

# UC Irvine

## UC Irvine Previously Published Works

### Title

Macromolecular crystal growth as revealed by atomic force microscopy

### Permalink

<https://escholarship.org/uc/item/41d9n6p6>

### Journal

Journal of Structural Biology, 142(1)

### ISSN

1047-8477

### Authors

McPherson, Alexander  
Kuznetsov, Yu G  
Malkin, Alexander  
[et al.](#)

### Publication Date

2003-04-01

### DOI

10.1016/s1047-8477(03)00036-4

### Copyright Information

This work is made available under the terms of a Creative Commons Attribution License, available at <https://creativecommons.org/licenses/by/4.0/>

Peer reviewed



ACADEMIC  
PRESS

Available online at [www.sciencedirect.com](http://www.sciencedirect.com)

SCIENCE @ DIRECT®

Journal of Structural Biology 142 (2003) 32–46

Journal of  
Structural  
Biology

[www.elsevier.com/locate/yjsbi](http://www.elsevier.com/locate/yjsbi)

# Macromolecular crystal growth as revealed by atomic force microscopy

Alexander McPherson,\* Yu.G. Kuznetsov, Alexander Malkin, and Marco Plomp

*Department of Molecular Biology and Biochemistry, University of California at Irvine, 560 Steinhaus Hall, Irvine, CA 92697-3900, USA*

Received 5 February 2003

## Abstract

Direct visualization of macromolecular crystal growth using atomic force microscopy (AFM) has provided a powerful tool in the delineation of mechanisms and the kinetics of the growth process. It has further allowed us to evaluate the wide variety of impurities that are incorporated into crystals of proteins, nucleic acids, and viruses. We can, using AFM, image the defects and imperfections that afflict these crystals, the impurity layers that poison their surfaces, and the consequences of various factors on morphological development. All of these can be recorded under normal growth conditions, in native mother liquors, over time intervals ranging from minutes to days, and at the molecular level.

© 2003 Elsevier Science (USA). All rights reserved.

*Keywords:* Virus crystals; Protein crystals; Crystallization; Probe microscopy; Molecular imaging

## 1. Introduction

The process of crystallization occurs in two stages, which we refer to as nucleation and growth. Because the former involves spontaneous, singular events that are unpredictable in time and place, nucleation is difficult to directly visualize and characterize by most imaging techniques, including atomic force microscopy (AFM). It is best studied using neutron or small-angle X-ray scattering from solution (Tardieu et al., 2001) or light scattering methods (Asherie et al., 1996; Malkin and McPherson, 1993, 1994; George and Wilson, 1994). Growth of crystals, on the other hand, occurs at predictable sites on the surfaces of existing crystals, and the size range and time scale are ideally suited to direct visualization and recording by AFM. As a consequence, in the past several years AFM has emerged as a powerful tool for the direct imaging and analysis of crystal growth events and their associated phenomena (McPherson et al., 1995, 2000, 2001). These are of substantial interest to X-ray crystallographers because the degree of order associated with different growth mechanisms, and

the defects arising from the incorporation of a myriad of different types of impurities, in large part determines or influences diffraction resolution, mosaic spread of reflections, and the mechanical stability of crystals. The application of AFM to the study of macromolecular crystal growth has been reviewed in some detail elsewhere (McPherson et al., 2000, 2001), but a summary here of results obtained from AFM studies may provide a useful physical context for understanding many of the day-to-day observations and problems of X-ray crystallographers and those who grow protein crystals.

## 2. How AFM works

AFM instruments, as shown schematically in Fig. 1, are conceptually simple in comparison with X-ray diffraction systems (Binnig et al., 1986). Instruments can be operated in either contact mode or tapping mode. In contact mode, a probe made of silicon nitride is placed in contact with the surface of interest and then translated in a systematic, raster mode over the surface. The AFM probe is a sharp stylus, similar to a minute phonograph needle, which has a tip radius of about 5 to 40 nm, though even sharper tips using carbon nanotubes

\* Corresponding author. Fax: 1-949-824-1954.

E-mail address: [amphers@uci.edu](mailto:amphers@uci.edu) (A. McPherson).

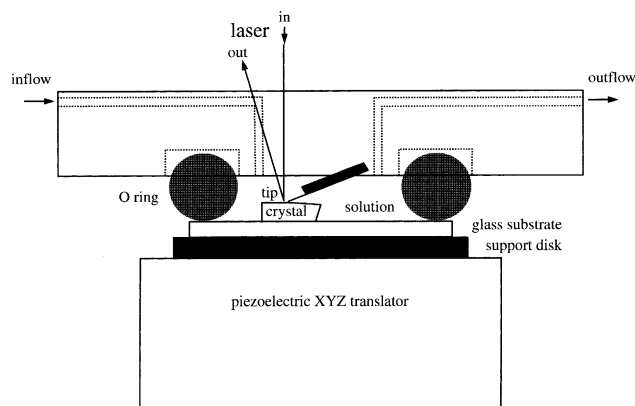


Fig. 1. Schematic diagram of an atomic force microscope. The vertical deflection of the cantilever tip is amplified through a reflected laser beam and reported by a photoelectric detector. Scanning takes place in a fluid-filled cell of about 75  $\mu$ l volume. The sample is translated in a raster manner by a piezoelectric positioner upon which the fluid cell is mounted.

are currently under development (Hafner et al., 2001; Wooley et al., 2000). The probe is mounted at the end of a short cantilever, typically 100–250  $\mu$ m in length, which has a low spring constant ( $<1$  N/m) to minimize the force between the tip and the sample. Scanning is achieved by translating the sample beneath the probe, using piezoelectric positioners, along a continuous sequence of raster lines beneath the probe. As the probe tip passes over the surface, it interacts through “aggregate atomic forces” with structural features on the surface. The encounters cause the probe to be displaced vertically. Exceedingly small displacements are amplified by deflection of a laser beam which is reflected from the upper surface of the probe, and these deflections are then detected by a split photodiode. Photoelectric circuitry converts the deflections into height information (Binnig et al., 1986). The resulting data, recorded as a digital topological image, can then be presented in a number of visual formats. Protein crystals can be investigated by AFM in their mother liquor, as they grow. The interaction of the cantilever tip with the surface does not appear to perturb or damage the sample, nor does it affect the events which transpire during crystal growth.

The AFM can be operated in either “height” or “deflection” mode. With the former, a feedback mechanism constantly adjusts the heights of the sample and maintains the cantilever position nearly constant. The system monitors the piezo height and produces the corresponding image information. In deflection mode, the piezo remains stationary and actual cantilever deflection data are recorded. When the AFM is operated in height mode quantitatively accurate information on surface morphology is obtained. Deflection mode of operation can be particularly useful for imaging surface features which vary widely in height over the field of observation.

Cantilevers can exert a significant pressure on the substrate surface and, as one might expect, the quality of the image depends on the degree of force employed. The greater the force between tip and surface, the more sensitive the probe is to height variations. On the other hand, too great a force will damage the surface. While this may not be a severe limitation for hard surfaces such as conventional crystals, it is a major consideration when dealing with relatively soft biological materials.

Problems arising from unfavorable probe–surface interactions have been obviated to some extent by the development of “tapping” mode instruments (Hansma et al., 1994). With tapping mode, the probe tip is not in continuous contact with the sample but oscillates up and down as it is scanned over the surface, essentially “tapping” its way and gently sensing the heights of obstacles it encounters. In tapping mode the vertical position of the sample is continually adjusted by the feedback mechanism to maintain the amplitude of the freely oscillating probe as a constant. Tapping mode minimizes contact between the probe tip and the sample surface and greatly reduces lateral forces. The tapping technique has proven to be a significant advance as it has permitted the visualization of materials that would otherwise be too soft to tolerate contact mode examination.

### 3. Sample preparation and data acquisition

AFM can yield images of crystal surfaces having remarkable clarity and detail. Scan fields may range in size from less than 20 nm up to about 150  $\mu$ m, with a spatial resolution on biological materials of a few nanometers and a height resolution better than 1 nm. Thus it provides precise visual detail over a size range that eludes most other techniques. Its application extends over the range of individual macromolecules, which are accessible by X-ray crystallography, to macromolecular assemblies amenable to electron microscopy, to living cells which can just be seen using light microscopy (Allen et al., 1997; Bustamante and Keller, 1995). Because visualization is carried out in a fluid environment, the specimens suffer no dehydration, as is generally the case with electron microscopy. Growing crystals can be observed over long periods so long as they remain immobilized. No fixing or staining is necessary. Contrast depends only on height variation.

The value of AFM does not lie in its imaging capability alone, but from the nonintrusive nature of the probe interaction with the sample surface. Because the specimen is ignorant of the probe’s presence, natural processes, such as transport and incorporation of molecules, appear to be minimally affected. Thus, the investigator can record not simply a single image, but a

sequence of images that may extend over many minutes, hours, or even days. This is ideal for the study of macromolecular crystal growth, which develops over just such periods. Imaging frequency depends on the scan rate of the probe. As scan speed increases, so does pressure on the sample surface that may produce damage, particularly for soft materials. For protein crystals, images are usually collected with a period of 0.5 to 5 min. For macromolecular crystal growth, a relatively slow process at low-to-moderate supersaturations, events on the surface impose no requirement for rapid scan speed. Thus an extended series of good-quality images is generally accessible to the investigator.

When AFM is carried out in fluid cells, the fluid can be changed during the course of experiments without appreciably disturbing the specimen. This is of real value in the study of protein crystallization because a common objective is to monitor growth processes under various degrees of supersaturation. Growth steps are visible on the surfaces of crystals, and because their advancement is relatively slow, their rate of progression over the surface can be recorded in a sequence of images. When rates are recorded as a function of temperature, salt

concentration, supersaturation, or some other variable, then growth step velocities can be used to deduce thermodynamic and kinetic parameters such as the step free energy and the kinetic coefficient (Kuznetsov et al., 1999a,b; Land et al., 1997; Malkin et al., 1996a, 1997a; Yau et al., 2000). In the best of cases, even individual protein molecules can be observed as they are recruited into advancing step edges (Kuznetsov et al., 1999a; McPherson et al., 2000).

In the AFM analysis of macromolecular crystallization some practical problems are common. Because biological crystals are fragile and often difficult to manipulate, and because scanning occurs in an aqueous environment, it may be difficult to fix the crystals to a substrate. This can be overcome by nucleating and growing the crystals directly on the substrate, i.e., in situ analysis, or by “clamping” larger crystals to the substrate beneath flexible carbon fibers. The greatest difficulty in obtaining images is the softness of the crystals and their susceptibility to scarring by the AFM tip. Tapping mode operation alleviates this problem in some instances, but even then softness may set the limit of resolution. Some crystals, such as lysozyme or thaumatin, are relatively hard and resistant to probe damage,

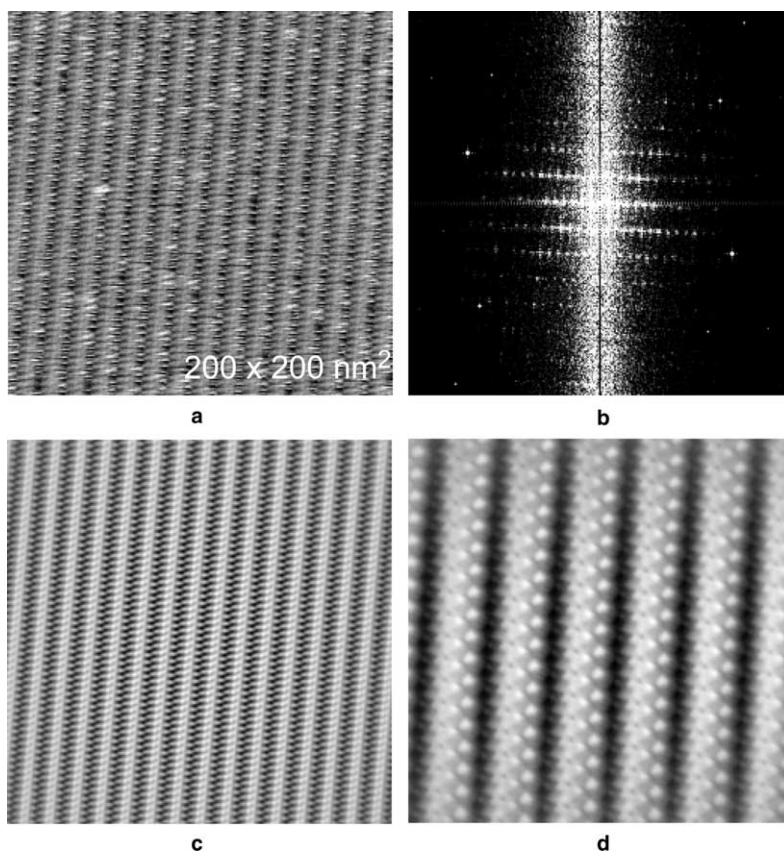


Fig. 2. Fourier filtering of an AFM image of a thaumatin crystal. In (a) is shown the raw AFM image and in (b) its Fourier transform, which contains high-order reflections that extend beyond 10 Å resolution. When intensity not corresponding to Bragg reflections is removed, the structure factors can be recombined through a Fourier synthesis to yield the filtered image in (c), which has in the process been both averaged and cleared of noise. A magnified area of the filtered image in (c) is shown in (d), in which individual thaumatin molecules become evident.

while crystals of large viruses, on the other hand, may be fragile and difficult to deal with.

With the exception of their softness and fragility, macromolecular crystals are otherwise excellent systems for studying the general phenomenon of crystal growth. The particle size is relatively large, 3- to 10-nm diameter for most proteins, many times that for viruses. This is an order of magnitude or more larger than conventional molecules that crystallize. Thus aggregates can be seen on the surfaces of crystals (Kuznetsov et al., 1999a; Land et al., 1997; Malkin et al., 1999a), and even the mobility of individual molecules on the crystal surface can be recorded. The kinetics of growth of macromolecular crystals are several orders of magnitude slower than for conventional crystals, thus the course of events during growth is compatible with the temporal resolution of the instrument. Unit cells are one to two orders of magnitude larger than for conventional crystals, and this dramatically enhances the definition of growth steps, dislocations, incorporation of impurities, and defect structure.

Macromolecular crystals are, of course, periodic. This is helpful because the eye averages when their images are examined, and otherwise minor features become evident. In addition, the underlying periodicity makes possible application of Fourier filtering and averaging processes that can yield improved images (Brisson et al., 1999; Kuznetsov et al., 1997). AFM may,

in the best of cases, yield lattice resolution images of crystals that even reveal some gross features of the macromolecules in the unit cells. From such images, it may be possible to deduce packing arrangements or even molecular orientations. Li et al. (1999) and Kuznetsov et al. (1999a), for example, used AFM to analyze packing arrangements on the faces of lysozyme and thaumatin crystals, respectively, and from these deduced the ordered pathways for molecule incorporation. Because height information is preserved, enantiomorphic space groups can be resolved. For large asymmetric units, as occur for example in virus or ribosome crystals, it may be possible to derive initial phase information from the particles seen in their fully hydrated, crystalline state.

The resolution of AFM images is visually apparent in direct images of crystal surfaces, particularly in the case of virus crystals. In many of these, for which the particle sizes range from 16 to 50 nm, considerable substructure is clearly evident, and even capsomeres can be directly seen on virions (Lucas et al., 2001; Malkin et al., 1999b; McPherson et al., 2000). An even better measure of resolution, however, can be obtained from Fourier transforms of raw AFM images, like that in Fig. 2, and the filtered reconstructions. In such transforms Bragg reflections occasionally extend beyond 10-Å spacings. Thus AFM provides the next step up from X-ray diffraction in structural biology.

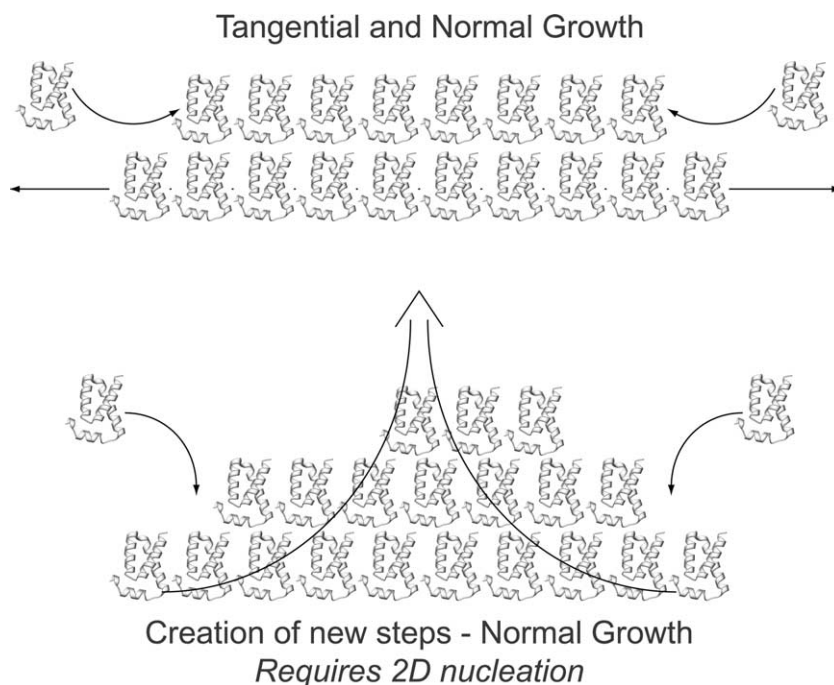


Fig. 3. Tangential and normal growth. Schematic diagram showing tangential growth by the addition of molecules to step edges, causing their lateral extension, and normal growth proceeding by the creation of nascent layers atop those already present. Growth normal to the surface requires two-dimensional nucleation on the crystal surface and therefore must overcome an energy barrier. Thus, normal growth may cease well before tangential growth.

#### 4. Mechanisms of crystal growth

As illustrated in Fig. 3, there are two processes that must occur for the growth of any crystal (Chernov, 1984; Chernov et al., 1988), which we refer to as crystal face growth and tangential growth. Growth normal to the surface proceeds by the initiation of new layers, or islands in most cases, which exhibit step edges to which new molecules can be added. Because creation of new layers requires, in the absence of dislocations (see below), the appearance of a new, ordered arrangement where none previously existed, i.e., atop the preexisting layer, it is a kind of nucleation event. Nucleation, which represents a phase transition, generally occurs with difficulty and requires surmounting an energy or probability barrier. Thus they are usually the rate-limiting steps in most physical and chemical processes, including crystallization. The initiation of new layers, then, is the slower, more difficult process in crystal growth.

Tangential growth refers to the recruitment of molecules into step edges and the extension of new layers over the surface. This is, relatively, a much easier process because the incorporation of a new individual is essentially a cooperative process favored by both the molecules composing the existing step edge and the new recruit. The energetics of the situation favors the union. Thus, once a nascent layer appears, its two-dimensional expansion may proceed unimpeded. Indeed, if we look at the surfaces of a crystal which has stopped growing, we see that there are no islands or step edges remaining on the surface, it is flat. The last available step edge has expanded over the surface to the very limits of the crystal, while the barrier to the formation of new layers cannot be overcome.

A property that dominates virtually all aspects of crystal growth, macromolecular and otherwise, is the degree of supersaturation of the mother liquor. Virtually all kinetic and thermodynamic variables are dependent upon supersaturation (Chernov, 1984; Chernov et al., 1988). This includes the probability of forming critical nuclei, that is, the birth of a new crystal; initiation of new layers on an existing surface; the velocity of step movement on the surface (tangential growth); the incorporation of impurities (Chernov, 1984; Chernov et al., 1988; Rosenberger et al., 1996) and a host of other processes. Even the particular kind of mechanism that is employed in growth on a crystal surface is dependent on supersaturation. Supersaturation in turn is, of course, a function of an array of experimental variables such as salt concentration, protein concentration, temperature, or other physical and chemical factors. It is also dependent on the underlying physical and chemical properties of the protein molecules and the manner by which they interact with one another.

There are four principal mechanisms that have been described for the development of the faces of macro-

molecular crystals (Malkin et al., 1995). It should be noted, however, that different faces of a single crystal, being nonidentical, might simultaneously employ different mechanisms for development. Furthermore, a single face may exhibit more than one mechanism at the same time, and the type of mechanism may change as some experimental variable, such as temperature, is altered (Kuznetsov et al., 2001a; Ng et al., 1997). Thus, when only one or a few observations of a growth mechanism are available for a particular crystal, this by no means implies that other mechanisms are not involved under other conditions of supersaturation. Most crystals, it seems, utilize all mechanisms at one time or another, though some one mechanism may be favored.

Over a broad range of supersaturation, most protein crystals generate step edges, new growth layers, through a process of two-dimensional nucleation. Undoubtedly guided by the underlying lattice, molecules from solution adhere to the surface and organize themselves into ordered arrays consistent with the preexisting layer. These molecules are also free to leave the surface as well, but when the organized array exceeds some critical size, the balance of events changes to favor addition, and the growth island persists and expands by recruitment of molecules from solution into its step edges. The first event, two-dimensional nucleation, provides growth normal to the surface, the latter process, tangential growth. Crystal surfaces growing by two-dimensional nucleation often appear to be littered with growth islands as seen in Fig. 4. In many cases, the shapes of the growth islands reflect the geometries of the morphological face on which they are present. This is also illustrated in Fig. 4. AFM allows the investigator to observe the changes in the islands as a function of time, thereby permitting the calculation of step movement rates which, at specified supersaturations, provide important thermodynamic parameters (Chernov, 1984; Chernov et al., 1988; Kuznetsov et al., 1995, 1999a, Land et al., 1997; Plomp et al., 2001).

Using AFM, the heights of growth steps on surfaces can easily be measured to a precision of a few angstroms, and frequently they correspond to a single unit cell dimension, though this depends on the symmetry elements present in the crystal. Crystals tend to grow by initiating and completing discrete crystallographic unit cells at the step edges rather than starting and filling cells here and there on an island or along an edge. The question as to whether molecules add individually to the advancing step edge or by ordered aggregates corresponding to an entire unit cell, or discrete portions thereof, can be answered by examining changes in the fine structure of step edges in high-magnification, high-resolution images. This has been done in the case of thaumatin crystals and it revealed that step edges do appear to advance by addition of individual molecules and not by the addition of pre-formed assemblies or clusters (Kuznetsov et al., 1999a).

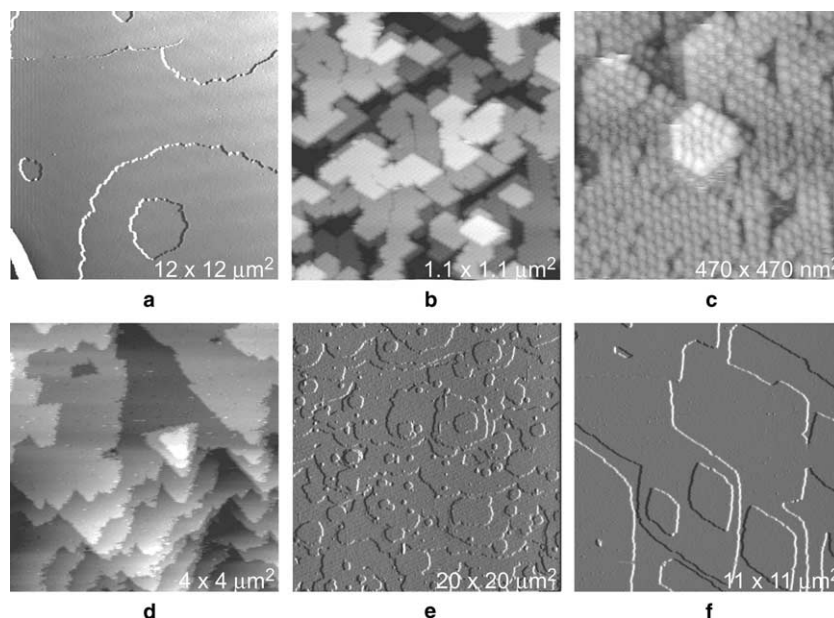


Fig. 4. Examples of two-dimensional nuclei, or islands, on the surfaces of growing macromolecular crystals: (a) Orthorhombic satellite tobacco mosaic virus (STMV), (b and c) a cubic STMV crystal, (d) a turnip yellow mosaic virus crystal, (e) a thaumatin crystal, and (f) a crystal of glucose isomerase.

The latter process could occur in some instances, but it is not a dominant mechanism.

The heights of growth islands are not always a single crystallographic unit cell, however, and some interesting exceptions have been recorded in those cases in which the crystal possesses some screw axis or some combination of screw axes. Orthorhombic crystals of beef liver catalase, for example, develop by formation of growth islands corresponding in height to exactly one half-unit cell (Malkin et al., 1997b). Thus layers of molecules corresponding to the “bottom halves” and “top halves” of unit cells are deposited alternately.

The shapes of two-dimensional islands depend largely on the surface energies associated with their various edges, but their orientations are specified by the symmetry of the crystal. This is particularly evident when screw axis symmetry is present. For example, when a  $2_1$  axis is present perpendicular to a surface, then the sequential growth islands assume orientations that are alternately  $180^\circ$  disposed to one another. As seen in Fig. 5a, this was the case for orthorhombic beef liver catalase crystals (Malkin et al., 1997a). In Fig. 5b is a trigonal crystal of trypsin viewed along the  $3_1$  axis and in Fig. 5c, a tetragonal crystal of Bence Jones protein seen along the  $4_3$  axis. In those two cases the growth islands are oriented alternately at  $120^\circ$  and  $90^\circ$ , respectively (Plomp et al., 2003).

Two-dimensional islands, whose development with time can be recorded, as in Fig. 6, generally do not advance at equal rates in all directions. This is so because the step edges present a different structure, that is, display different bonding possibilities in different directions. Molecules from solution are recruited into step edges at

different points on the island boundaries according to the energetics of association at those points and, therefore, at different rates. Impurities, which affect the rate of step advancement as well, are also incorporated with different affinities at the periphery of the growth islands, and these too alter recruitment rates, leading to further asymmetric shapes for the islands.

An interesting question in all areas of crystal growth, including that of macromolecules, is what size a nucleus must be in order to persist and develop. Nucleation, of course, represents a phase transition and is, therefore, of significance even beyond crystallization phenomena. Critical nuclear size is dependent on the particular molecule, the intermolecular interactions driving association, and the degree of supersaturation. While it is difficult to use AFM to study the formation of three-dimensional “critical nuclei” directly from solution, the two-dimensional nuclei that form on existing crystal surfaces, and which give rise to new growth layers, are readily visualized (Malkin et al., 1993, 1996b, 1995; Yau and Vekilov, 2000). Because these exhibit properties of nuclei that initiate new crystals, their analysis is of substantial interest. Using AFM to observe the number of unit cells comprising individual two-dimensional nuclei and simply recording whether they persist over time or disappear, and by carrying out these observations at different supersaturations, the sizes of critical nuclei as a function of supersaturation can be determined. This has been done for several crystals and has yielded some important quantitative information related to the bonding energies and assembly properties that govern the formation of nuclei.

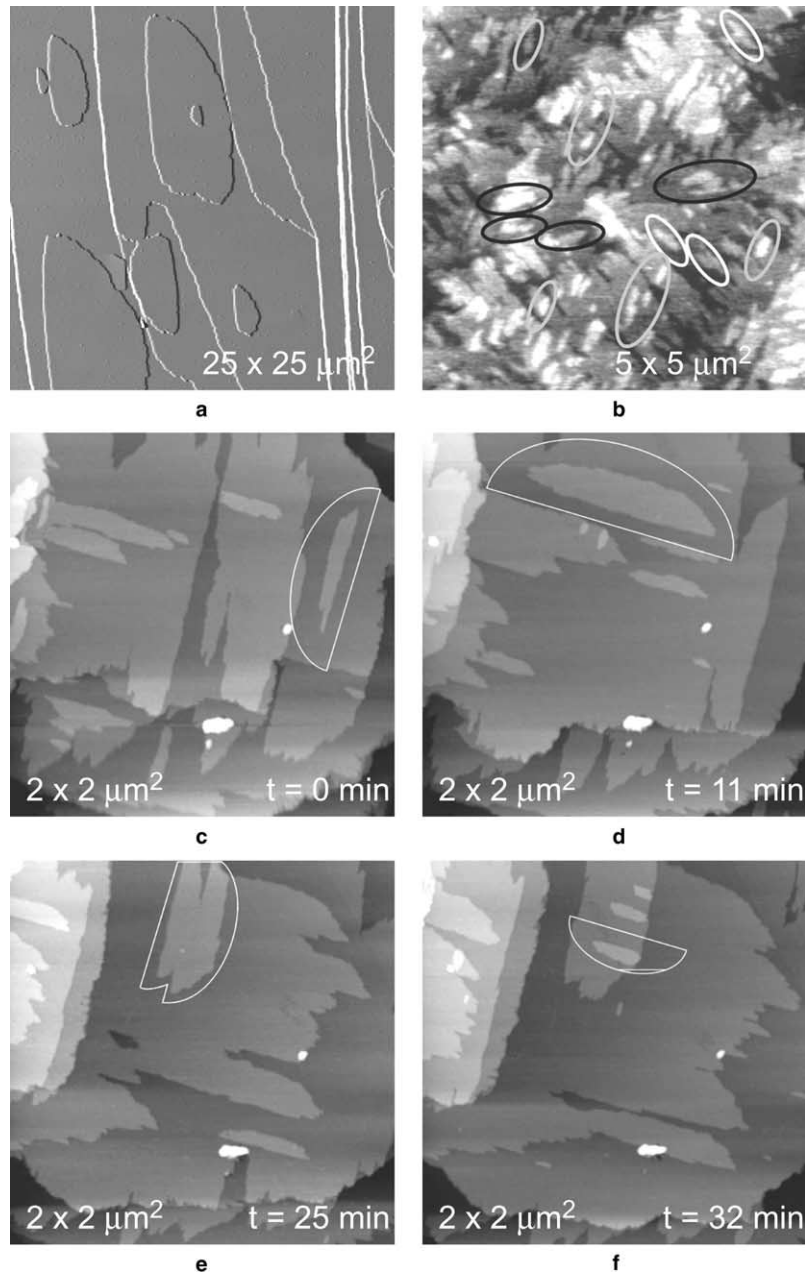


Fig. 5. Illustration of the asymmetric shapes and rotating orientations assumed by two-dimensional nuclei or islands on growing crystal surfaces perpendicular to a crystallographic screw axis. (a) A beef catalase crystal with a perpendicular  $2_1$  axis, (b) a trigonal beef trypsin crystal with a perpendicular  $3_1$  axis, and (c–f) the four positions assumed by the asymmetric islands in tetragonal Bence Jones protein crystals for the surface exhibiting a perpendicular  $4_3$  axis.

Another mechanism, very common to crystals of conventional molecules, is growth through the creation of step edges at dislocations in the lattice (Burton et al., 1951; Frank, 1949). These arise when, for one reason or another, perhaps incorporation of a contaminant or misincorporation of one or several molecules, a discontinuity occurs along the direction normal to the surface. At such points, steps are continuously propagated in a spiral about the dislocation, hence their name, screw dislocations. The salient difference between growth by

this mechanism and growth through formation of two-dimensional islands is that face growth arising from spiral dislocation step generation is more readily facilitated since no two-dimensional nucleation event is necessary to initiate a new layer. The spirally produced step edges continually provide nascent layers and the crystal grows almost exclusively by tangential addition of molecules to step edges.

An array of screw dislocations from various crystals is shown in Fig. 7. In most cases, the spirals, like the



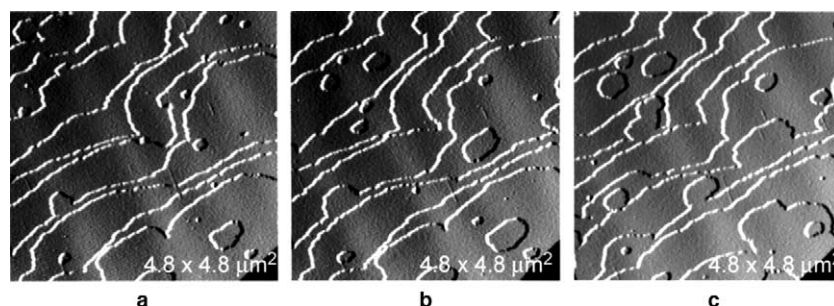


Fig. 6. A sequence of three images recorded at 2-min intervals of a small area on the surface of a growing thaumatin crystal showing the development with time of two-dimensional islands and their merging to create new growth layers.

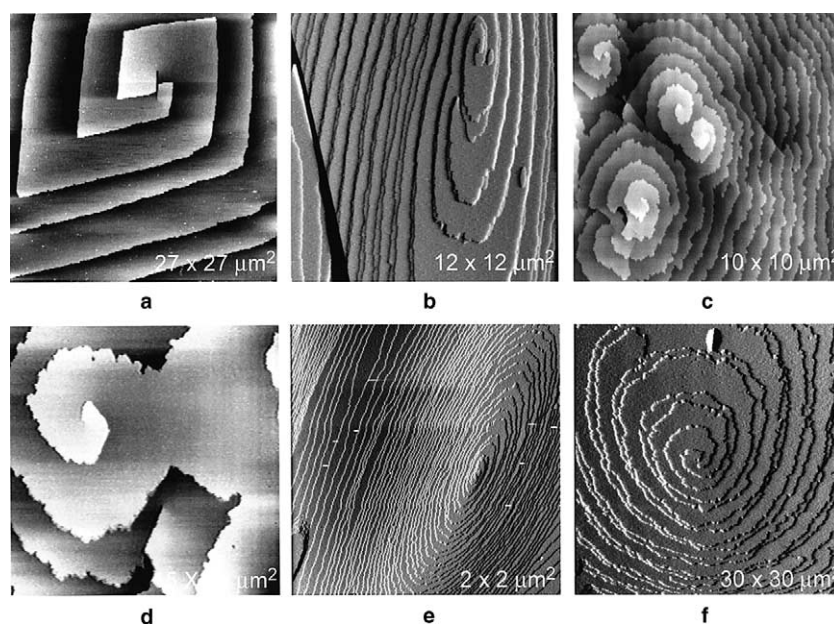


Fig. 7. AFM images of screw dislocations on the growth surfaces of a variety of macromolecular crystals. (a) Glucose isomerase, (b) lysozyme, (c, d) canavalin, (e) lysozyme, and (f) beef trypsin.

growth islands discussed above, are asymmetric in shape and for the same reasons. Spirals may be left-handed or right-handed depending on the nature of the dislocation, and a single crystal surface will often exhibit both. Spirals may be single or double at the dislocation, and these more complex spirals may also have either hand. Although screw dislocations have been observed on the surfaces of nearly all protein and nucleic acid crystals examined, they have not been seen on any virus crystal to date. AFM allows us to record the development of screw dislocations just as we can do so for two-dimensional islands.

Not all protein crystals have an equal propensity to form screw dislocations, presumably due to differences in their mechanical properties. Some crystals, like those of beef liver catalase, exhibit none at all (Malkin et al., 1997a), while the surfaces of rhombohedral canavalin crystals are crowded with them (Ko et al., 2001; Land et al., 1997; Malkin et al., 1995). The appearance of

screw dislocations appears to be crystal dependent, a function of its mechanical properties, rather than dependent on molecular structure. While rhombohedral canavalin is thick with screw dislocations, the orthorhombic and hexagonal forms of the same protein are virtually dislocation free.

On many crystals, particularly at higher supersaturations at which growth is rapid and tends to become somewhat disorganized, large macrosteps, like those illustrated in Fig. 8, consisting of stacks of growth layers, are common. Although individual layers grow independently by molecular addition to their step edges, except through competition of their diffusion fields (Land et al., 1997), the macrosteps tend to move like organized waves over the surfaces of crystals. It is remarkable that when the growth layers of one macrostep encounter those of another, the corresponding individual layers within the two macrosteps seem to merge and form a flawless union.

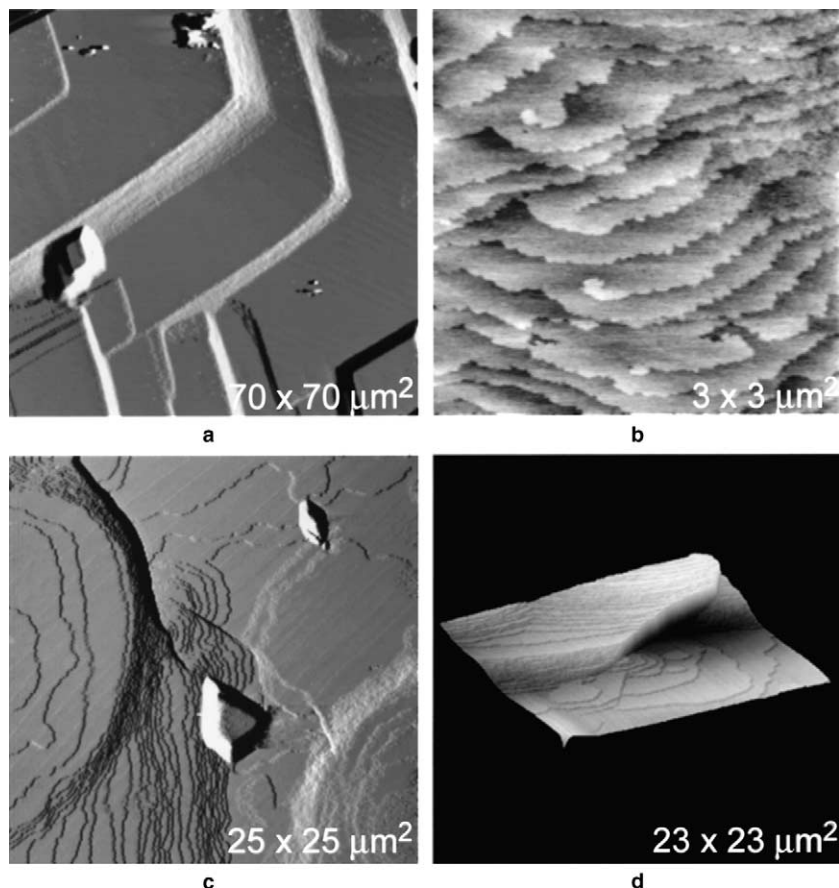


Fig. 8. AFM images of macrosteps on the surfaces of growing macromolecular crystals. (a) Glucose isomerase, (b) brome mosaic virus, (c) cubic STMV, and (d) thaumatin. In (d) the AFM image is rendered in three dimensions, which is an alternative mode of presentation available for any AFM image.

Most crystals, including macromolecular crystals, grow by addition of smooth layers, one atop another, through addition of molecules to the edges of the layers. As described above, these layers may be generated by two-dimensional nucleation or by screw dislocations. Another mechanism for crystal development, called normal growth, does not proceed by layer addition, but by random recruitment of molecules at arbitrary sites on the surface. In a sense, molecules are joining the lattice everywhere on the surface at once. This kind of crystallization is characteristic, for example, of conventional crystals grown from the melt. This leads to atomically “rough” surfaces as opposed to the atomically “smooth” surfaces yielded by layer growth.

Macromolecular crystals have been observed by AFM to grow by this normal mechanism, in which cases the surface appearance becomes extremely rough and irregular as shown for the ferritin crystals in Fig. 9. Growth by this mechanism, in the regime of high supersaturation, is disorganized and produces, as one might expect, crystals of poor quality, though ultimate size is not necessarily constrained. Ferritin crystals, for example, grow to very large size.

A mechanism that may be unique to macromolecular crystal growth is illustrated in Fig. 10. It has not been described previously for conventional crystals, and it

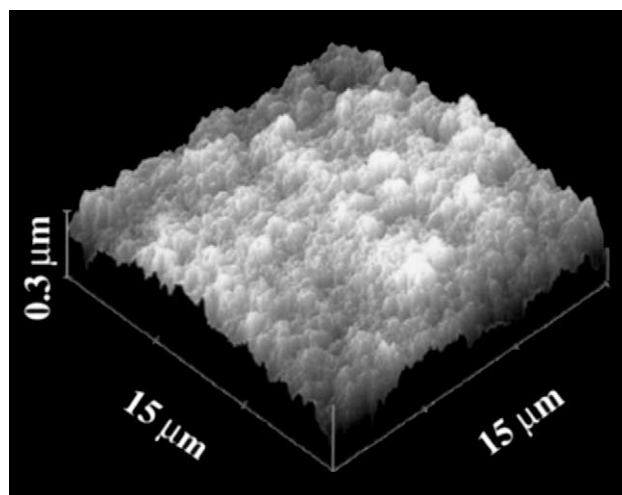


Fig. 9. An AFM image of a small area of a growing crystal of horse spleen ferritin under conditions of very high supersaturation under which the normal mechanism dominates. The surface is extremely irregular and rough and the crystals obtained under these conditions diffract very poorly.

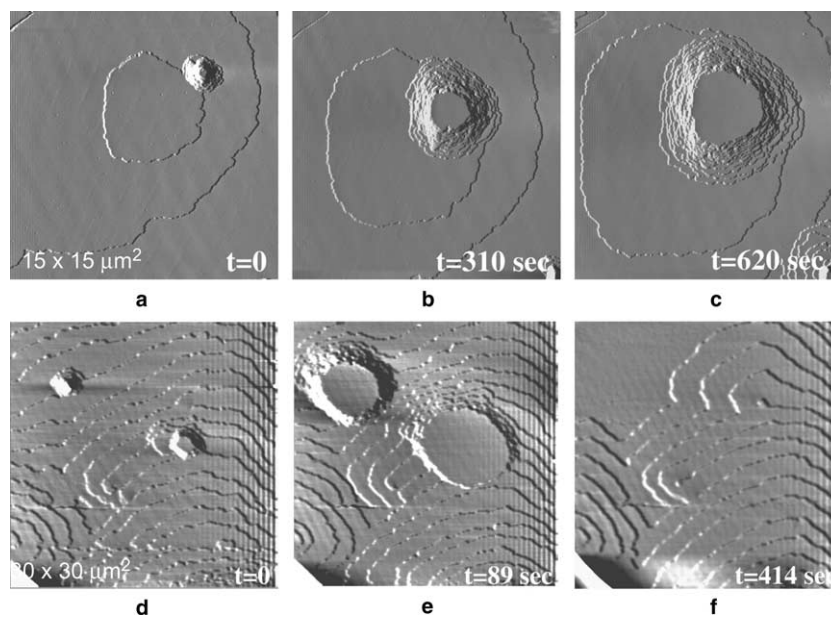


Fig. 10. Sequences of images showing the development with time of three-dimensional nuclei on the surfaces of growing macromolecular crystals. In (a–c) is shown a stack of growth layers on a cubic STMV crystal at the border of a tangentially expanding two-dimensional island. In (d–f) are shown two three-dimensional nuclei that expand tangentially and ultimately merge in a faultless manner (f) into continuous layers.

likely arises as a consequence of the unique properties of concentrated macromolecular solutions, i.e., mother liquors. For virtually all of the protein, nucleic acid, and virus crystals investigated, the spontaneous appearance of vast, multilayer stacks of growth layers on crystal surfaces has been observed. Often these hillocks, whose characteristic shapes frequently reflect the gross morphologies of their crystals, are ten to a hundred or more layers in height. Each layer of the stack provides step edges and, therefore, sources for tangential growth and the formation of new layers. Growth by this mechanism, which has been termed growth by three-dimensional nucleation, can in some cases be the dominant growth mechanism (Malkin et al., 1995). It is noteworthy that, when tangential growth of layers proceeds simultaneously from several multilayer stacks on the surface of a crystal, the corresponding layers from the various stacks ultimately encounter one another, merge, and knit in a completely contiguous manner.

An intriguing question is the origin of these multilayer stacks. One possibility was that they arise from microcrystals preformed in solution that sediment on the surfaces and continue to grow. Such a phenomenon fails, however, to explain the perfect alignment of all of the stacks with the underlying lattice and with one another. A second explanation, for which there is now substantial and persuasive evidence, suggests that they arise from liquid protein phase droplets that exist in concentrated macromolecular solutions (Asherie et al., 1996; Kuznetsov et al., 1998; Lui et al., 1995). These liquid protein phase droplets are composed of many thousands of molecules exhibiting short-range order,

mediated principally by nonspecific hydrophobic interactions and random arrangements of hydrogen bonds. They are, in a sense, very large, disorganized protein aggregates seeking to become ordered. Because of the extraordinary concentration of molecules in the droplets, they are locally hypersaturated. When the droplets contact existing crystal surfaces, the lattice serves as an epitaxial substrate to guide and promote crystallization in the molecules above. These form a crystal layer, inspire crystallinity in the molecules above them, and so forth, propagating a continuous series of growth layers, a multilayer stack. For some crystals for which the process was relatively slow, this phenomenon has been visually recorded (Kuznetsov et al., 1998).

The existence of a liquid protein phase in concentrated protein solutions, the multilayer stacks discussed here being one manifestation, has been dealt with in greater detail elsewhere, and it is the source of much current interest in the field of colloids as well as crystal growth (Asherie et al., 1996; Haas and Drenth, 1999; Kuznetsov et al., 1999b; Lui et al., 1995; Ten Wolde and Frenkel, 1997). It was one of the more unexpected results to emerge from AFM studies of macromolecular crystal growth.

As discussed elsewhere (Malkin et al., 1996a,b; McPherson et al., 1996) macromolecular crystals are remarkably forgiving of contaminants, and they incorporate an extensive variety of impurities into their interiors. The catalog of those impurities is continuing to expand. Impurities may produce a diverse array of lattice defects, or they may create virtually no serious imperfections in the crystal at all. This largely depends on the nature of the

macromolecule, the type of impurity, and the physical forces responsible for maintaining the lattice. Generally, however, the effects of impurity incorporation are evident in AFM images, and these dislocations or distortions of the lattice are important, for they may significantly affect the diffraction properties of the crystal.

Some common effects of impurity incorporation are seen in Fig. 11. Step edges, whatever their source, are usually extremely rough and irregular, though ideally, they should be smooth. The roughness is a consequence of impurity molecules incorporating into the step edges and locally “pinning” step edge progression, thereby resulting in gaps and teeth in advancing layers (Chernov, 1984; Chernov et al., 1988). The ubiquity of irregular step edges attests to the very high level of impurities present in macromolecular crystals compared with most conventional crystallizing systems.

Vacancies, empty unit cells, partially filled unit cells, and even lines of missing particles or unit cells are also common and may represent up to 1% of all of the unit cells in a protein crystal (Malkin et al., 1996a). These are particularly evident in virus crystals

where single particles, because of their high symmetry, may correspond to an entire crystallographic unit cell. Lattice absences are present, however, in all macromolecular crystals. While individual absences may not drastically affect the diffraction or physical properties of crystals, their effects being rather localized, they are numerous.

More serious imperfections that produce long-range disorder, affecting many molecules in a crystal, are planar defects like those seen in Fig. 12. These are dislocation planes that may extend through hundreds or thousands of molecular layers in crystals and which displace great numbers of molecules in their immediate neighborhoods.

Planar dislocations in large numbers can form networks and create a system of domains within crystals. The relative number and displacements of these domains determine the mosaic character of crystals and the consequent mosaicity of the diffraction patterns. In conventional crystals these planar defects, and the long-range discontinuities they produce, are commonly referred to as grain boundaries.

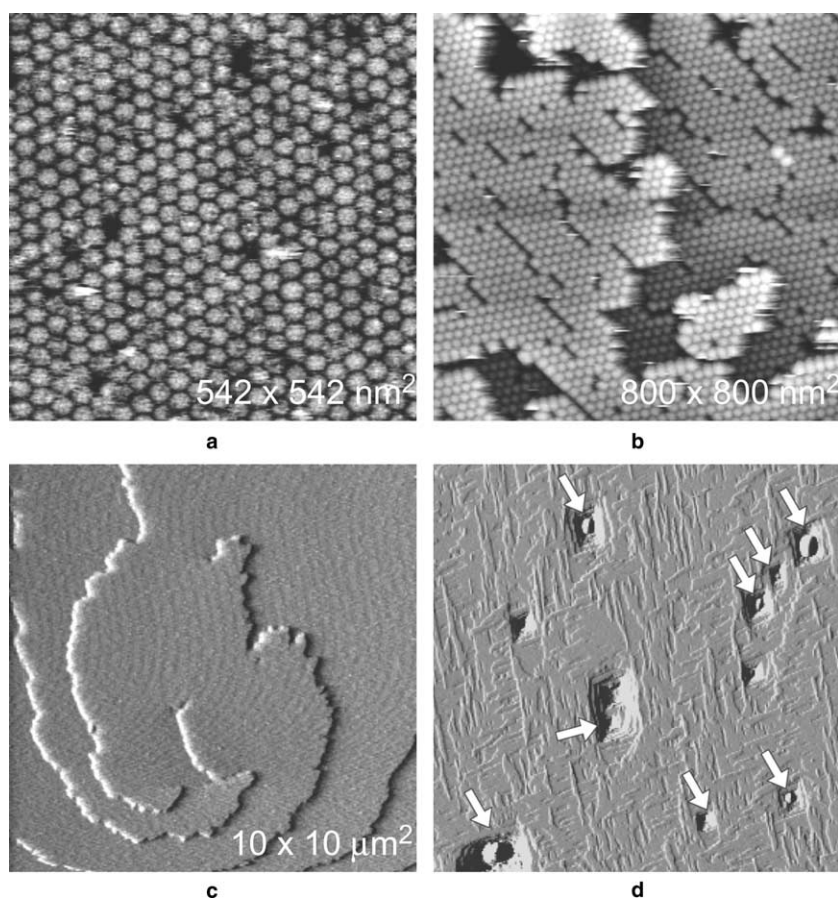


Fig. 11. Consequences of impurity incorporation are evidenced by the single absences, and lines of absences of particles in (a) crystals of brome mosaic virus and (b) orthorhombic STMV crystals. In (c) the very rough and irregular step edges emanating from a screw dislocation on the surface of a trypsin crystal are a result of the high level of contaminants present in the mother liquor. In (d), particulate impurities can be found in etch pits created on the surfaces of Bence Jones protein crystals at undersaturated conditions.

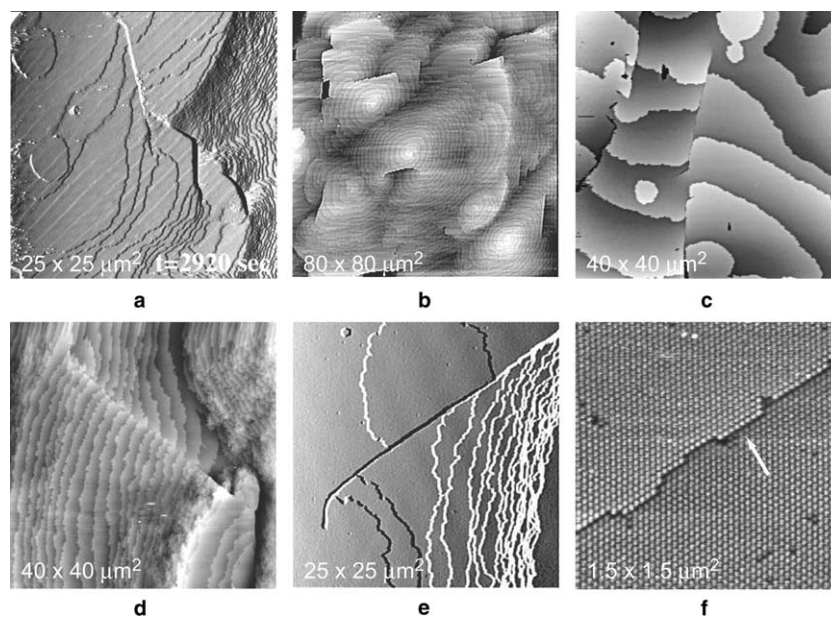


Fig. 12. Examples of stacking faults found in several macromolecular crystals. (a) Cubic STMV, (b) canavalin, (c) orthorhombic STMV, (d) trypsin, (e) thaumatin, and (f) cucumber mosaic virus.

Macromolecules and their crystals, because they are derived from biological sources, and because of their forgiving nature, are subject to an exotic variety of impurities seldom encountered in conventional systems (McPherson et al., 1996), and some are illustrated by the images in Fig. 13. We find not only foreign proteins and denatured or degraded macromolecules, but also their aggregates and even their microcrystals. All of these, as has been shown elsewhere (Malkin et al., 1996a,b; McPherson et al., 1996), can be incorporated in their entirety into growing protein, nucleic acid, or virus crystals. These are in addition to the dust particles and other foreign inorganic materials that may contaminate conventional mother liquors.

There have been some interesting, recent additions to the list of biologically derived impurities that enter into macromolecular crystals. For example, helical fibers, most probably actin or actin complexes remaining from dead microbes which contaminate macromolecular solutions, have been observed to be incorporated directly into the lattices of both protein and virus crystals (Kuznetsov et al., 2001b). These fibers are quite extraordinary because they always assume crystallographic directions by lying in the valleys between lattice rows. In being incorporated, they produce linear occlusions about themselves, and very distinctive linear defects that may affect hundreds of neighboring particles.

Another finding is that virus preparations are surprisingly microheterogeneous and frequently contain many versions that are both larger and smaller than the normal population. That is, a population of 28-nm-di-

ameter  $T = 3$  icosahedral particles may contain many  $T = 1$  particles of 17 nm diameter, as well as particles of higher  $T$  number and greater diameter. The most extraordinary thing is that even these unattractive misfits are nonetheless incorporated into growing virus crystals, somehow making sufficient suitable intermolecular interactions to ensure their stable entry into the lattice. Because of their size incompatibility, however, the anomalous particles can be incorporated only with the creation of various kinds of local vacancies and defects. This unique property of virus crystals to incorporate aberrant particles leads to the production of a vast array of packing defects and domain structures that are unknown in conventional crystals (Lucas et al., 2001; Malkin and McPherson, 2002).

In early studies, using AFM not only as an imaging device but as a mechanical tool as well, it was shown that scarring the impurity-contaminated surface of inactive, nongrowing lysozyme crystals could restore the appearance of two-dimensional growth islands (see McPherson, 1998). More recently, the surface morphology of Bence Jones protein crystals was investigated by Plomp et al. (2003), during growth and dissolution, using AFM. It was demonstrated that over a wide supersaturation range, impurities absorbed on crystalline surfaces and ultimately formed impurity absorption layers that prevented further growth of the crystal. At low undersaturations, this impurity absorption layer prevented dissolution. At greater undersaturation, dissolution took place around large contaminants incorporated into the crystal, leading to etch pits with impurity-free bottoms. Upon restoration of

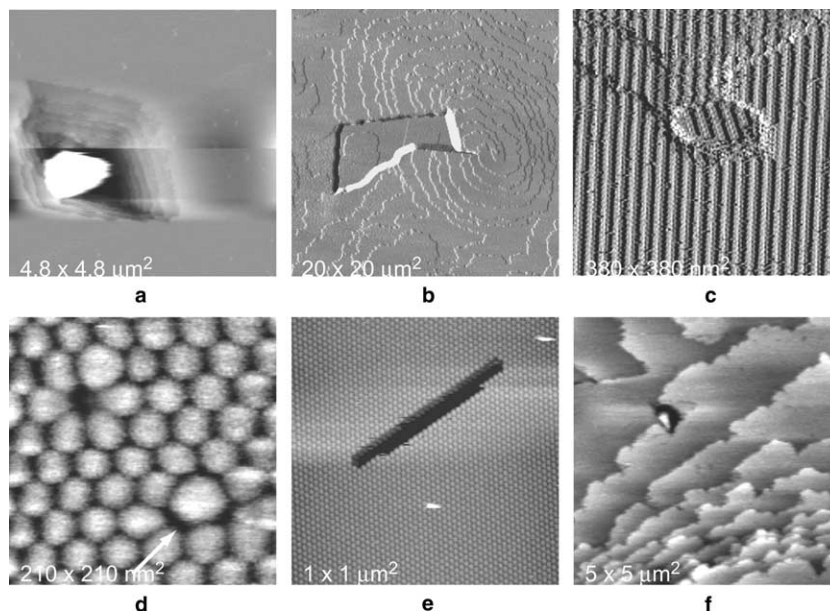


Fig. 13. Examples of the incorporation of unusual impurities and contaminants into growing macromolecular crystals. In (a) a dust particle is revealed at the bottom of an etch pit on the surface of a thaumatin crystal; in (b) incorporation of a large microcrystal into a growing canavalin crystal is shown. In (c), incorporation of a misaligned two-dimensional growth island into a thaumatin crystal is shown. In (d) is illustrated the incorporation of aberrant, oversized virus particles (one marked with an arrow) into a crystal of cucumber mosaic virus, in (e), occlusion evidencing the incorporation of a biologically derived fiber into a growing orthorhombic STMV crystal, and in (f) the incorporation of a large dust particle at a moving step edge on the surface of a rhombohedral canavalin crystal.

Table 1  
Lattice properties

Lattice	Tendencies
<b>Rigorous</b> Characterized by strong, numerous, geometrically well-defined bonds. Lattice is highly selective in terms of particle size and conformation and requires stringent particle homogeneity. Crystal faces have high surface free energies $\alpha$ and long-range order is very precise.	Accumulates lattice stress over long distances More sensitive to incorporation of impurities and anomalous particles Contains few linear and planar defects Low solvent content Low elasticity or deformability Mechanically strong but brittle, sensitive to freezing Growth is sensitive to purity Low kinetic coefficient $\beta^a$ Diffracts to high resolution with low overall thermal factor Has low mosaicity Does not readily dissolve Strong optical properties Thermodynamically favored
<b>Nonrigorous</b> Characterized by weak, sparse, and imprecise bonding. Lattice is very tolerant in terms of particle size and conformation and readily incorporates aberrant particles and contaminants. Crystal faces have low surface free energies $\alpha$ and long-range order is poor.	Dissipates lattice stress over long distances Less sensitive to incorporation of impurities and anomalous particles Contains many linear and planar defects; forms domains High solvent content High elasticity and deformability Mechanically weak and soft, less sensitive to freezing Growth less sensitive to purity High kinetic coefficient $\beta^a$ Diffracts to only low resolution with high overall thermal factor Has high mosaicity Readily dissolves Weak optical properties Kinetically favored

<sup>a</sup>The kinetic coefficient  $\beta$  is a measure of the rate of the ensemble of processes (e.g., transport, attachment) that lead to incorporation of a molecule into the crystal.

supersaturation conditions, two-dimensional nucleation resumed on the impurity-free bottoms of the etch pits. After new growth layers filled in the etch pits, they proceeded to cover the impurity-poisoned top layers of the crystal faces. This led to resumption of growth. Formation of impurity-absorption layers is very likely responsible for the termination of growth of macromolecular crystals that has been widely noted (Durbin and Feher, 1996; Feher, 1986). Growth–dissolution–growth cycles based on these observations could, in principle, be used to produce larger crystals that otherwise would have stopped growing due to impurity poisoning.

AFM studies demonstrate that macromolecular crystals have a broad range of properties and that they vary, as do conventional crystals, according to the molecules that comprise them and the interactions by which the molecules interact with one another. It is possible to describe two extremes between which most crystal lattices fall, and associate with those two extremes some defining characteristics. At one end of the spectrum are crystals whose macromolecules form numerous, strong, geometrically well-defined bonds in three dimensions. As a consequence, their lattices are highly selective in terms of particle size, integrity, and conformation. That is, they depend upon stringent particle or molecular homogeneity. They have a high surface free energy  $\alpha$ , and their long-range order is very precise. At the other end of the spectrum are crystal lattices whose macromolecules form sparse, weak, and geometrically imprecise bonds. Their lattices are promiscuously tolerant in terms of molecular size and conformation, and they readily incorporate aberrant particles and contaminants. The crystals have a low surface free energy, and long-range order is poor.

The properties that we might associate with the two kinds of lattices are shown in Table 1. From that collection of properties, that context, a tentative conclusion emerges. A rigorous lattice is sensitive to impurities and their incorporation produces high local stress and therefore much defect formation. In addition, stress due to microinhomogeneities in molecules or particles accumulates over long distances, and that stress is ultimately relieved by formation of severe defects, which include stacking faults, absences, screw dislocations, and the formation of domains. Nonetheless, within domains the lattice is very highly ordered, and as a result, the crystal diffracts to high resolution with low mosaicity.

A nonrigorous lattice, in contrast, is tolerant and indiscriminate in the molecules and particles it incorporates. It contains few severe defects and suffers little lattice strain. On the other hand, its accommodation produces poor long-range order and therefore low-resolution diffraction and poor mosaicity. The rigorous lattice acts as a filter to reject questionable particles, and it therefore grows slowly but diffracts well, despite its internal scars. The nonrigorous lattice accepts all who

come, suffers no wounds, but diffracts poorly as a result. Most lattices, of course, lie somewhere in between.

## References

- Allen, S., Davies, M.C., Roberts, C.J., Tendler, S.J.B., Williams, P.M., 1997. Atomic force microscopy in analytical biotechnology. *Trends Biotechnol.*, 15.
- Asherie, N., Lomakin, A., Benedek, G.B., 1996. Phase diagram of colloidal solutions. *Phys. Rev. Lett.* 77, 4832–4835.
- Binning, G., Quate, C.F., Gerber, C., 1986. Atomic force microscope. *Phys. Rev. Lett.* 56, 930–933.
- Brisson, A., Bergsma-Schutter, W., Oling, F., Lambert, O., Reviskine, 1999. Two dimensional crystallization of proteins on lipid monolayers at the air–water interface and transfer to an electron microscopy grid. *J. Cryst. Growth* 196, 456–470.
- Burton, W.K., Cabrera, N., Frank, F.C., 1951. The growth of crystals and the equilibrium structure of their surfaces. *Philos. Trans. R. Soc. London A* 243, 299–310.
- Bustamante, C., Keller, D., 1995. Scanning force microscopy in biology. *Phys. Today* 48, 32–38.
- Chernov, A.A., 1984. *Modern Crystallography. Crystal Growth*, Vol. III. Springer, Berlin.
- Chernov, A.A., Rashkovich, L.N., Smoliskii, I.L., Kuznetsov, Y.G., Mkrtchyan, A.A., Malkin, A.I., 1988. In: Givargizov, E.I., Grinberg, S.A. (Eds.), *Growth of Crystals*, Vol. 15. Consultant Bureau, New York, pp. 43–91.
- Durbin, S.D., Feher, G., 1996. Protein crystallization. *Annu. Rev. Phys. Chem.* 47, 171–204.
- Feher, G., 1986. Mechanisms of nucleation and growth of protein crystals. *J. Cryst. Growth* 76, 545–546.
- Frank, F.C., 1949. The influence of dislocation on crystal growth. *Discus. Faraday Soc.* 5, 48–54.
- George, A., Wilson, W.W., 1994. Predicting Protein Crystallization from a Dilute Solution Property. *Acta Crystallogr. D* 50, 361–365.
- Haas, C., Drenth, J., 1999. Understanding protein crystallization on the basis of the phase diagram. *J. Cryst. Growth* 196, 388–394.
- Hafner, J.H., Cheung, C.L., Wooley, A.T., Mieber, C.M., 2001. Structural and functional imaging with carbon nanotube. *Prog. Biophys. Mol. Biol.* 77, 73–110.
- Hansma, P.K., Cleveland, J.P., Radmacher, M., Walters, D.A., Hillner, P.E., Bezaniilla, M., Fritz, M., Vie, D., Hansma, H.G., Prater, C.B., Massie, J., Fukunage, L., Gurley, J., Elings, V., 1994. Tapping mode atomic force microscopy in liquids. *Appl. Phys. Lett.* 64, 738–740.
- Ko, T.-P., Kuznetsov, Y.G., Malkin, A.J., Day, J., McPherson, A., 2001. X-ray diffraction and atomic force microscopy analysis of twinned crystals: Rhombohedral canavalin. *Acta Crystallogr. D* 57, 829–839.
- Kuznetsov, Y.G., Konnert, J., Malkin, A.J., McPherson, A., 1999a. The advancement and structure of growth steps on thaumatin crystals visualized by atomic force microscopy at molecular resolution. *Surf. Sci.* 440, 69–80.
- Kuznetsov, Y.G., Malkin, A.J., Greenwood, A., McPherson, A., 1995. Interferometric studies of growth kinetics and surface morphology in macromolecular crystal growth: canavalin, thaumatin and turnip yellow mosaic virus. *J. Struct. Biol.* 114, 184–196.
- Kuznetsov, Y.G., Malkin, A.J., Land, T.A., DeYoreo, J.J., Barba, A.P., McPherson, A., 1997. Molecular resolution imaging of macromolecular crystals by atomic force microscopy. *Biophys. J.* 72, 2357–2364.
- Kuznetsov, Y.G., Malkin, A.J., McPherson, A., 1998. Atomic force microscopy studies of phase separations in macromolecular systems. *Phys. Rev. B* 58, 6097–6103.

- Kuznetsov, Y.G., Malkin, A.J., McPherson, A., 1999b. AFM studies on the mechanisms of nucleation and growth of macromolecular crystals. *J. Cryst. Growth* 196, 489–502.
- Kuznetsov, Y.G., Malkin, A.J., McPherson, A., 2001a. The influence of precipitant concentration on macromolecular crystal growth mechanisms. *J. Cryst. Growth* 232, 114–118.
- Kuznetsov, Y.G., Malkin, A.J., McPherson, A., 2001b. Self repair of biological fibers catalyzed by the surface of a virus crystals. *Proteins* 44, 392–396.
- Land, T.A., DeYoreo, J.J., Lee, J.D., 1997. An in situ AFM investigation of canavalin crystallization kinetics. *Surf. Sci.* 384, 136–155.
- Li, H., Perozzo, M.A., Konnert, J.H., Nadarajah, A., Pusey, M.L., 1999. Determining the molecular-packing arrangements on protein crystal faces by atomic force microscopy. *Acta Crystallogr. D* 55, 1023–1035.
- Lucas, R.W., Kuznetsov, Y.G., Larson, S.B., McPherson, A., 2001. Crystallization of bromo mosaic virus and  $T = 1$  bromo mosaic virus particles following a structural transition. *Virology* 286, 290–303.
- Lui, C., Lomakin, A., Thurston, G.M., Hayden, D., Pande, A., Pande, J., Ogun, O., Asherie, N., Benedek, G.B., 1995. Phase separation in multicomponent aqueous-protein solutions. *J. Phys. Chem.* 99, 454–461.
- Malkin, A.J., McPherson, A., 1993. Crystallization of pumpkin seed globulin: Growth and dissolution kinetics. *J. Cryst. Growth* 133, 29–37.
- Malkin, A., McPherson, A., 1994. Light scattering investigations of nucleation processes and kinetics of crystallization in macromolecular systems. *Acta Crystallogr. D* 50, 385–395.
- Malkin, A.J., McPherson, A., 2002. Novel mechanisms for defect formation and surface molecular processes in virus crystallization. *J. Phys. Chem.* 106, 6718–6722.
- Malkin, A.J., Cheung, J., McPherson, A., 1993. Crystallization of satellite tobacco mosaic virus. I. Nucleation phenomena. *J. Cryst. Growth* 126, 544–554.
- Malkin, A.J., Kuznetsov, Y.G., Glantz, W., McPherson, A., 1996a. Atomic force microscopy studies of surface morphology and growth kinetics in thaumatin crystallization. *J. Phys. Chem.* 100, 11736–11743.
- Malkin, A.J., Kuznetsov, Y.G., Land, T.A., DeYoreo, J.J., McPherson, A., 1995. Mechanisms of growth for protein and virus crystals. *Nat. Struct. Biol.* 2, 956–959.
- Malkin, A.J., Kuznetsov, Y.G., Lucas, R.W., McPherson, A., 1999a. Surface processes in the crystallization of turnip yellow mosaic virus visualized by atomic force microscopy. *J. Struct. Biol.* 127, 35–43.
- Malkin, A.J., Kuznetsov, Y.G., McPherson, A., 1996b. Defect structure of macromolecular crystals. *J. Struct. Biol.* 117, 124–137.
- Malkin, A.J., Kuznetsov, Y.G., McPherson, A., 1996b. Incorporation of microcrystals by growing protein and virus crystals. *Proteins: Struct. Funct. Genet.* 24, 247–252.
- Malkin, A.J., Kuznetsov, Y.G., McPherson, A., 1997a. An in situ AFM investigation of catalase crystallization. *Surf. Sci.* 393, 95.
- Malkin, A.J., Kuznetsov, Y.G., McPherson, A., 1997b. An in situ investigation of catalase crystallization. *Surf. Sci.* 393, 95–107.
- Malkin, A.J., Kuznetsov, Y.G., McPherson, A., 1999b. In situ atomic force microscopy studies of surface morphology, growth kinetics, defect structure and dissolution in macromolecular crystallization. *J. Cryst. Growth* 196, 471–488.
- McPherson, A., 1998. *Crystallization of Biological Macromolecules*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- McPherson, A., Malkin, A.J., Kuznetsov, Y.G., 1995. The science of macromolecular crystallization. *Structure* 3, 759–768.
- McPherson, A., Malkin, A.J., Kuznetsov, Y.G., 2000. Atomic force microscopy in the study of macromolecular crystal growth. *Annu. Rev. Biophys. Biomol. Struct.* 29, 361–410.
- McPherson, A., Malkin, A., Kuznetsov, Y.G., Koszelak, S., 1996. Incorporation of impurities into macromolecular crystals. *J. Cryst. Growth* 168, 74–92.
- McPherson, A., Malkin, A.J., Kuznetsov, Y.G., Plomp, M., 2001. Atomic force microscopy applications in macromolecular crystallography. *Acta Crystallogr. D* 57, 1053–1060.
- Ng, J.D., Kuznetsov, Y.G., Malkin, A.J., Keith, G., Giegé, R., McPherson, A., 1997. Visualization of nucleic acid crystal growth by atomic force microscopy. *Nucleic Acids Res.* 25, 2582–2588.
- Plomp, M., McPherson, A., Larson, S.B., Malkin, A.J., 2001. Growth mechanisms and kinetics of trypsin crystallization. *J. Phys. Chem. B* 105, 542–551.
- Plomp, M., McPherson, A., Malkin, A.J., 2003. Repair of impurity-poisoned protein crystal surfaces. *Proteins*, 50, 486–495.
- Rosenberger, F., Vekilov, P.G., Muschol, M., Thomas, B.R., 1996. Nucleation and crystallization of globular proteins—What we know and what is missing. *J. Cryst. Growth* 168, 1–27.
- Tardieu, A., Finit, S., Bonnet, F., 2001. Structure of the macromolecular solutions that generate crystals. *J. Cryst. Growth* 232, 1–9.
- Ten Wolde, P.R., Frenkel, D., 1997. Enhancement of protein crystal nucleation by critical density fluctuations. *Science* 277, 1975.
- Wooley, A.T., Cheung, C.L., Hafner, J.H., Lieber, C.M., 2000. Structural biology with carbon nanotube AFM probes. *Chem. Biol.* 7, 193–204.
- Yau, S.T., Petsev, D.N., Thomas, B.R., Vekilov, P.G., 2000. Molecular level thermodynamic and kinetic parameters for the self assembly of apoferritin molecules into crystals. *J. Mol. Biol.* 303, 667–678.
- Yau, S.T., Vekilov, P.G., 2000. Quasi-planar nucleus structure in apoferritin crystallization. *Nature* 406, 494–497.