molecular weight 600 ("PEG 600"); 4 M $(NH_4)_2SO_4$; a 50−50 mixture of Hit Sol. and 4 M $(NH_4)_2SO_4$; 5.8 M NaCl; a 50−50 mixture of Hit Sol. and 5.8 M NaCl, and the stock solution of the protein being tested (see Table 1 for concentrations and buffers).

Automation and Volumes Dispensed. We used an Oryx8[14] robot (Douglas Instruments) throughout the study. We dispensed vapor diffusion sitting-drop experiments to 96-well two- or three-drop plates (SwissCI). Drop volumes were 0.3 μL of protein plus 0.3 μL of screen for all nonseeding experiments, while we used 0.3 μL of protein, 0.29 μL of screen and 0.01 μL of seed stock for all seeding experiments except where we used 0.1 μL of seed stock (with 0.3 μL of protein, 0.2 μL of screen) as noted below. Optimization was carried out with the Oryx8 robot using "2-D Grid" designs (see Supporting Information).

Table 1. Proteins Used in This Study

protein	supplier	product code	conc used (mg/mL)	protein buffer	"receptive" conditions found	crystal observation period (days)
glucose isomerase	Hampton Research	HR7-102	33	10 mM tris, 1 mM $MgCl_2$	2	1
hemoglobin (bovine)	Sigma Aldrich	H2500	60	50 mM Na acetate	4	6
thaumatin	Sigma Aldrich	T7638	30	50 mM Na acetate	6	10
thermolysin	Sigma Aldrich	T7902	15	50 mM Na acetate, 14 mM NaOH	6	4
trypsin (porcine)	Sigma Aldrich	T7418	30	2% (w/v) benzamidine	2	3
xylanase	Macro Crystal		36	43% glycerol, 0.2 M phosphate	5	7

Identification of Receptive Conditions. We identified Receptive Conditions (see the definition below Table 2) as follows: first, we set up three plates (two drops per well) with regular screens (i.e., no additive or seeds added) in order to eliminate conditions that crystallized at least once in the absence of seeds. We then set up a plate (again two drops per well) with the addition of crushed seed crystals suspended in IPA and another with seed crystals suspended in 100% PEG 600. We identified Receptive Conditions if (1) none of the six wells that were set up without the addition of seed crystals produced crystals, but (2) three out of the four wells with added seed crystals (suspended in IPA and PEG) produced crystals. Hemoglobin proved to be difficult to crystallize, and microseeding experiments were set up using the conventional method of suspending seed crystals in the Hit Solution, with nine wells per condition. We identified Receptive Conditions if none of the wells in the regular screens contained crystals, but all nine of the seeded wells did. Some proteins crystallized more quickly than others; for each protein, a cutoff period for observation of 1−7 days was used that gave reliable Receptive Conditions (see "Crystal Observation Period" of Table 1). The Receptive Conditions are shown in Table 2.

Identification of Protein Crystals. We used a battery of tests to distinguish protein crystals from salt crystals during the experiments where Receptive Conditions were identified (see above). All crystals grown were well formed with straight edges, with the exception of hemoglobin, where crystals could be identified by color and clarity. We photographed putative crystals of the colorless proteins in a darkroom using UV light (wavelength 280 nm) with a microscope with normal glass optics and a Panasonic DMC-FX12 compact camera, using a 30 s "Starry Sky" setting. UV was provided by a UV Pen-280 (Douglas Instruments) with a 2 mm thick UG11 filter (Schott) to cut out visible light. Crystals that fluoresced with emission in the visible spectrum were identified as protein.[15] When preparing seed stock, we crushed all large crystals with a glass probe, thereby subjecting them to the "crunch test": crystals that produced a click that could be heard and felt were eliminated as salt crystals. The identification of protein crystals was confirmed with brightly colored (Jena Biosciences) and fluorescent[16] dyes, and the cross-linking agent glutaraldehyde (Fluka). Glutaraldehyde in high concentrations turns protein crystals brown or golden (see below).

In the cases of crystals of the proteins concanavalin A, trypsin, and thaumatin, we used an interesting novel method of making the distinction, which is a modification of the method of Pusey et al.[17] We covalently labeled 50 μL aliquots of the proteins with the fluorescent dye DyLight 350 NHS Ester (from Thermo), following the manufacturer's instructions except that we used higher protein concentrations (30 mg/mL for trypsin and concanavalin A, 36 mg/mL for xylanase). We added 20 nL samples of labeled protein to wells containing putative protein crystals *after the crystals had grown*. We photographed crystals in a darkroom by illuminating with the UV Pen-280 or with an FL4BLB UV lamp (Luxina), which has a peak wavelength of 370 nm. As shown in Figure 2, crystals fluoresced brightly and were unambiguously identified as protein rather than salt. (The DyLight kits are very easy to use because all resins, columns, etc. are provided. We chose the label that is excited at 350 nm because it is not necessary to use a filter since most cameras have built-in UV filters.) The advantages of the method are (1) since it allows protein to be seen directly, it does not give false positives or negatives (except when the drop contains a lot of precipitate, see below). (2) It cannot interfere with the crystallization process. (3) Labeled protein need only be prepared if crystal identification by other methods fails; (4) even needles and small crystals can be identified. The method does not work well when the drop contains a lot of protein precipitate, which may absorb the labeled protein before it can reach the crystals. Note also that protein sometimes coats salt crystals in crystallization experiments, giving a superficially similar appearance. Such cases can, however, easily be distinguished by comparing UV images with visible light images because the protein coating is outside the salt crystal.

Once Receptive Conditions were established, we identified protein crystals using the UV Pen-280 only.

Preparation of Seed Stocks. We harvested all seed crystals from sitting drop plates except for xylanase, where we harvested the initial batch of seed crystals from microbatch-under-oil. All seed crystals (after the initial batch) were obtained from the conditions shown in bold and outlined in blue in Table 2 (i.e., crystals from microseeding experiments were used to prepare seed stocks for subsequent rounds of microseeding).

Seed stock suspensions were generated as follows: (1) a Seed Bead[18] (Hampton Research) was placed in an Eppendorf tube on ice. (2) The well containing the crystals to be harvested was opened by cutting the tape, and 20 μL of reservoir solution from the well (or other solution) was placed in the tube. (3) Using a glass probe (with a 0.25 mm bead melted on the end) and observing with a microscope, the crystals were thoroughly crushed in the well. (4) 2 μL of reservoir solution (or other stabilizing solution) was transferred from the tube to the drop with seed crystals, crystals were suspended by withdrawing and dispensing from the pipet tip several times, and the mixture was transferred back to the tube using a slightly higher volume setting on the pipet to ensure all solution was transferred. (5) Step 4 was repeated to ensure that all crystals were picked up. (6) The tube and Seed Bead were vortexed for 2 min, the tube was placed back on ice, the bead was removed, and the stock was used immediately.

Since the Oryx8 crystallization system uses contact dispensing (the tip touches the well during each dispensing operation), it was not necessary to centrifuge the seed stock to remove the larger seed crystals, which may cause blockages in noncontact systems. During experiments, the seed stock is held in the Oryx robot in one channel of the dispensing tip at ambient temperature. When not in use, all seed stocks were kept frozen at −15 °C.

We also prepared a seed stock for cross-seeding with crystals of 15 proteins that were unrelated to any of the test proteins. These proteins (PDB codes) comprised N1 (3CL2) and N2 neuraminidase, a mutant of N1 (3CKZ), erythrocyte binding antigen, H3 hemagglutinin, Ser/Thr protein kinase, polycomb EED protein (3IJC), a post synaptic density protein (2RJI), the flavoprotein soxF, the cell envelope protein MtrF, rabbit hemorrhagic disease virus, and other mutations of these proteins. The seed stock was prepared as follows: (1) crystals of the first protein were crushed in their drops; (2) 3.5 μL of 100% PEG was added to each

Table 2. "Receptive" Crystallization Conditions[a] Identified

Protein	Screen	Solution	Composition
Glucose isomerase	JCSG+	2-2	2 M $(NH_4)_2SO_4$, 0.2 M NaCl, 0.1 M Na MES, PH 6.5
Glucose isomerase[b]	**JCSG+**	**2-43**	**25%(w/v) PEG 3350, 0.2 M $(NH_4)_2SO_4$, 0.1 M bis-tris**
Hemoglobin	JCSG+	2-25	30%(w/v) Jeffamine ED-2001, 0.1 M Na HEPES, PH 7.0
Hemoglobin	**JCSG+**	**2-33**	**30%(w/v) PEG 2000 MME, 0.1 M K thiocyanate**
Hemoglobin	JCSG+	2-34	30%(w/v) PEG 2000 MME, 0.15M K bromide
Hemoglobin	JCSG+	2-44	25%(w/v) PEG 3350, 0.2 M NaCl, 0.1 M bis-tris, PH 5.5
Thaumatin	Structure screen 1	7	30%(w/v) PEG 4K, 0.2 M ammonium acetate, 0.1M Na citrate, PH 5.6
Thaumatin	Structure screen 1	9	20%(v/v) IPA, 20%(w/v) PEG 4K, 0.1 M Na citrate, PH 5.6
Thaumatin	Structure screen 1	14	30%(w/v) PEG 8K, 0.2 M $(NH_4)_2SO_4$, 0.1 M Na cacodylate, PH 6.5
Thaumatin	Structure screen 1	15	20%(w/v) PEG 8K, 0.2M magnesium acetate, 0.1 M Na cacodylate, PH6.5
Thaumatin	Structure screen 1	32	2 M $(NH_4)_2SO_4$, 0.1 M tris, PH 8.5
Thaumatin	**JBScreen Membrane 3**	**D5**	**1.5 M Li2SO4, 0.1 M Na HEPES, PH 7.5**
Thermolysin	JCSG+ (2:1 water)	1-2	20%(w/v) PEG 3K, 0.1 M Na citrate, PH 5.5
Thermolysin	JCSG+ (2:1 water)	1-21	20%(w/v) PEG 6k, 0.1 M citric acid, PH 5.0
Thermolysin	JCSG+ (2:1 water)	2-18	10%(v/v) MPD, 0.1 M bicine, PH 9.0
Thermolysin	**JCSG+ (2:1 water)**	**2-19**	**0.8 M succinic acid, PH 7.0**
Thermolysin	JCSG+ (2:1 water)	2-21	2.4 M Na malonate, PH 7.0
Thermolysin	JCSG+ (2:1 water)	2-22	0.5%(w/v) Jeffamine ED-2001, 1.1 M Na malonate, 0.1 M Na HEPES, PH 7.0
Trypsin	**JBScreen Membrane 3**	**D3**	**1.5 M NaCl, 0.1M Na acetate, PH 4.6**
Trypsin[c]	JBScreen Membrane 3	D3	1.5 M NaCl, 0.1M Na acetate, PH 4.6
Trypsin	JBScreen Membrane 3	D6	2 M NaCl, 0.1 M Na citrate
Trypsin	JBScreen Membrane 3	D6	2 M NaCl, 0.1 M Na citrate
Xylanase	Structure screen 1	32	2 M $(NH_4)_2SO_4$, 0.1 M tris, PH 8.5
Xylanase	Structure screen 1	37	30%(w/v) PEG 4K, 0.2 M Na acetate, 0.1 M tris, PH 8.5
Xylanase	**Structure screen 1**	**45**	**4 M Na formate**
Xylanase	JBScreen Membrane 3	B5	3.5 M $(NH_4)_2SO_4$, 0.25M NaCl, 50mM Na/K phosphate, PH 7.5
Xylanase	JBScreen Membrane 3	D4	1.5 M K phosphate, PH 7.0

[a] Receptive Conditions were defined as conditions that do not give crystals without the addition of seeds but generally give crystals when seed stock is added (see text). [b] For each protein, the conditions shown in **bold** and outlined in blue were used to provide seed crystals for the experiments of Figures 4, 6, 7, and 8 (Supporting Information) and Table 4. They are also the Hit Solutions used to suspend the seed crystals. [c] The two conditions identified for trypsin were repeated.

drop, then aspirated and transferred to an Eppendorf tube on ice; (3) step 2 was repeated twice more; (4) steps 1−3 were repeated for all 15 proteins in turn (pooling the seed stock), resulting in about 150 μL of seed stock in a single tube; (5) the seed stock was vortexed with a seed bead.

Quantification of Crystal Seeding Activity. In each round of microseeding and nucleation experiments described below (see Figures 4, 6, 7, and 8 (Supporting Information) and Table 4), we set up nine crystallization drops for each Receptive Condition, adding the seed stock to be tested to each drop. At the end of the Crystal Observation Period, we recorded the number of wells that contained at least one crystal, and we estimated the average number of crystals per well.

Direct Observation of Crystal Stability in Various Solutions. We investigated the stability of crystals in Hit Solution, IPA, PEG, $(NH_4)_2SO_4$, NaCl, and the protein stock by direct visual observation as follows: (1) crystals were grown in sitting drops using seed stock made with Hit Solution; (2) crystals were photographed; (3) the mother liquor was wicked away from drops using the corner of a laboratory wipe,

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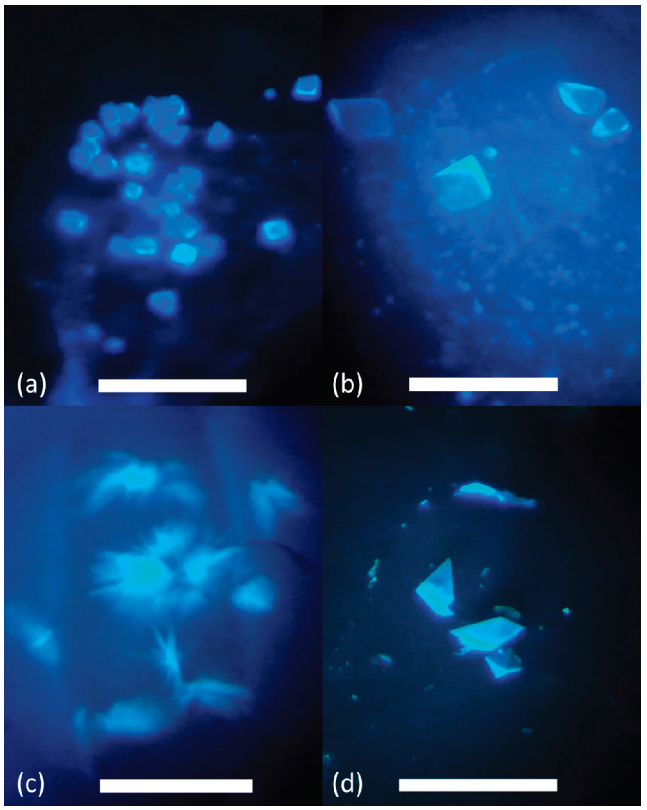


Figure 2. An unambiguous method of distinguishing protein crystals from salt crystals. A 20 nL sample of protein that has been covalently labeled with a fluorescent dye is added to wells *after* crystals have grown. The labeled protein is absorbed into the surface layers of protein crystals, which fluoresce brightly when illuminated with UV light (around 370 nm), while salt crystals do not fluoresce. Three examples are shown: (a) concanavalin A, (b) thaumatin, (c) trypsin. In (a) and (b), the label is clearly localized in the outer layers. For comparison, the conventional approach is shown, (d), where crystals of unlabeled proteins that contain tryptophan and other aromatic residues (here thaumatin) fluoresce uniformly when illuminated at 280 nm. Note that (without label) most proteins do not fluoresce nearly as brightly as thaumatin. The sharper focus of (d) is not significant. Scale bars are 0.5 mm in length. Camera exposure times: (a−c), 15 s; (d) 60 s.

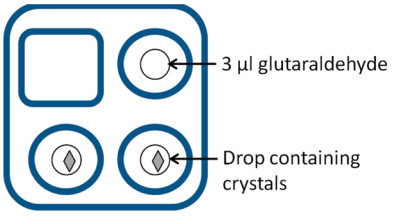


Figure 3. The physical layout used for cross-linking seed crystals with 0.08% glutaraldehye. Crystals were crushed, then glutaraldehyde was diffused into the drops containing crystals.

leaving intact crystals in the wells; (4) 10 μL of the solution to be tested was added to each drop, wicked away, then a further 10 μL was added; (5) crystals were observed at high magnification and photographed after 1 day (as well as after 1 min and 1 h for comparison). (6) After 1 day, one of five possible outcomes was recorded for each well: "OK" meant that crystals remained unchanged, "grew" indicated that crystals retained sharp corners and increased in size, "cracked" crystals retained their general outline but cracks became visible and crystals often became opaque, "shattered" crystals first cracked then fell apart, while "dissolved" meant that crystals disappeared (although sometimes precipitate was visible in their previous positions).

Preparation of Cross-Linked Seed Stocks. We produced cross-linked seed stocks essentially by the method of Lusty.[19] We used 3-Well Crystallization Plates by SWISSCI for cross-linking as shown in Figure 3, growing crystals of glucose isomerase, hemoglobin, or trypsin in two wells of each chamber. After growth, we crushed crystals thoroughly within the drops using a glass probe, and then added 3 μL of 0.08% glutaraldehyde (Fluka) in water to the unused well (Figure 3). **Note that goggles, protective clothing, and gloves should be used when handling glutaraldehyde.** (We tested 0.8%, 0.08%, and 0.008% solutions. The seed stocks produced all worked equally well. However, 0.8% glutaraldehyde caused some crystals to turn slightly yellow or brown. Lusty reported that crystals that turned brown or yellow were cross-linked too much and did not diffract as well as clear crystals. We therefore used 0.08% for our experiments.). We resealed the plate with tape for 90 min allowing the glutaraldehyde to diffuse into the drops containing crushed crystals. We withdrew 10 μL of Hit Solution from the crystallization reservoir and put it in an Eppendorf tube on ice, and then followed the procedure above for the Preparation of Seed Stocks.

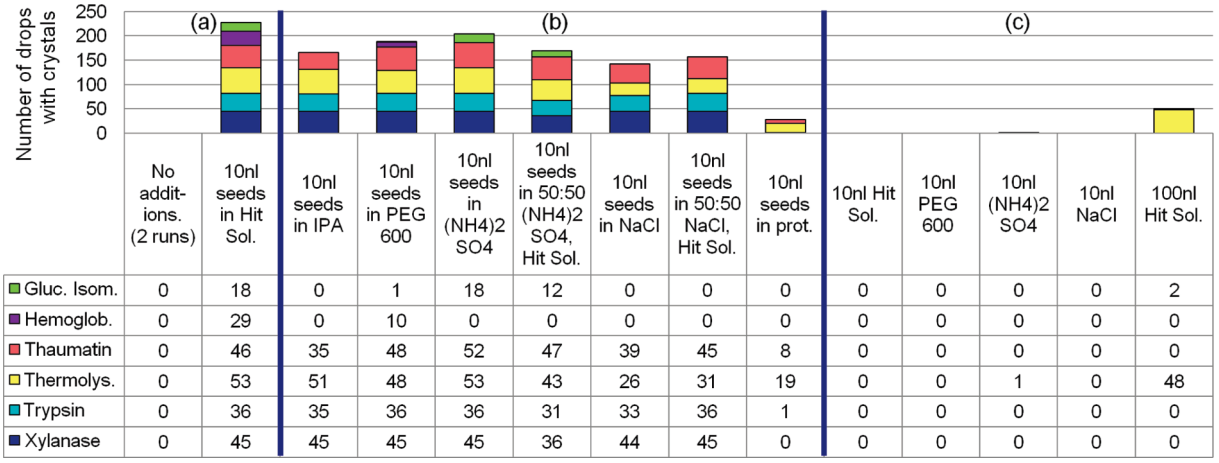
Preparation of Seed Stocks from Microfluidic Devices. We grew seed crystals of all six proteins in the Crystal Former HT 96-channel device, by Microlytic North America Inc., and in the Counter-Diffusion Screening Kit[20] (24 conditions with 0.2 mm ID capillaries), by Triana Science and Technology. We also grew seed crystals of glucose isomerase in a Topaz 1.96 diffraction-capable chip[21] from Fluidigm Corporation. We grew crystals without seeding using conditions that had previously been found to give crystals in all cases except for hemoglobin and xylanase, where seed stocks were added.

We harvested crystals grown in Crystal Former chips by removing the sealing film on the back of the chip under a microscope, crushing the crystals in the chip with a probe, flushing the crushed crystals with 5 μL of Hit Solution, and transferring the resulting crystal mixture to an Eppendorf tube on ice. We repeated the last step, giving 10 μL of seed stock, which we vortexed without a Seed Bead for 2 min.

In order to harvest crystals grown in capillaries, we scratched each capillary with a glass cutter about 20 mm from the end that was blocked with putty and carefully snapped it off. We placed a 5 μL drop of the Hit Solution onto a glass slide and pushed the crystals out of the capillary with a fine wire (provided by the Hamilton Company for unblocking syringe needles) into the drop. We then crushed the crystals with a glass probe and transferred them to an Eppendorf tube on ice. We rinsed the slide with a further 5 μL, giving 10 μL of seed stock, which we vortexed without a Seed Bead for 2 min.

Before harvesting crystals grown in the Topaz chips, we released the pressure in the protein and containment lines by cutting along the center (protein) line of the chamber containing crystals with a scalpel. We made two further parallel cuts, through the top layer of the silicone elastomer only, on either side of the chamber containing crystals by following the dashed lines marked on the plate. These cuts joined the first cut, allowing a rectangular flap of elastomer to be peeled back by separating the top and bottom layers. This exposed the crystals, which we crushed with a probe, flushed with 4 μL of Hit Solution, and extracted with a pipet. We transferred the mixture to a tube on ice and repeated the flushing and transfer to ensure that no crystals were lost. We then agitated the solution with a probe to break up the crystals further.

Heterogeneous Nucleation. We used two heterogeneous nucleants: bioglass ("Naomi's Nucleant") from Molecular Dimensions Ltd., and synthetic zeolite (Molecular Sieves 5A 1/16) from Wako Pure Chemical Industries Ltd. Both materials were used in two ways: first, we transferred one particle (about 0.25 mm) to each well of a crystallization plate using



	No addit-ions. (2 runs)	10nl seeds in Hit Sol.	10nl seeds in IPA	10nl seeds in PEG 600	10nl seeds in (NH4)2 SO4	10nl seeds in 50:50 (NH4)2 SO4, Hit Sol.	10nl seeds in NaCl	10nl seeds in 50:50 NaCl, Hit Sol.	10nl seeds in prot.	10nl Hit Sol.	10nl PEG 600	10nl (NH4)2 SO4	10nl NaCl	100nl Hit Sol.
Gluc. Isom.	0	18	0	1	18	12	0	0	0	0	0	0	0	2
Hemoglob.	0	29	0	10	0	0	0	0	0	0	0	0	0	0
Thaumatin	0	46	35	48	52	47	39	45	8	0	0	0	0	0
Thermolys.	0	53	51	48	53	43	26	31	19	0	0	1	0	48
Trypsin	0	36	35	36	36	31	33	36	1	0	0	0	0	0
Xylanase	0	45	45	45	45	36	44	45	0	0	0	0	0	0

Figure 4. A comparison of the number of wells with crystals that were obtained using various seed stocks. In each run we set up a total of 243 wells using 6 test proteins in "Receptive Conditions" (see text and footnote to Table 2). Hit Sol. indicates the solution that was in the reservoir of the well that the seed crystals were taken from. (a) The first two columns confirm that Receptive Conditions had indeed been identified. (b) The next seven columns show results when we added 10 nL of seed stock, suspending the crushed seed crystals in the solutions shown. (c) The remaining five columns show control experiments where solutions without suspended seeds were added. See Reagents and Screens Used, above, for the concentrations of solutions used.

Table 3. Observations of the Crystals' Stability in Various Solutions

Protein	Crystals in Hit Sol.	Crystals in IPA	Crystals in PEG 600	Crystals in $(NH_4)_2SO_4$	Crystals in NaCl	Crystals in protein stock
Gluc. Isom.	**OK**[a]	Cracked	Shattered	**Cracked**	Dissolved	Dissolved
Hemoglobin	**OK**	Cracked	**OK**	Dissolved	Dissolved	Dissolved
Thaumatin	**OK**	**Cracked**	**OK**	**OK**	**OK**	**Grew**
Thermolysin	**OK**	**OK**	**Shattered**	**OK**	**Dissolved**	**Grew**
Trypsin	**OK**	**OK**	**Dissolved**	**OK**	**OK**	Dissolved
Xylanase	**OK**	**OK**	**Cracked**	**OK**	**OK**	Dissolved

[a] Mother liquor was removed and crystals were incubated for one day before observation. See Reagents and Screens Used, above, for the concentrations of solutions used. Combinations that correspond to seed stocks that gave crystals in at least two wells in Figure 4 are shown in bold and outlined in blue. "OK" means that crystals remained unchanged.

forceps. This was a labor-intensive operation, but it was made easier when we reduced the static on the plates by placing them in a humid environment at 4 °C. Second, we ground both types of particles on a glass plate with a glass pestle (the flange of a Hamilton syringe) as finely as possible and suspended the resulting powder in 50 μL of 100% PEG 600. We added 10 nL of this suspension to crystallization experiments as usual.

Collection of Precipitate for Seeding. For each protein, we set up regular crystallization screens (0.3 + 0.3 μL) and identified drops that contained precipitate but no visible crystals. We added 2 μL of 100% PEG 600 to each drop identified, mixed the PEG with the precipitate, withdrew 2 μL of the mixture and transferred it to an Eppendorf tube. Eventually, we obtained six tubes, each containing approximately 10−20 μL of mixed precipitates of one of the six proteins. In the cases of trypsin and thermolysin, very few or no wells contained precipitate. We therefore set up experiments with a high concentration of these proteins (60 mg/mL) and left the plates on the bench for about 20 min without sealing them with tape. When the wells became cloudy, we collected precipitate as described above. We set up microseeding experiments with Receptive Conditions using these seed stocks, attempting to nucleate each protein with mixed precipitates of the same protein. Each experiment (plate) required less than 2 μL of the suspension.

RESULTS AND DISCUSSION

Comments on Our Experimental Approach. To increase the statistical significance of our results, we identified and focused on "Receptive Conditions". We defined these as conditions that consistently gave crystals in the presence of seed crystals, but consistently failed to support crystallization without seed crystals. By screening six proteins with and without the addition of seed stocks, we identified 25 Receptive Conditions (shown in Table 2). Note that the Receptive Conditions are a subset of the metastable conditions that may have been present: first, some metastable conditions may not have given crystals because the seed crystals added were incompatible. Second, some metastable conditions gave inconsistent results and were excluded.

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We set up 34 runs in a single side-by-side experiment, varying only the seed stock or nucleant. (Over 15 000 drops were set up and their results were recorded.) This gave very reproducible results and allowed us to make direct comparisons of the various seeding techniques.

The salt solutions $(NH_4)_2SO_4$ and NaCl were used to suspend seed crystals because data mining of the PDB[22] showed that these are the two most popular salt precipitants in successful crystallization experiments. We used PEG because it is the most popular organic precipitant, using a molecular weight of 600 for convenience (it is a liquid at room temperature). We selected IPA as a popular low-molecular weight organic precipitant.

Suspension of Seed Crystals in Alternative Solutions. Figure 4b shows that seed crystals can be suspended in a variety of high concentration precipitants. For example, thaumatin seed stocks were more effective when suspended in PEG and $(NH_4)_2SO_4$ than in Hit Solution. Overall, however, the Hit Solution was the most effective.

We included the 50:50 mixtures of Hit Solution and concentrated precipitants with the idea that this would be a good compromise because the Hit Solution might stabilize crystal contacts while the high precipitant concentrations might minimize dissolution. However, these solutions did not give improved seeding in practice.

There was some interesting variation in the behavior of the seed crystals of the different proteins. The seed crystals that were grown in ionic precipitants (thaumatin, thermolysin, trypsin, and xylanase) were effective when suspended in all solutions tested except the corresponding protein stock. Once formed, these crystals survived even when in contact with a variety of ions and precipitants.

In contrast, crystals of the two proteins where the main precipitant was PEG were sensitive to the presence or absence of particular solutes. Seed crystals of glucose isomerase were grown in PEG 3350 supplemented with 0.2 M $(NH_4)_2SO_4$, and all seed stocks that contained $(NH_4)_2SO_4$ worked for this protein, while all others were inactive or gave only one hit. Hemoglobin crystals were grown in PEG 2000 MME, and both the seed stocks containing PEG 600 worked, while all others were inactive. We conclude that in some cases the solution for suspension must contain a particular precipitant or additive.

Predicting the Effectiveness of Seed Crystals. It is instructive to compare the data of Figure 4b with Table 3, which shows observations under the microscope of the stability of crystals in various solutions. When it was observed that crystals either grew or were stable, they could in all cases be used to make active seed stocks with the solution in question (the 20 cases marked "OK" or "grew"). When, however, crystals cracked, shattered, or dissolved (16 cases), predictions could not be made: in roughly 60% of cases the resulting seed stocks were inactive, but in 40% of cases they were active in spite of the crystals' visible deterioration.

Preseeding the Protein Stock. Figure 4b shows one other important experiment. Seed crystals are sometimes crushed and mixed with the protein stock solution in routine crystallization screens. This approach ("pre-seeding the protein stock") avoids the need for special hardware and software to handle the seed stock separately in automated experiments. As indicated by the last column of Figure 4b, this method generated some extra crystals in our hands, but it was far less successful than the other seed solutions used, completely failing with three of the six proteins tested. It worked best with thaumatin and thermolysin. Table 3 shows that crystals of these two proteins grew when they were

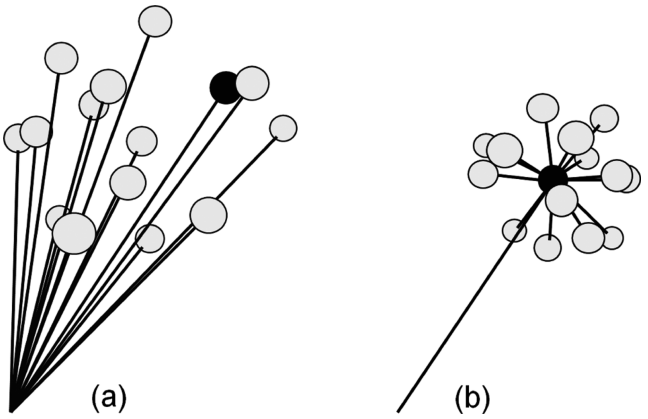
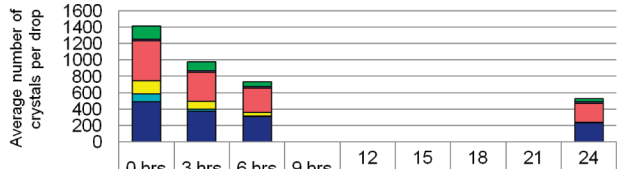


Figure 5. (a) A screening experiment can be thought of as a cloud of points that land randomly in the multidimensional crystallization space (3D representation). (b) A (random) additive experiment can be thought of as a smaller cloud of points around a center point. If the points are placed around a condition that has already yielded crystals — indicated by the black sphere — the chance of obtaining crystals, especially well-formed crystals, is increased. The cost, however, is that a smaller region of crystallization space is sampled in the additive experiment.



	0 hrs	3 hrs	6 hrs	9 hrs	12 hrs	15 hrs	18 hrs	21 hrs	24 hrs
Gluc. isom.	166	113	63						36
Hemoglob.	19	18	19						20
Thaumatin	489	357	293						228
Thermolysin	158	96	46						11
Trypsin	95	23	6.0						0.1
Xylanase	490	372	309						229

Figure 6. The stability of seed stocks in Hit (reservoir) Solution was investigated over various periods, using the same set of Receptive Conditions (Table 2).

soaked in protein solution (a surprising and interesting result), so one would expect these seed stocks to be stable. A seed stock where crystals are suspended in the protein stock would be near the point marked "SPS" in Figure 1. One might expect this point to be outside the metastable zone because very little precipitant is present. These two proteins seem to be unusual in that the metastable zone extends further to the left (lower precipitant) than is usual.

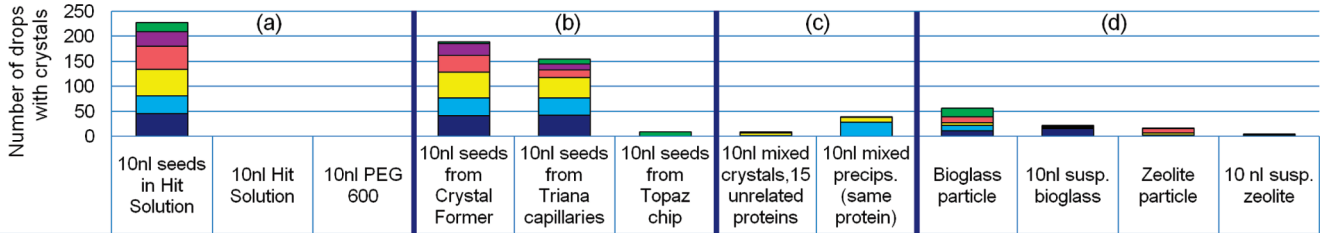
Figure 4c shows additional "control" experiments that confirm that the solutions used to suspend the seed stocks did not themselves cause significant crystallization. Note however that larger volumes (100 nL) of Hit Solution did cause nucleation by an additive effect as shown in the last column, especially in the case of thermolysin. Since adding 10 nL of Hit Solution gave no crystals, these results confirm that reducing the volume of seed stock added can reduce the additive effect.

Attempts to Increase the Diversity of Crystals. Typically, one-third of the precipitant in each well of an rMMS experiment comes from the seed stock.[4] This increases the likelihood of producing crystals that are similar to the seed crystals, because a

Table 4. Microseeding Experiments Carried out with Cross-Linked Crystals

protein	seeds in Hit Sol[b]	seeds in PEG 600	X-linked seeds in PEG, used immediately	X-linked seeds in PEG, 1 wk 20 °C	seeds in NaCl	X-linked seeds in NaCl, used immediately	X-linked seeds in NaCl, 1 wk 20 °C
gluc. isom.	18[a]	1	9	9	0	9	9
hemoglobin	10	10	7	0	0	0	0
trypsin	36	36	36	13	33	36	25

[a] The number of wells where crystals grew is shown. Seed crystals were crushed, cross-linked in their wells (where shown), and suspended in the solution shown. [b] Data from Figure 4 are included in columns 1, 2, and 5 for comparison.



	10nl seeds in Hit Solution	10nl Hit Solution	10nl PEG 600	10nl seeds from Crystal Former	10nl seeds from Triana capillaries	10nl seeds from Topaz chip	10nl mixed crystals,15 unrelated proteins	10nl mixed precips. (same protein)	Bioglass particle	10nl susp. bioglass	Zeolite particle	10 nl susp. zeolite
Gluc. Isom.	18	0	0	3	10	9	0	0	17	0	1	2
Hemoglob.	29	0	0	24	12		0	0	0	0	0	0
Thaumatin	46	0	0	33	15		2	1	12	3	8	0
Thermolysin	53	0	0	51	40		6	10	5	2	5	2
Trypsin	36	0	0	36	35		0	28	11	2	0	0
Xylanase	45	0	0	41	42		1	0	11	15	2	0

Figure 7. Using seed stocks from unconventional sources and heterogeneous nucleation. Experiments again used Receptive Conditions (Table 2). (a) Data from Figure 4 are shown for comparison. (b) Seed stocks were harvested (using Hit Solution) from a microfluidic device, the Crystal Former by Microlytic, and from capillaries from a crystal screening kit by Triana. Glucose isomerase crystals harvested from the Topaz chip by Fluidigm were also used. (c) A seed stock was used where crushed crystals of 15 unrelated proteins were suspended in 100% PEG 600, and seed stocks were prepared by harvesting precipitates (containing no visible crystals) from crystallization screens. (d) Heterogenous nucleation was carried out with Bioglass and with synthetic zeolite. Both materials were used both as particles (approximately 0.25 mm) and in suspension.

smaller volume of crystallization space is searched (Figure 5). We attempted to show that crystals with different space groups could be obtained by reducing the volume of the seed stock added and suspending seed crystals in PEG. We were, however, unable to demonstrate a beneficial effect with our six test proteins. This is discussed in the Supporting Information to this paper.

The Stability of Seed Stocks. As noted above, we had received reports that seed stocks sometimes become inactive when they are kept at room temperature for a few hours. In order to investigate the stability of seeds further, we next incubated seed stocks in Hit Solution at 20 °C for various periods. We set up experiments as soon as each seed stock was made, and after 3, 6, and 24 h (Figure 6). The hemoglobin seed stock was completely stable, with no decrease in the number of crystals. At the other extreme, the trypsin seed stock was unstable, with the number of crystals decreasing to 0.1% of the starting number. The number of crystals for all proteins taken together decreased to 38% of the starting number. Note that the rate of loss of seed crystals in the first 3 h was greater than in later periods (the histogram in Figure 6 is concave). Our interpretation of this profile is that the seed crystals have a variety of sizes and that there are more small seed crystals than larger ones. During early times, the small crystals are lost relatively quickly. Later, protein molecules may be leaving the surfaces of crystals at the same rate, but the rate of loss of nuclei is lower because the remaining crystals are larger. This effect may mislead crystallizers into believing that their seed stocks are stable when in reality only a small percentage of the original seeds remain after storage.

The Supporting Information shows another set of experiments where the stability of seed stocks was tested in a variety of solutions.

Cross-Linking Seed Crystals. One way to increase the stability of protein crystals is to cross-link them. Cross-linking is easily accomplished by diffusing glutaraldehyde into seed stocks. As shown in Table 4, our three test proteins gave different results. Glucose isomerase seed crystals retained their activity in PEG and NaCl only when cross-linked, remaining active for at least a week. This confirmed the utility of the approach. Cross-linking did not improve stability with hemoglobin, while trypsin seed crystals, in spite of cross-linking, lost their activity slowly in both PEG and NaCl. Clearly cross-linking helps in some cases, but not all. In these examples and several others (data not shown), however, we only came across one case where cross-linking significantly reduced nucleation activity.

Seed Crystals from Microfluidic Devices. The next stage of the project (Figure 7) looked at several options that can be tried when crystals cannot be obtained by conventional methods.

Microfluidic devices often produce crystals when other methods fail, but it can be difficult to translate the conditions to, for example, vapor diffusion or microbatch-under-oil setups.[23] Harvesting seed crystals and using them for rMMS experiments is a way around this difficulty.

We harvested seed stocks from the Crystal Former by Microlytic, a capillary-based system by Triana, and the Topaz system by Fluidigm and tested the resulting seed stocks (Figure 7b). The seed stocks from all three systems (columns 4−6) were nearly as successful as the regular seed stocks taken from sitting drop

experiments (column 1). In all cases crystals were grown. The first two are clearly useful sources of seed crystals. Since only one experiment was carried out with the Topaz chip this result is less easy to interpret, but apparently it also works well.

Cross-Seeding and Seeding with Precipitates. We also investigated crystal nucleation with mixtures of crushed crystals of unrelated proteins. Previous experiments have shown that, for example, crushed lysozyme crystals are generally ineffective in inducing crystallization of unrelated target proteins. However, crystals of mutated proteins and homologues from different species have occasionally been used to nucleate target proteins. For example, Eichele et al.[24] were able to crystallize pig mitochondrial aspartate aminotransferase by adding seed crystals of the equivalent chicken mitochondrial enzyme. Moreover, seed crystals with one crystal form can sometimes nucleate crystals with different crystal forms. Stura et al.[25] reported four examples of such "epitaxial jumps" (including both homoepitaxy and heteroepitaxy). These jumps were instigated by varying the crystallization conditions or by cross-seeding with crystals of related proteins or complexes. In all four cases the unit cell parameters changed dramatically, and in all but one case the space group changed. In another example, Obmolova et al.[6] were able to crystallize an antibody/antigen complex using seed crystals of the antigen complexed with an antibody that differed in 40 residue positions. Crystals with a different space group were produced. These examples suggest that an exact match of crystal unit cells is not required for effective nucleation. This should not be surprising when one considers that each face of a seed crystal presents a different two-dimensional (2D) array of repeating molecular structures. For effective nucleation, there does not have to be a match of the three-dimensional structures, only an (approximate) match between a 2D array on one surface of the seed crystal and a 2D array associated with one of the structural planes of the crystal being nucleated.[25] We therefore reasoned that if crystals of many proteins were added to a crystallization trial there was a reasonable chance that a seed crystal with an appropriate crystal structure could nucleate a crystal of the target protein. We were aware that a very sensitive and low-noise system might be required to detect the effect.

Column 7 of Figure 7 shows that a mixture of the crushed crystals of 15 proteins gave crystals of two (maybe three) of the test proteins. This was much less effective than homogeneous seeding, but the intriguing possibility exists that the success rate may increase with the number of different proteins' crystals included in the stock. A mixture of seed crystals of, say, 500 proteins could be as effective as regular rMMS seeding.

Suspended precipitates harvested from wells with no visible crystals (column 8) initiated crystal growth of at least two of our test proteins.

Heterogeneous Nucleation. Another option for cases where no seed crystals are available is the use of heterogeneous nucleants (Figure 7d). We used bioglass and synthetic zeolite, testing both materials in two ways: as particles and in suspension in 100% PEG 600. The most successful option was the bioglass particles (column 9), which worked with five of the six proteins tested. The zeolite particles crystallized four of the six proteins. Note, however, that zeolites can be used for dehydration, rather like silica gel. The crystallization in those drops may therefore have been caused by dehydration rather than nucleation on the surface of the zeolite. (We noted that several drops with large lumps of zeolite had crystals, whereas drops with many small particles did not although the surface areas were comparable.)

Using the materials as particles was a much more labor-intensive option than using them as suspensions (which were dispensed automatically), but the particles were in both cases much more effective than the suspensions.

CONCLUSIONS

Our extensive investigations have enabled us to answer the following questions that we had in mind at the start of the project.

1. How can we carry out rMMS microseeding in a way that will give the maximum number of crystal hits? We found that the Hit Solution (the solution in the reservoir of the hit that was used to make the seed stock) is generally the most effective for suspending seed crystals. Moreover, our results show that some seed stocks will become inactive if the composition of the solution that the seed crystals are suspended in changes. We therefore recommend suspending seed crystals in Hit Solution for routine rMMS.

2. How can we produce crystals with different space groups? Consideration of the volume of "crystallization space" occupied by random screens and by additive experiments suggests that reducing the volume of seed stock added and suspending seeds in "neutral" precipitants such as PEG will increase the likelihood of producing crystals with different crystal contacts and space groups (see Supporting Information). We were, however, unable to demonstrate this.

3. How can we reduce the number of salt crystals that arise in rMMS experiments? rMMS experiments where the seed crystals are suspended in Hit Solutions that contain significant concentrations of ions may well produce salt crystals when combined with random screens. This tendency can be reduced by suspending seed crystals in, for example, NaCl (since most sodium and chloride salts are soluble) or PEG.

The stability of uncrushed crystals can be tested by incubating them in various precipitant solutions before making a selection. If crystals remain unchanged after one day, then it is likely that an effective seed stock can be made using the solution in question.

4. How can we use microseeding for crystallizing protein complexes? An obvious strategy for nucleating crystals of a protein complex is to use seed crystals of the corresponding apoprotein in rMMS experiments. The crystallization of complexes is, however, favored by low-salt conditions.[7] Where possible, therefore, seed stocks for protein complexes should be made up in organic precipitants such as PEG. (Although we used PEG 600, higher molecular weight PEGs such as PEG 3000 may be preferable because Stura et al. have shown that low molecular weight PEG is more likely to interact specifically with protein.[25])

Moreover, it may be helpful to test the individual ingredients of the Hit Solution to see if any of them destabilize the complex. Here biophysical methods such as isothermal titration calorimetry, fluorescence anisotropy titration, and dynamic light scattering[26] can be used. Bear in mind that even "low-salt" conditions may contain ions (including carboxylic acids, buffers and multivalent ions) and low molecular weight organics (including alcohols) that may competitively bind to the protein, disrupting the complex. Before use, the solutions can be tested as described above by visual observation of crystal stability for one day. The objective here is to find *a solution that stabilizes seed crystals but does not disrupt the target complex.*

5. How can we stabilize seed stocks? Some seed stocks are unstable, such as our trypsin stock, which lost 99.8% of its activity

after storage at room temperature for 24 h. In such cases, crystallizers may however observe some seeding activity and conclude that their seed stocks still "work", incorrectly assuming that they have not deteriorated. D'Arcy[4] showed that seed stocks generally withstand several cycles of freezing and thawing. We recommend freezing seed stocks immediately after use. (Freezing several small aliquots of the seed stock avoids multiple freeze−thaw cycles when the stock is used on different occasions. Making a series of diluted seed stocks when the seed stock is generated is also helpful; the diluted seed stocks can be used later if many small crystals grow.) In addition, seed crystals can usually be cross-linked without significant loss of activity although cross-linking only protected seed crystals in one of three cases.

An alternative approach guarantees the stability of seed stocks. When plenty of seed crystals are available, crushed crystals can be harvested in their own mother liquor from several large drops to make a seed stock without the addition of any other solution. Here it is helpful to use a drop-setter that can dispense very small volumes of seed stock (down to say 10 nL) without significant wastage.

6. Is it helpful to "preseed" the protein stock? Adding crushed seeds to the protein stock may be beneficial in some cases, but in general it is much less effective than adding a separate seed stock. Again, a visual check of stability is helpful.

7. Can we harvest seed crystals from microfluidic devices and capillaries? We have shown that seed crystals harvested from these devices work well in microseeding experiments.

8. Can we encourage crystal nucleation if we have not yet obtained our first crystals? Mixtures of precipitates harvested from unsuccessful crystallization conditions gave extra crystals of three of the proteins that we tested. This method may be useful, for example, when granular precipitates are observed. In a separate approach, mixtures of crushed crystals of unrelated proteins also gave extra crystals of at least two of the proteins that we tested. We hope that increasing the number of different proteins in the seed stock may increase the success rate.

Crystals of five of our six test proteins could also be nucleated by adding bioglass particles (zeolite was less effective in our hands). This method appears to be helpful, but it is labor-intensive. An easier alternative is to crush bioglass, suspend it in PEG, and dispense it automatically. This method is less effective but it can be used routinely more easily.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. A discussion of rMMS and additive experiments, and of approaches to producing crystals with different space groups; additional data and further analysis of the stability of seed stocks in a variety of stabilizing solutions. This material is available free of charge via the Internet at http://pubs.acs.org.

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