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Incorporation of Microcrystals by Growing Protein and Virus Crystals

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ABSTRACT **In the course of time-lapse video and atomic force microscopy (AFM) investigations of macromolecular crystal growth, we frequently observed the sedimentation of microcrystals and three-dimensional nuclei onto the surfaces of much larger, growing protein or virus crystals. This was followed by the direct incorporation over time of the smaller crystals into the bulk of the larger crystals. In some cases, clear indications were present that upon absorption of the small crystal onto the surface of the larger, there was proper alignment of the respective lattices, and consolidation proceeded without observable defect formation, i.e., the two lattices knitted together without discontinuity. In the case of at least one virus crystal, cubic satellite tobacco mosaic virus (STMV), addition of three-dimensional nuclei and subsequent expansion provided the principal growth mechanism at high supersaturation. This process has not been reported for growth from solution of conventional crystals. In numerous other instances, the lattices of the small and larger crystals were obviously misaligned, and incorporation occurred with the formation of some defect. This phenomenon of small crystals physically embedded in larger crystals could only degrade the overall diffraction and materials properties of macromolecular crystals.** © 1996 Wiley-Liss, Inc.

Key words: atomic force microscopy, canavalin, satellite tobacco mosaic virus, dislocation, two-dimensional nuclei

INTRODUCTION

We previously reported on the use of time-lapse video microscopy for the monitoring and investigation of macromolecular crystal growth.[1,2] Among the many interesting observations was that in most crystallization samples foreign particles, fragments of precipitate, and other small objects occasionally sedimented onto the surfaces of growing crystals and were subsequently incorporated into the crystals. The most intriguing situations, however, were when the objects themselves were small crystals. In our video recordings, the small crystals adsorbed to the surfaces of the larger, both continued to grow, but eventually the smaller was incorporated into the bulk of the larger with little visible external trace.

Using in situ atomic force microscopy (AFM) we have been able to examine this phenomenon of microcrystal capture and recruitment in several different protein and virus systems. Our studies demonstrate that a variety of microcrystals, three-dimensional nuclei, and molecular clusters can be incorporated into large growing crystals. There appear to be two distinct cases: where the lattices of the smaller and larger crystals are aligned, and when they are misaligned, and these two situations produce quite different consequences for the growing crystal.

MATERIALS AND METHODS

Canavalin is a protein composed of three identical subunits of M_r = 47,000 organized around an exact 3-fold axis of symmetry. It is the major storage protein of Jack Bean seeds and its structure is known from X-ray diffraction analysis.[3] Canavalin crystallizes in four different forms[4]; that utilized in these studies was the most common, rhombohedral form (space group $R3$, $a = b = 135$ Å, $c = 75$ Å).

STMV is an icosahedral, T = 1 satellite virus of the classical rod-shaped tobacco mosaic virus (TMV). It has a diameter of 17 nm and a molecular weight of $M_r \approx 1.4 \times 10^6$. The structure of STMV is known from X-ray diffraction analysis.[5] STMV crystallizes in at least three different crystal forms; that studied here was the cubic form (space group $I23$, $a = b = c = 257$ Å).

For AFM studies seed crystals of the macromolecules were nucleated in 3 μl droplets on electron microscopy grids cemented to plastic discs. These were then transferred to the closed fluid cells of a Nanoscope E (Digital Instruments) atomic force microscope and the cell immediately flooded with mother liquor of known supersaturation. Observation was begun immediately and continued on the growing crystals for several hours to several days depending on the intent of the experiment. The temperature was 25° ± 2°C.

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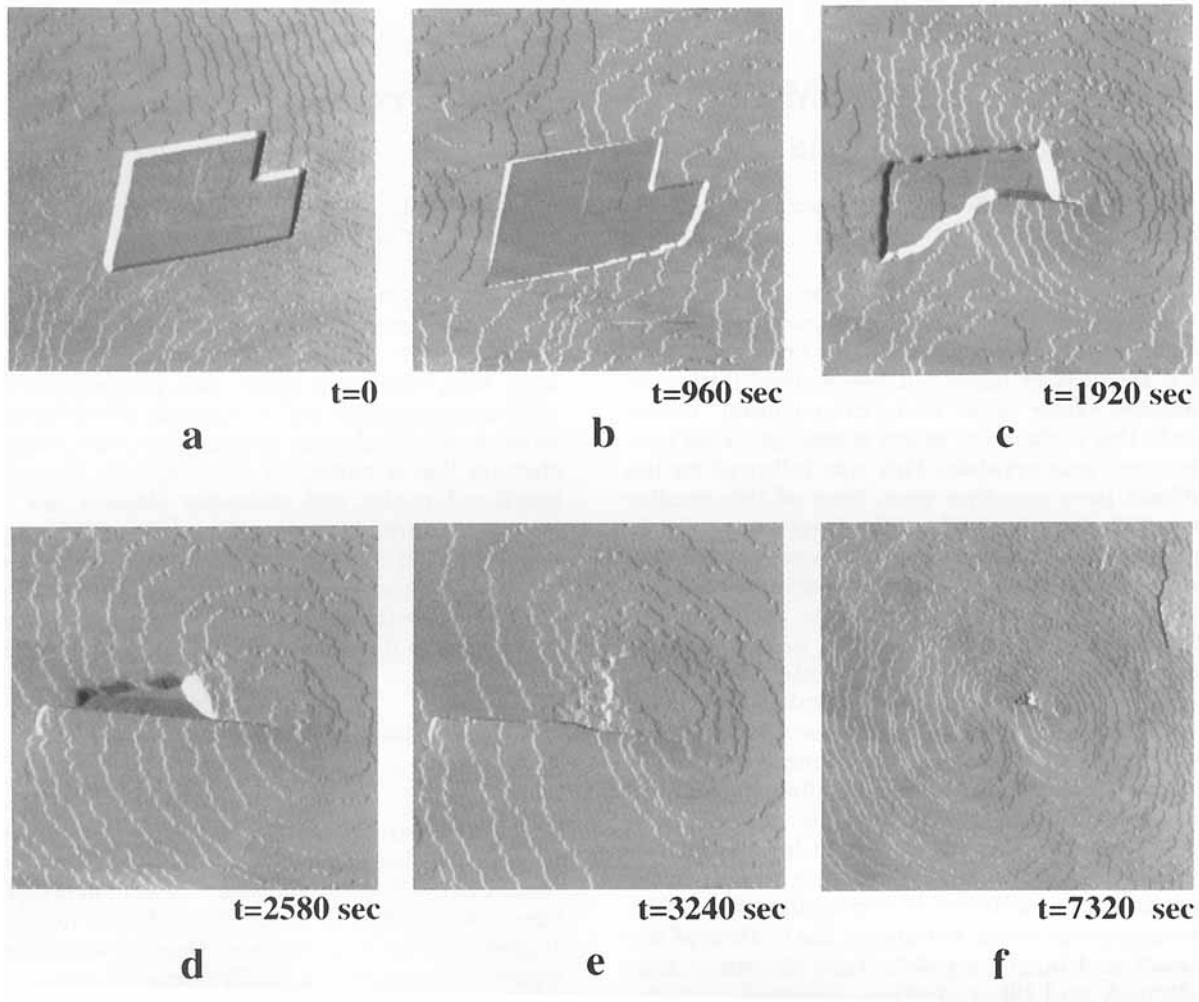

Fig. 1. A series of six AFM images (**a–f**) showing the direct incorporation of a microcrystal of 15 μm × 8 μm into a larger, growing crystal of the protein canavalin. A lattice mismatch clearly exists for the two crystals. As the sequence progresses and the small crystal is consumed by the larger, both a planar defect and spiral dislocations form and propagate in the larger crystal. The scan areas are (a) 28 × 28 μm^2, (b) 25 × 25 μm^2, (c) 20 × 20 μm^2, (d,e) 15 × 15 μm^2, and (f) 50 × 50 μm^2.

RESULTS AND DISCUSSION

The AFM sequence of Figure 1 illustrates the sedimentation of a small, 15 × 8 μm flat canavalin crystal, of thickness about 0.3 μm, on the broad surface of a larger, growing crystal. This series of images spans 122 min during which time the small crystal is nearly inactive while the larger continues to grow. The successive layers and growth steps are clearly evident on all sides of the smaller crystal.

As the sequence proceeds the pattern of growth steps changes with time as the entire crystal accumulates molecules from the surrounding solution into the step edges which are advancing with a rate of 9×10^{-6} cm/s. The face normal growth rate was 4.5×10^{-8} cm/s, which corresponds to the deposition of one molecular layer in approximately 16 sec. Evident, too, is the gradual, and then rapid disappearance of the small canavalin crystal into the growing, larger crystal. There is clearly a mismatch between the respective lattices. Midway through the sequence one can see a very large macrostep advancing over the top of the smaller crystal from the lower right, and the initial formation of a double spiral screw dislocation at the right end of the small crystal. Consumption of the small crystal ends with formation of a planar defect in frames c–f, terminating in the spiral dislocation. Finally, after the small crystal has been totally incorporated, in the last frame, we see two interacting, residual spiral dislocations, one right handed and one left handed. The incorporation was not without consequence. A triangular impression about 3 μm square and about 500 Å deep persisted for several hours.

From these images, it is clear that there is no dissolution or restructuring at the lattice interface, and that the lattice of the larger crystal accommo-

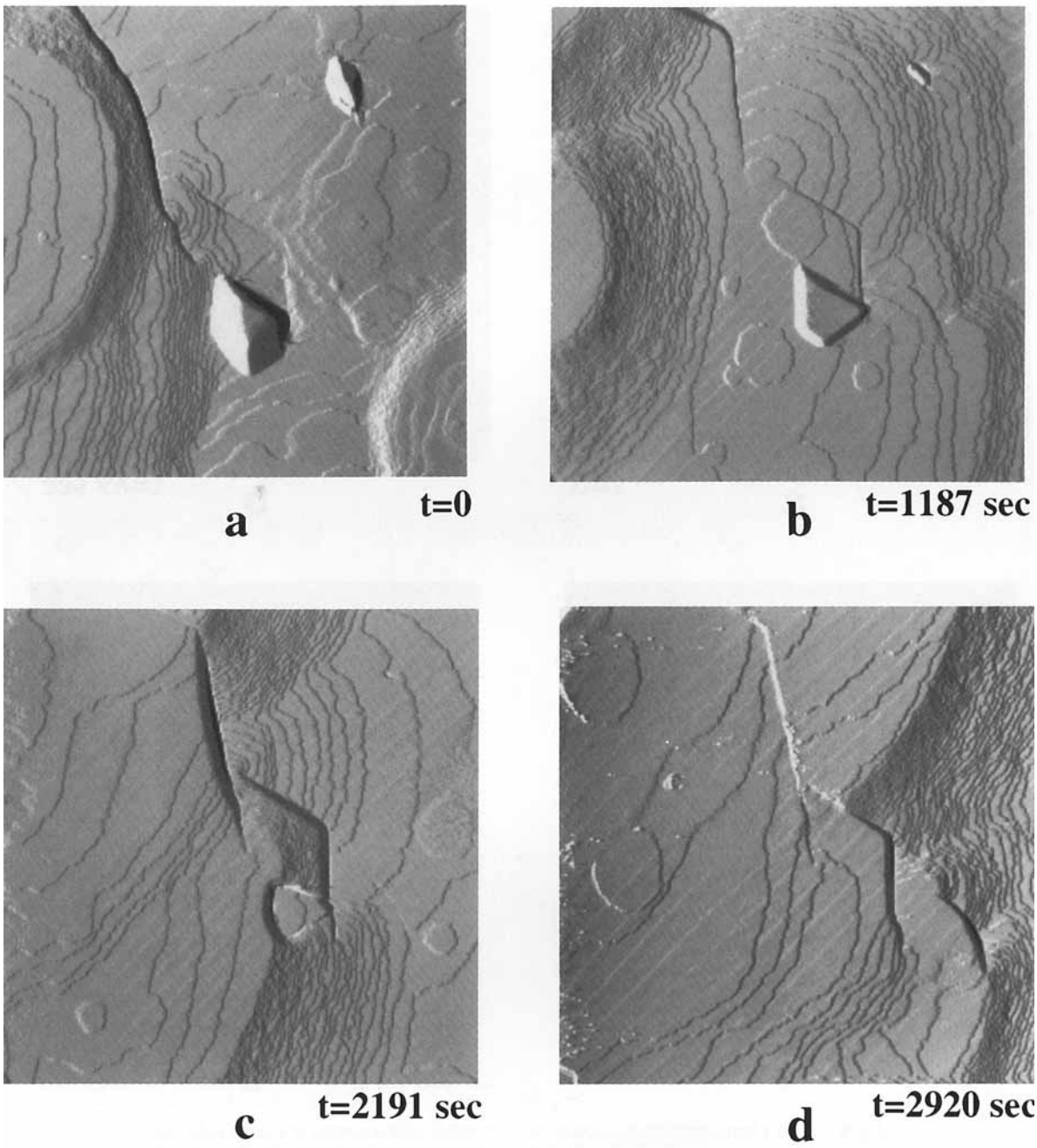

Fig. 2. In **(a)** two microcrystals have sedimented on the (111) face of a growing, cubic satellite tobacco mosaic virus (STMV) crystal. The surface structure of the larger crystal is very complex and step trains, two-dimensional nuclei, macrosteps, planar defects, and growth plateau are evident everywhere. In **(b)**, the smallest of the two nuclei has virtually disappeared leaving in **(c)** a cavity above its incorporation site. The same process occurs for the larger of the two as well. A cavity can be seen forming around it in **(c)** due to step retardation, and it too has completely disappeared by frame **(d)**. The scan areas are 25 × 25 μm^2.

dates interruption by that of the smaller, but only with formation of defects. The small, misoriented crystal is intact and embedded in the bulk of the larger.

The sequence of AFM images of Figure 2 presents a similar series of events, but this time the crystal is cubic, and composed of the spherical plant virus STMV. In the first frame two small crystals have sedimented on the surface of a much larger, actively growing virus crystal. A dense bunching of growth steps arising from a growth plateau is seen to the left and numerous two-dimensional nuclei are seen over the remaining surface. The tangential growth rates of macrostep and elementary step advance-

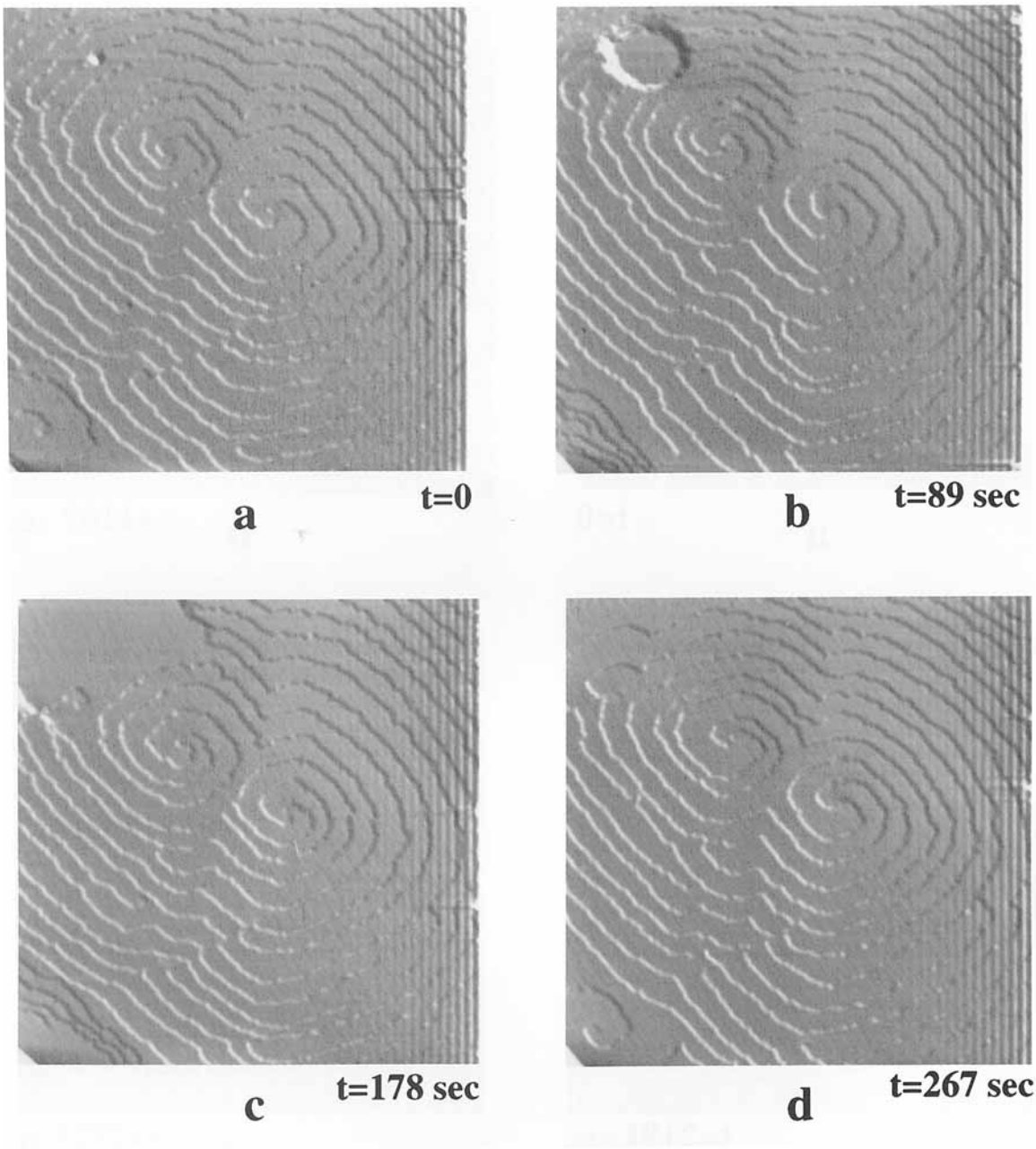

Fig. 3. In **(a)** a three-dimensional nucleus has sedimented on the surface of a larger canavalin crystal in the immediate vicinity of two right-handed, double spiral dislocations. The three-dimensional nucleus grows as the spirals develop further in **(b)** and its step edge merges with that from a spiral dislocation in **(c)**. Complete consolidation and merging of the two lattices is complete in frame **(d)**. The scan areas are 30 × 30 μm^2.

ments were 10^{-6} cm/s. As growth progresses with the recruitment of new STMV particles the step bunch to the left, a virtual macrostep, moves to the right. A major, planar defect is also visible. By the middle of the sequence the small crystal in the upper right has nearly disappeared, and the second small crystal (lower center) is becoming submerged. Note in passing that the growth plateau at the far left has now grown, by successive two-dimensional nucleation, far above the plane of the crystal face.

In frame c of Figure 2, it is noteworthy that the small crystals have completely disappeared into the interior, but that the growth of steps around them leave cavities due to the stress energy caused by incorporation. The chemical potential of the crystal in this region is higher because of the stress energy which effectively reduces local supersaturation.

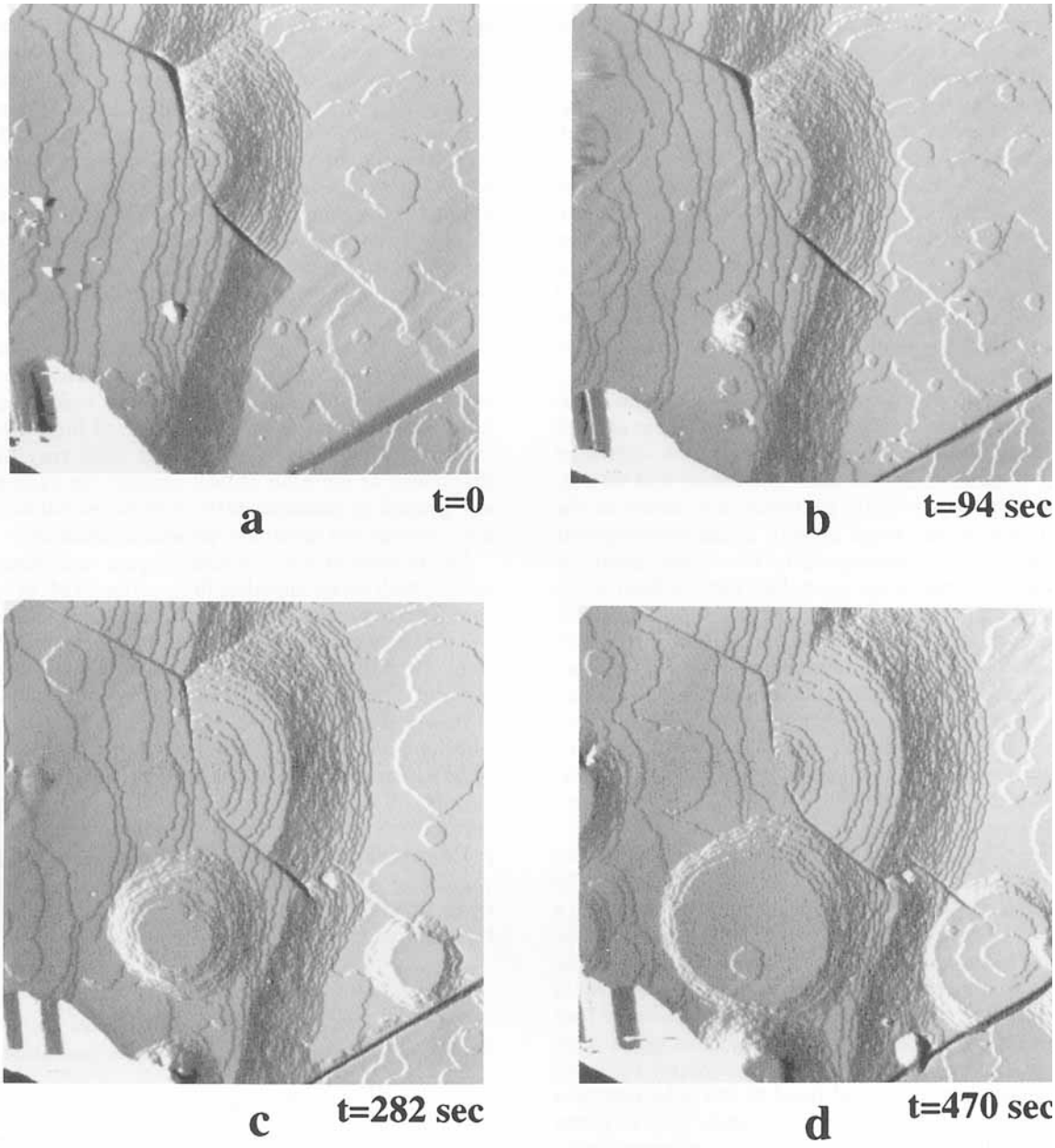

Fig. 4. A series of AFM images showing the adsorption of a three-dimensional nucleus on the (111) face of a growing, cubic STMV crystal **(a)** and its development (**b** and **c**) into a plateau **(d)**. A second similar process also takes place on the lower surface to the right from a second three-dimensional nucleus which has adsorbed there. This process of absorption of three-dimensional nuclei in a manner consistent with the underlying lattice, and their development, provides the major growth mechanism for these crystals. The scan areas are 30 × 30 μm^2.

Thus, step advancement over incorporated microcrystals is slower than for other areas of the crystal. These are subsequently overgrown by growth steps. In the last frame, it is evident that the virus crystal lattice was sufficiently elastic to accommodate the small, misaligned crystal without the cost of significant defects. We have also obtained numerous sequences where noncrystalline, foreign particles, such as dust, sedimented on the growing surfaces, and these too were incorporated by the same processes illustrated in Figures 1 and 2.

To this point, small crystals sedimenting on larger presented lattice mismatches and resulted in defects. This is not always the case, however, and it is not the most interesting. Lattice matches between very small microcrystals, which we refer to as three-dimensional nuclei, and larger crystals to which they adhere, frequently occur for both protein and

virus crystals. In these cases growth proceeds without noticeable discontinuity and the lattices of the smaller and large crystals can be seen to actually merge and knit in a contiguous fashion.

This is seen for canavalin in the sequence of Figure 3 where a three-dimensional nucleus has adsorbed to the surface of a larger canavalin crystal. Although the orientation of the lattice of the microcrystal is initially unclear, both the large and small crystals continue to grow, and it is then apparent that the lattices do indeed exhibit a common orientation. With time, the edges of the small crystal merge with the advancing growth steps of the larger, and consolidation occurs without formation of any visible defect.

The situation presented in Figure 4 is more striking. Here, a cubic STMV crystal is growing at high supersaturation. Not only a few, but a continual flux of small, three-dimensional nuclei and microcrystals are observed to sediment and absorb to the (111) face of the large crystal. These microcrystals proceed to grow, principally by two-dimensional nucleation normal to the crystal surface, to form large, spreading growth plateaus. Observation of this process over an extended time period confirmed that, while surface growth occurred everywhere by more conventional two-dimensional nucleation, the addition and incorporation of three-dimensional nuclei were the predominant sources of growth. This growth mechanism has not previously been described for any conventional, small molecule crystals grown from solution.[5]

The (111) face of cubic STMV exhibits 3-fold symmetry, so there are three equivalent orientations by which a three-dimensional nucleus could absorb to a larger crystal with the correct lattice orientation. Nonetheless, it is difficult to explain how so many crystals adhering to the large crystal surface could be properly oriented. Statistics would indicate that the vast number of these should be misaligned. This may be due, in part, to the AFM technique. Particles or objects which are not fixed to the substrate or a growing crystal, but are free to move, may be swept away by the cantilever tip on each scan over an area. Thus microcrystals having improper orientations to form good bonds at the lattice interface may be lost, leaving only those with the correct disposition. On the other hand, there may also be some orienting force exerted by the underlying lattice that directs approaching, or adsorbed crystals into an optimal match. This is speculative, admittedly, but not implausible given the complex electrostatic surfaces involved and the possibility of two-dimensional and rotational diffusion.

From the investigations presented here, which are fairly representative of others that we have carried out, we can draw a number of conclusions. First, small crystals and three-dimensional nuclei are routinely captured and incorporated directly into larger growing crystals. As one would expect, this occurs increasingly at higher supersaturations. It occurs without dissolution and restructuring at the lattice interfaces, i.e., the smaller crystals are incorporated intact. In most cases the smaller crystals are misaligned with respect to the larger crystal lattice and their incorporation proceeds with significant, even extensive defect generation that includes various types of spiral dislocations, planar dislocations, and macrosteps.

In some cases, small and larger mates exhibit a mutual lattice orientation, and incorporation proceeds by the knitting together of the two crystals without noticeable defect generation. At high supersaturation, this process may occur with very high frequency, as for cubic STMV crystals for example, and growth by addition of three-dimensional nuclei may become the dominant growth mechanism.

The process of microcrystal capture and incorporation, such as we visualize here using AFM, is very rare for conventional crystals. In those cases the lattice strain is generally intolerable and major defects form. Growth by addition of three-dimensional nuclei and microcrystals having preferred orientations is unheard of in conventional crystals grown from solution. Biological macromolecule crystals appear to be unusually elastic and accommodating in this regard. Nonetheless, the creation of extensive, severe defects, as we visualized here for most cases, could not help but weaken the mechanical strength of such crystals, introduce major internal deformations, and degrade the useful properties of the crystals such as their ability to diffract X-rays.

ACKNOWLEDGMENTS

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