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A Straightforward and Robust Method for Introducing Human Hair as a Nucleant into High Throughput Crystallization Trials

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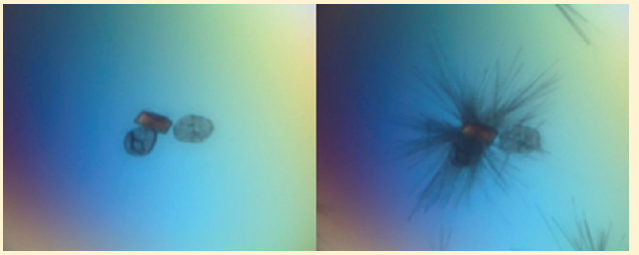
ABSTRACT: Growing X-ray grade crystals of a specific protein is a process of trial and error. Usually, hundreds or even thousands of conditions are screened in order to identify useful crystallization conditions. Heterogeneous nucleants have been shown to increase the success rate of crystallization trials, and (human) hair has previously been identified as a promising nucleant. Here, we describe and evaluate a method for preparing crystallization plates that are locally coated with fragments of human hair, allowing automated, high-throughput crystallization trials in a fashion that is entirely compatible with standard hanging or sitting drop crystallization techniques. We assessed the effect of these nucleants on the crystallization of 11 different proteins in more than 4000 crystallization trials. We found additional crystallization conditions for 10 out of 11 proteins when using the standard JCSG+ screen (96 different conditions). In total, 34 additional crystallization conditions could be identified (13.1% of the total number of successful crystallizations). The increase in crystallization conditions ranged between 33.3% (two additional conditions were identified for myoglobin on top of four homogeneous crystallizations) to 1.2% (we identified a single additional condition for insulin, which crystallized in 85 out of 96 conditions); the median increase in crystallization hits was 14%. On the basis of these numbers, we conclude that the inclusion of human hair fragments in high throughput crystallization screens may be beneficial. The method is inexpensive, straightforward with standard equipment and uses materials available in any crystallization lab. Furthermore, initial experiments with the crystallization of membrane proteins on hair show the technique may also be beneficial for growing membrane proteins.



1. INTRODUCTION

Crystallization of biological macromolecules is still one of the major bottlenecks in X-ray crystallography.[1−4] The process of obtaining diffraction-grade crystals comprises a screening and an optimization step.[2] First, nucleation of a microscopic crystallite must occur. Such a nucleus often exists of only a few hundred molecules and is not visible with an optical microscope. The screening step aims at identifying suitable nucleation conditions. It should provide directions for further crystal optimizing experiments, required for producing well diffracting single crystals.[5]

A number of studies have shown that often the initial nucleation phase of macromolecular crystals is the limiting step in the formation of X-ray grade crystals. The reason is that, while the protein should be in the metastable phase for crystal growth, much higher levels of saturation are needed for nucleation.[6] Spontaneous formation of a crystal nucleus by the ordered aggregation of supersaturated proteins (homogeneous nucleation) is an unlikely event,[7] but it is well-known that surfaces can help in the formation of crystal nuclei.[8]

The first report of a nucleant inducing nucleation of macromolecules on heterogeneous material was the epitaxial growth of protein crystals on minerals, published more than 20 years ago.[9] Other candidate nucleants followed (e.g., zeolites, silicates, and dried seaweed) and have been tested for multiple proteins.[10−13] Previous results have proven that hydroxy-apetite, cellulose, horsehair, and dried seaweed, showed increased hits when added to Sparse Matrix crystallization screening. The observed increase in crystallization was 35% when horsehair was added to 10 test proteins before screening with standard methods.[10,11] Specially designed bioglass (amorphous-mesoporous bioactive gel-glass) has recently been found to be a nucleation-inducing material with greater universality than other studied materials before.[14−17] Porous materials as nucleants for protein crystallization have been intensively studied for years. In short, results and calculations indicate that the cavities of nucleants might entrap the protein molecules and thereby support nucleation and crystal growth.[18]

For many years, natural hairs (cat's whiskers, horse hair, human hair) have been used successfully in macromolecular crystallization for transferring crystal seeds (i.e., critical sized nuclei) into solutions.[4] The microstructure of hair contains

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Table 1. Proteins Used for Screening Experiments

protein	concentration (mg/mL)	buffer (mM)	pH	ions (mM)	source
lysozyme	10	acetate (20)	4.6		Fluka
glucose isomerase	22	tris (6)	7	$(NH_4)_2SO_4$ (90)	Hampton Research
trypsin	50	tris (20)	8	$CaCl_2$ (3)	Sigma
Ccp-Cc	38	H_2O			Qamar et al.[25]
insulin	30	Ches (25)	8.4		Sigma
myoglobin	25	Tris (10)	7.4		Sigma
(ov)albumin	20	Tris (10)	7.4		Sigma
alpha-lactabumin	25	Tris (10)	7.4		Sigma
alpha-chemotrypsin	50	Tris (10)	7.4		Sigma
alcohol dehydrogenase	20	Tris (20)	7.4	$MgCl_2$ (1)	Sigma
pepsin	25	Tris (10)	7.4		Sigma

overlapping cuticles and thus is ideal for trapping the microfragments of a crystal.[19] Horsehair was shown to be effective as a nucleant for three model proteins as well as for a difficult to crystallize Fab-D protein.[6] Recently, the nucleation properties of human hair have been investigated, showing that various surface properties of human hair (cavities within the cuticles, an ordered keratin surface, preferred entrapment of protein at specific sites resulting in concentration gradients of protein) all contribute to effective protein crystal nucleation.[19]

Ideally, heterogeneous nucleants in initial screening should be combined with automated, miniaturized crystallization.[20,21] In this study, a method is described that introduces fragments of human hair in miniaturized high throughput crystallization experimentation. The method is straightforward and can be preformed in any crystallization lab with materials readily available. To keep the nucleating properties of the hair surface and surface patterning intact, thin slices of hair were produced with a microtome. As an alternative, we also screened using hair fragments that were too small to distinguish by optical microscopy and were obtained by crushing hair in liquid nitrogen, using a mortar and pestle.

Our method was tested by screening 11 proteins with 96 crystallization conditions (JCSG+ screen[22,23]) in wells that either contained hair particles (of either type: crushed or sliced) and comparing them to experiments that did not contain heterogeneous nucleants. The results show an increase in hits in initial screening of 12% when drops containing either sliced or crushed hair are compared to drops without purposefully added nucleants. This percentage of increase is lower than previous results;[10,11] however, the total number of additional hits is significantly higher. These differences are probably due to the different screen that was used as well as different proteins. Since heterogeneous nucleation often resulted in small, needle-shaped crystals, the described high-throughput method promoted growth of crystals suitable for electron diffraction.[24]

Since the crystallization of membrane proteins are known to be problematic,[26] we also performed initial crystallization experiments using hair as a nucleant with the membrane protein diacylglycerol kinase (DgkA). The fact that DgkA crystallized on the hair surface suggests the method to also be beneficial for membrane proteins, but in the case of membrane proteins data on additional proteins needs to be gathered.

2. EXPERIMENTAL SECTION

2.1. Preparation of Hair Particles. Three different hair samples were collected, prepared, and cut. A small tuft with a length of 2 cm was washed extensively with petroleum ether to remove any remaining proteins and lipids. The hair was then cut using a microtome (Leica) into pieces with a diameter ranging between 25 and 45 μm, and a length of 25 μm. The hair slices were extensively washed with Milli-Q water to remove any polymers and resuspended in ethylene glycol. Of the three different types of hair used, the hair with a diameter of 25−30 μm showed the best properties for dispensing with our dispensing robot.

2.2. Preparation of Crystallization Plates Dusted with Hair Particles. A suspension containing around 12 hair particles per microliter was used for dispensing. The hair fragments stay in suspension for a few minutes in ethylene glycol before they aggregate and then precipitate. The hair slices were added to designated wells on Pzero 3550 plates (Corning) using an ORYX 6 robot and XStep software (both Douglas Instruments). In each well, 0.5 μL of hair suspension (around six particles) was deposited and 0,1 μL of water using the second microtip was added in order to speed up ethylene glycol evaporation at 37 °C. After 3 days, both ethylene glycol and water had evaporated and the plates were ready for experiments. In order to have reproducible amount of particles, all wells were imaged and occasionally wells with less than three hair particles were reprocessed as described above.

2.3. Preparation of Crushed Hair with Liquid Nitrogen. For crushed hair, thicker hair was used with a diameter of around 100 μm. Hair was crushed using a mortar under liquid nitrogen until a powder was formed. Around 25 mL of ethanol was added to form a suspension of the crushed hair powder. The suspension was mixed and transferred to tubes (Eppendorf) with 500 μL of this solution. After evaporation of ethanol, for each experiment the protein solution was added to these tubes in order to mix with crushed hair before dispensing with an ORYX crystallization robot into clean crystallization plates.

2.4. Scanning Electron Microscopy. Scanning electron microscopy (SEM) was preformed on the sliced hair particles as well as the crushed hair to determine the surface structure of hair particles. Experiments were preformed on a Nova Nanosem 200 (FEI). After a 20 nm layer of evaporated carbon was applied on the particles, images were obtained at different magnifications. The goal was to investigate whether the properties of hair that are known to promote nucleation[19] remained intact after preparing the hair for high throughput experiments.

2.5. Crystallization Screening. Crystallization experiments were carried out using the sitting drop vapor diffusion technique in Pzero 3550 plates (Corning). Rockmaker software (Formulatrix) was used for designing the experiments. We used a Genesis (Tecan) for dispensing the screening solutions in the reservoirs. The ORYX 6 (Douglas Instruments) crystallization robot was used for transferring 200 nL of reservoir solution and 200 nL of protein solution in sitting drop wells. Plates were stored at 18 °C and imaged using the automated imaging system Rock Imager (Formulatrix). The JCSG+ screen (Qiagen) was

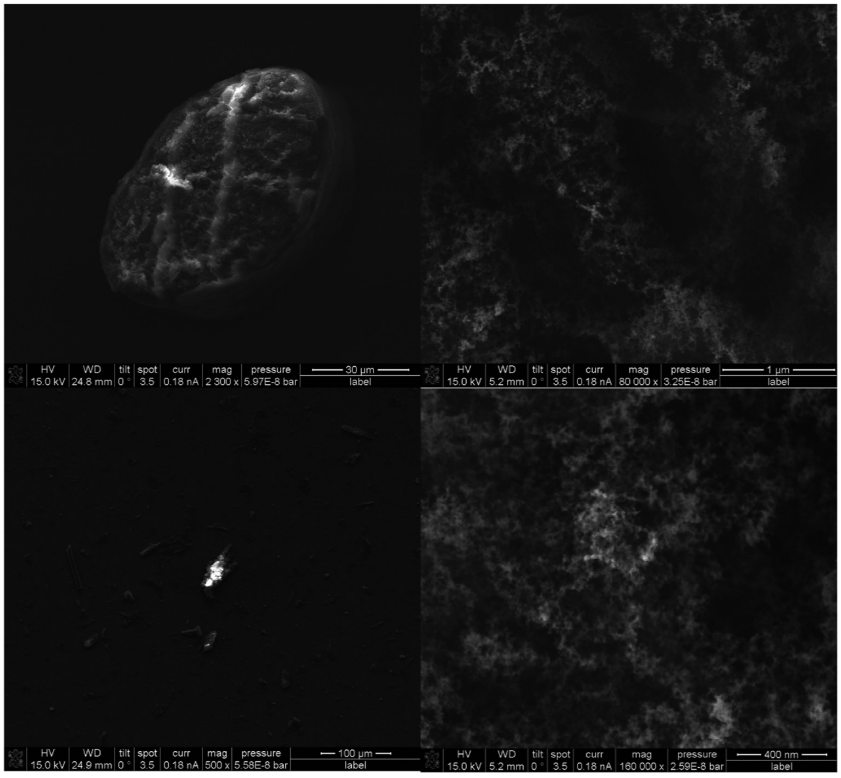

Figure 1. SEM images of hair. Top left: hair particle sliced using microtome. Top right: high magnification image of hair slice showing spongy center that promotes nucleation. Bottom left: Image of scattered crushed hair particles. Bottom right: high magnification image of crushed hair shows spongy properties are maintained.

used for random screening experiments and chemicals used for grid experiments were purchased from Sigma.

A list of the proteins used in the screening experiments and their source is shown in Table 1. In all cases, the proteins were not further treated nor were any inhibitors used. By direct dissolution of dry material most of the proteins were made up as 10 mg/mL stock solutions unless a concentration more suitable for crystallization was described in the literature. For most proteins, the buffer used was 10−20 mM TRIS buffer at pH 7.4, and salt ions were kept to a minimum to prevent false positives in the form of salt crystals.

2.6. Crystallization Scoring and Validation. Crystallization plates were imaged using an automated imaging system but scored manually. To prevent false positive hits several precautions were taken. The control conditions were set up in duplicate; if in one of the two (or in both) controls a crystal was observed, the condition was labeled as a hit. All images were recorded using a polarization filter to check birefringence. If it was still unclear if a hit was a protein crystal or a false positive, UV images were collected using an Olympus BX51 microscope. UV imaging distinguishes between protein and salt crystals since the tryptophane residues cause the crystals to fluoresce under UV-light.

2.7. *Escherichia coli* Diacylglycerol Kinase (DgkA) Crystallization on Human Hair. His-tagged DgkA was expressed in BL21 strains. Growth and expression methods were implemented as previous described.[27] The DgkA protein was purified and solubilized with *n*-decyl-beta-D-maltopyranoside (DM) according to the literature.[28] Crystallization was performed above a 75 μL reservoir buffer containing 33% (v/v) PEG 300, 0.05 M glycine pH 9.65, 0.1 M NaCl. Crystallization drops consisted of 2 μL of reservoir solution and 2 μL of 16 mg/mL DgkA. Human hair was prepared as described above (without slicing) and added to crystallization experiments in NeXtal evolution μplate from Qiagen.

3. RESULTS AND DISCUSSION

3.1. Scanning Electron Microscopy. The scanning electron microscopy images (Figure 1) prove that when hair is sliced with a microtome it retains cuticles on the edges. These cuticles have been found to be beneficial for nucleation.[19] Additional positive effects of cutting hair in small slices could be caused by the increased ratio between the cortex surface (the cutting planes of the sliced hair) and the cuticle surface. This cortex surface, including the medulla, is porous and shows cavities of many different sizes. These cavities have been known to induce nucleation.[6] With the method described here for including hair particles in microcrystallization drops, it is now also possible to control the amount of sliced hair particles in the drop.

The SEM images of crushed hair (Figure 1) show that when hair is crushed, particles are obtained of 20 μm and smaller. When using this method, there will be hundreds of these particles scattered around the drop. The spongy structure of the keratin fibers in the center of the hair containing the cavities is maintained. A major advantage of using crushed hair is that due to the small size of the particles they could easily be added to the protein prior to setting up experiments. A disadvantage is that sometimes the particles are too small to see with the optical microscope, making it is impossible to check how many particles are present in the drop.

3.2. Hair Slices Induce Nucleation. The nucleation ability of protein crystals on sliced hair was tested using a single buffer condition with different amounts of hair fragments. In a total of 288 drops with increasing amounts from zero to up to 45 hair particles, lysozyme was crystallized. It was expected that higher

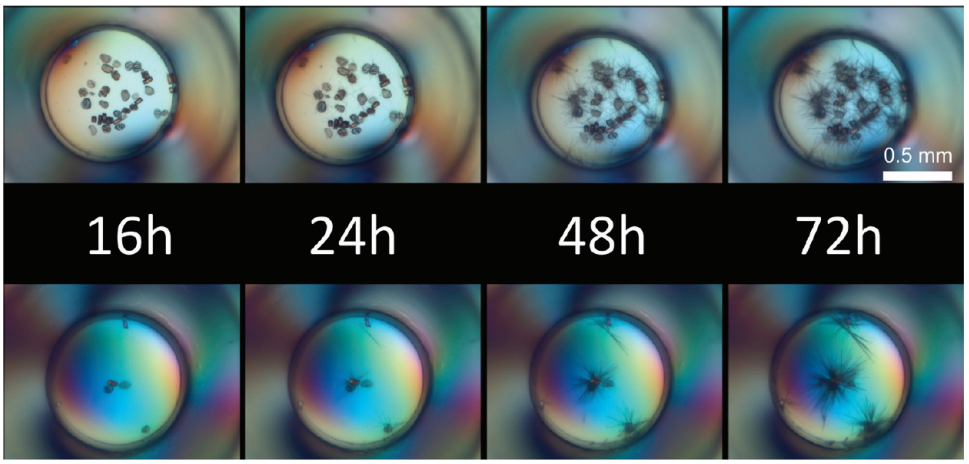

Figure 2. Lysozyme needles growing in a condition with five hair particles (below) as well as in a condition with approximately 30 particles (above).

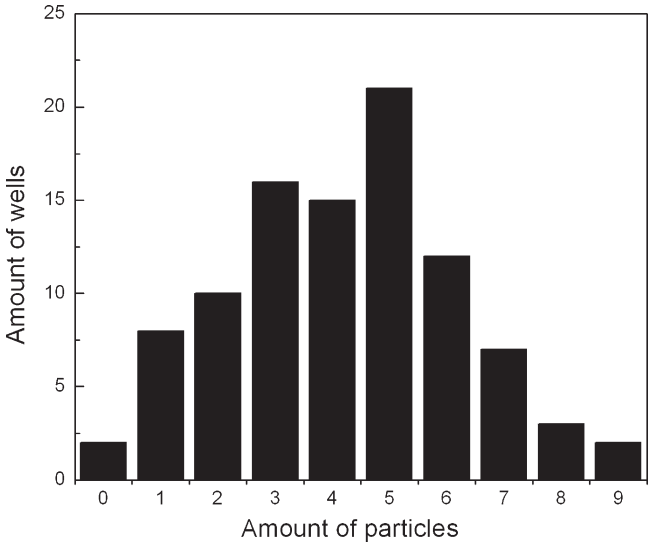

Figure 3. Amount of sliced hair particles dispensed in each well of a 96-well plate.

amounts of hair particles might disrupt nucleation and crystal formation by capturing a substantial proportion of the protein molecules on the hair surface, thereby reducing the protein concentration. However, also in all drops with more than 40 particles crystal formation was observed. Time-lapse images show that needle-shaped crystals start growing from the hair particles (Figure 2). The accuracy of dispensing these particles is shown in Figure 3.

The needle-shaped crystals seem to prefer growing on the cuticle surface, confirming previous observations on long strands of hair.[19] However, needle-shaped crystals were also observed to be growing from the spongy surface at the cutting planes of the hair particles. Hair slices of 25 μm in length and an average a radius of 25 μm (with an approximately equal ratio between hair cuticle surface and edge surface) were used. Problems were encountered when using our robotics to dispense hair with a larger radius. For subsequent experiments, only a few hair particles per trial were introduced in the crystallization experiments, in order to prevent disruption of the crystallization process by excess absorption of protein.

3.3. Heterogeneous Crystallization with JCSG+ Screen Using Hair As a Nucleant. The influence of the crushed and sliced hair on 11 different proteins was tested using the broad JCSG+ Screen. In total, including controls, we scored in excess of 6000 crystallization trials. Table 2 shows the results of screening with either sliced hair or crushed hair. The control conditions are in duplicate; if in one of the two (or in both) controls a crystal was observed, the condition was labeled as a hit. Furthermore, in certain crystallization solutions both crushed hair and sliced hair induced nucleation and crystal formation for the same protein. Subtracting these two events if they were found to overlap yields the number of unique extra crystallization hits, which are also included in Table 2.

We found additional crystallization conditions for 10 out of 11 proteins when using the standard JCSG+ screen (96 different conditions). Pepsin crystallized only in two conditions, and the inclusion of nucleants did not affect this outcome, but of the remaining proteins, 34 additional crystallization conditions could be identified (13.1% of the total number of successful crystallizations). The increase in crystallization conditions ranged between 33.3% (two additional conditions were identified for myoglobin on top of four homogeneous crystallizations) down to 1.2% (we identified a single additional condition for insulin, which crystallized in 85 out of 96 conditions); the median increase was 14%.

UV imaging proved a useful tool to determine whether crystals were protein crystals or false positives in the form of salt crystals. Figure 4 shows five crystals of which doubt emerged whether they were protein crystals or salt. UV illumination of the proteins removed this doubt, as tryptophane fluorescence clearly lights up the crystals. If the crystals had been salt, they would not have shown UV fluorescence.

3.4. Experiments at Suboptimal Conditions. Experiments with sliced and crushed hair were set up at suboptimal conditions using lysozyme. It was clear that at suboptimal conditions crystal growth could be promoted by adding heterogeneous nucleants. Figure 5 shows an example: no crystals grow in a suboptimal crystallization condition without hair. The exact same condition containing hair slices yields a single crystal after 36 h and a total of two crystals after 60 h. In the well with crushed hair, there are a lot more nucleation sites scattered over the well. In this well, a lot more crystals can be observed at a much shorter time starting around 12 h and the crystals keep growing until 60 h. This

Table 2. Additional Hits in JCSG+ Screening for 11 Different Proteins after Adding Hair Particles[a]

proteins	conditions crystallized in control	additional conditions crystallized with crushed hair	additional conditions crystallized with sliced hair	additional unique conditions crystallized
insulin	85	1	1	1
lysozyme	17	7	3	7
trypsin	34	2	0	2
Ccp-Cc complex	43	4	3	5
myoglobin	4	2	1	2
(ov)albumin	11	0	3	3
glucose isomerase	31	2	6	8
α-lactabumin	12	2	0	2
alcohol dehydrogenase	9	1	0	1
α-chemotrypsin	11	1	2	3
pepsin	2	0	0	0
total	259	22	19	34

[a] Additional hits were counted if a protein crystallized in a condition where it did not crystallize without the addition of the hair particles. A condition was considered unique if the extra condition with crushed hair and with sliced hair were different. Ten out of eleven proteins produced a wider range of crystallization hits after hair particles were introduced in crystallization trials.

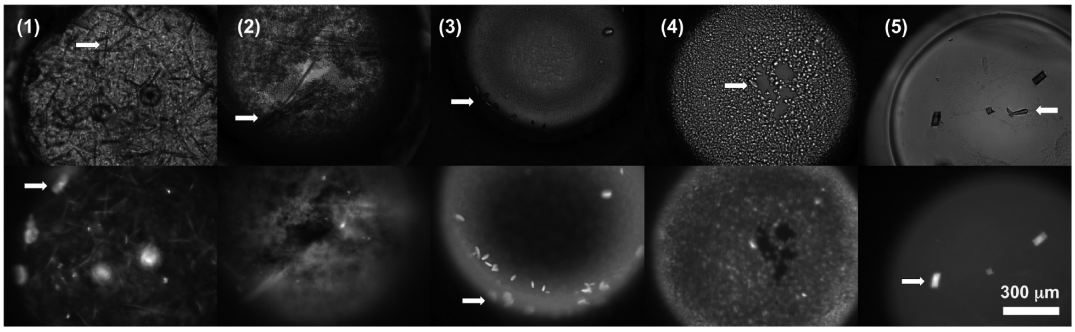

Figure 4. Bright field (above) and UV (below) images of protein crystals suspected of being false positives. In the bright field images the arrows point to the suspected crystals in the UV these crystals fluoresce. In the UV images the arrows point to fluorescent hair slices. 1. The small needles are glucose isomerase crystals. 2. Crystals of alpha lactoglobin at right and bottom left, under a cloud of precipitation. 3. Small crystalline discs of alcohol dehydrogenase around bottom edge of plate where hair (bigger discs) are also present. 4. Small crystals of insulin, the biggest crystals fluoresce the most in UV light. 5. Salt crystals; these crystals do not fluoresce.

indicates that increased heterogeneous nucleation increases the growth of crystals in conditions that under normal circumstances do not yield crystals. When too much heterogeneous nucleation sites are included smaller crystals and irregular crystal growth are observed.

3.5. Heterogeneous Crystallization Using a Membrane Protein. DGKA crystals grew overnight at 20 °C, using conditions described in section 2.7. An image of DgkA crystals grown on human hair is shown in Figure 6. From the image it is clear that nucleation is taking place on the hair as most crystals grow on the surface. Heterogeneous crystallization may also be beneficial for membrane proteins. Experiments performed with hair slices also showed crystallization localized around the chips as is visible in Figure 6; UV imaging proved the crystals to be protein.

4. DICUSSION/CONCLUSION

Overall a versatile high-throughput method is described for introducing hair as heterogeneous nucleants in microcrystallization. The method is straightforward and can be done in almost any crystallization lab with available materials. The nucleants showed an increase in number of crystallization hits in standard screening experiments, compared to controls without the specific addition of heterogeneous nucleants. These additional crystallization conditions would have been missed without addition of nucleants, even upon repeated testing with the same screening solutions. Initial experiments show that the method may also be beneficial for membrane protein crystallization, but further experiments need to be done in order to validate this observation with additional proteins and extensive screening. Scanning electron microscopy revealed that both crushed hair and sliced hair show a surface containing small cavities suitable for crystal nucleation. The advantage of using sliced hair particles over crushed hair is that the hair slices can easily be seen by light microscopy, and the crushed hair cannot. This makes it easier to check and control the amount of nucleants per well. However, making the plates containing hair slices is more work and a microtome is needed. If a microtome is not available, the crushed hair method is the only option. The increase in hits of both methods is similar, though can vary per protein; thus, ideally a plate should be set up with both methods.

Human keratin is one of the major contaminants in mass spectroscopy,[29] and its most likely source is dander (shed fragments

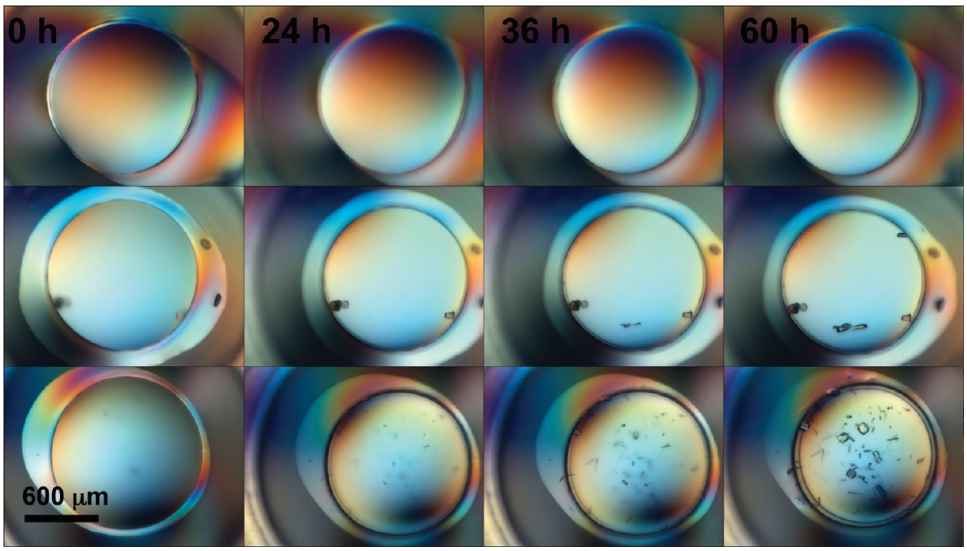

Figure 5. Lysozyme crystals growing in a suboptimal condition (0.1 M Na-acetate buffer pH 4.2, 1 M NaCl) at a concentration of 8 mg/mL. Images of a well containing crushed hair (bottom row), a well with hair slices (middle row) and of a control with no added heterogeneous nucleants. Images were taken at 0, 24, 36, and 60 h.

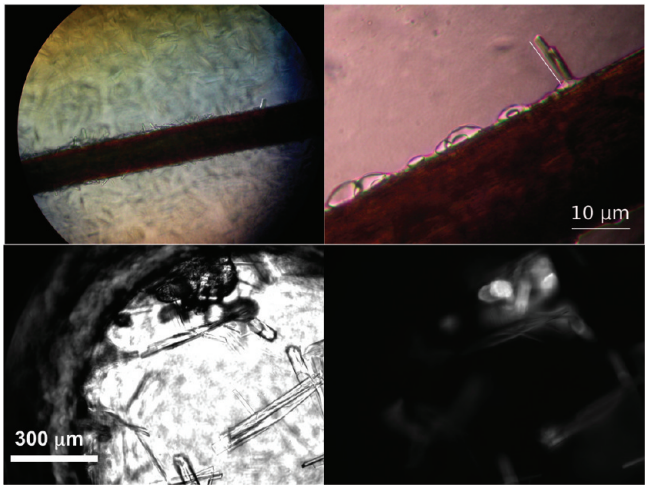

Figure 6. Image of DgkA crystals growing on human hair as a nucleant. Top left: small crystal growing on a human hair and bigger crystal growing in the drop solution. Top right: nucleation of DgkA on human hair and small crystal from nucleation. Bottom left: DGKA crystals growing around hair chips. Bottom right: UV-illuminated image of DGKA around hair chips proves crystals are protein.

of skin) and hair fragments of the experimenters. We did not perform our crystallization experiments in a clean-room facility, and hence we cannot exclude the possibility that a proportion of the hits we observed in the control experiments (to which we did not deliberately added hair fragments) may in fact have been induced by ambient, keratinatious dust. In that case, the increase in nucleation due to human hair fragments that we report here may even be an underestimation. In any case, our experiments indicate it may not be a good idea to work in a clean-room environment for protein crystallization experiments, or to be very rigorous about filtering all crystallization buffers. Our results also lend some credence to aspects of folklore in protein crystallography (for instance: "Good crystallographers have beards") that indicate that the experimenter may influence the outcome of protein crystallization trials in unexpected ways.

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