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The incorporation of large impurities into virus crystals

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Virus crystals can incorporate a wide range of unusual impurities, not possible for conventional crystals, or even most protein crystals because of the large size of their constituent particles. These impurities include anomalous virions, satellite viruses and biological fibers. Examples of several of these unusual impurities are presented here, along with some of the consequences for the crystal lattices. The high solvent content, the forgiving character of the lattice and the plasticity of the virions allow these incorporations to be possible.

1. Introduction

Previous studies using atomic force microscopy have shown that protein crystals incorporate a wide variety of impurities, which frequently produce defects in the lattice. These, in turn, degrade the quality of diffraction data collected from the crystals. The impurities are not restricted to contaminating proteins, but arise from a host of chemical, biochemical, and physical sources (McPherson, 1998; McPherson *et al.*, 1996, 2000). These may include, among others, aggregates of macromolecules, particles, microcrystals from the mother liquor, and misoriented two-dimensional nuclei (McPherson *et al.*, 1995, 1996; Malkin *et al.*, 1997).

We have further examined a variety of virus crystals using atomic force microscopy and present here some of the unique kinds of impurities that are incorporated into these crystals. Virus crystals are in most ways very similar to protein crystals, particularly crystals of very large proteins or protein complexes, but exhibit a number of unusual properties. The virus particles are icosahedral and therefore both highly symmetrical and approximately spherical. Viruses which can be crystallized have relatively large diameters, varying from about 170 Å for the satellite plant viruses (Ban *et al.*, 1995), to 300 Å for the most commonly studied plant and insect viruses, up to 1000 Å for more complex animal and bacterial viruses (Kaper, 1975; Casjens, 1985; Rossmann & Arnold, 2001; Johnson & Rueckert, 1997). As a consequence, the spaces between particles in the lattice also tend to be quite large, the solvent content of the crystals high, the lattice contacts between particles tenuous and few. The crystals are inclined to be physically soft and fragile, and because of the high particle symmetry and the frequently high symmetry of the crystals which this engenders, the optical properties of virus crystals are usually almost non-existent.

The large spacings between particles in the lattice, however, have other consequences. Virus crystal lattices tend to be very forgiving in that they appear capable of absorbing severe defects and other structural insults without cessation, or even extensive perturbation of their growth. For example, planar defects and absences are quite common. Also, because of the sizes of the interparticle spacings, virus crystals can absorb and incorporate large impurities of a diverse, and even unexpected nature that are not found at all, or at best only infrequently in conventional molecule crystals and protein crystals.

Atomic force microscopy, as we and others have previously shown (McPherson *et al.*, 1996; Malkin *et al.*, 1996*a*; Malkin, Kuznetsov *et al.*, 1995; Vekilov, 2002) provides an excellent technique for analyzing impurity incorporation and defect formation in macromolecular crystals. Its resolution is in the 1–5 nm range, even better in the vertical direction. It can be applied to macromolecular crystal growth

in a non-invasive manner, *in situ*. The growth solution can be altered over time while under continuous observation, and in the case of virus crystals, single particles can be visualized as they enter or leave the lattice at step edges (Malkin & McPherson, 2002, 2004; Malkin *et al.*, 2002; Kuznetsov *et al.*, 2003).

In this brief paper we present some unusual and we believe striking examples obtained by AFM of the incorporation of large impurities into virus crystals, and the consequences they produce. There are undoubtedly others that have not yet been recorded but which will likely emerge from future investigations.

2. Materials and methods

The images presented here were recorded on Nanoscope III atomic force microscopes using oxide sharpened silicon nitride tips. All recordings were made using tapping mode (Hansma & Hoh, 1994) in the crystal's native mother liquor. The techniques used here have been detailed in previous papers (McPherson *et al.*, 2000; Kuznetsov *et al.*, 1997; Malkin, Land *et al.*, 1995) and need not be repeated. The growth conditions for the different virus crystals and their crystallographic unit cells have also been described for STMV (Koszelak *et al.*, 1989), TYMV (Canady *et al.*, 1995), CMV (Malkin & McPherson, 2002), BMV (Lucas *et al.*, 2001) and PMV (Makino *et al.*, 2005).

3. Results

In Fig. 1 are examples of the kinds of defects commonly found in virus crystals as a consequence of impurity incorporation. These include absences in the lattice, lines of absences, planar defects, and localized defects. The density of these defects in virus crystals is very high, and can be estimated to be 10^3 to 10^5 times greater than those present in conventional crystals, and probably 10 to 10^2 higher than in most protein crystals (Malkin *et al.*, 1996*a*; Malkin & McPherson, 2002). The question we sought to address is the nature of the impurities that produce these defects.

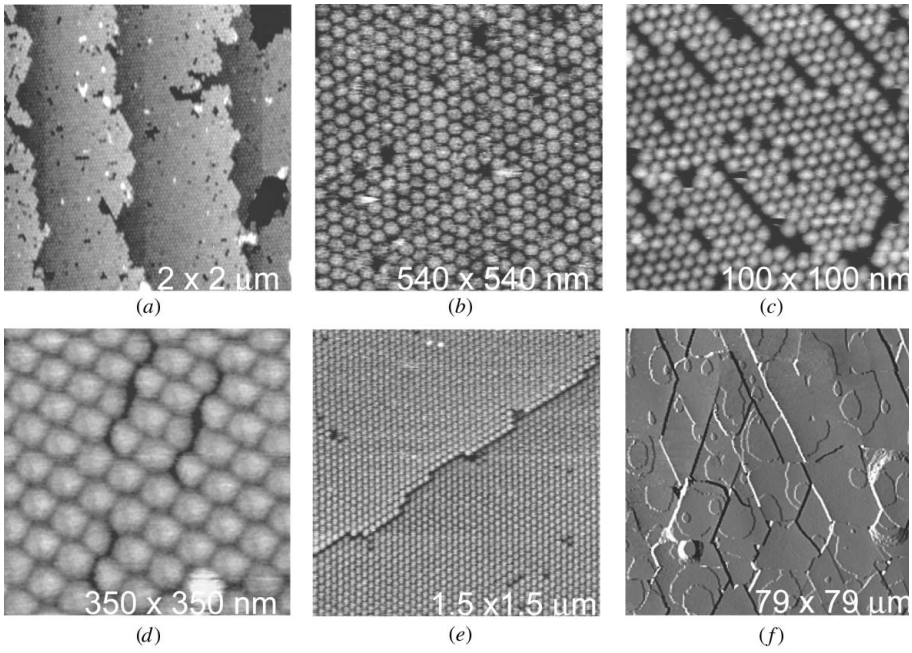


Figure 1
Examples of defects in virus crystals. In (*a*) are advancing step edges on a crystal of brome mosaic virus (BMV) and in (*b*) the surface of a BMV crystal. The layers are pocked with vacancies, and the step edges are extremely rough and irregular, reflecting the excessive incorporation of impurities as they progress. In (*c*) lines and clusters of absences characterize a satellite tobacco mosaic virus (STMV) crystal. In (*d*) are local dislocations of limited extent and in (*e*) a long planar dislocation on the surface of a cucumber mosaic virus (CMV) crystal. In (*f*), the network of stacking faults permeating an STMV crystal produce a mosaic block structure in the crystal.

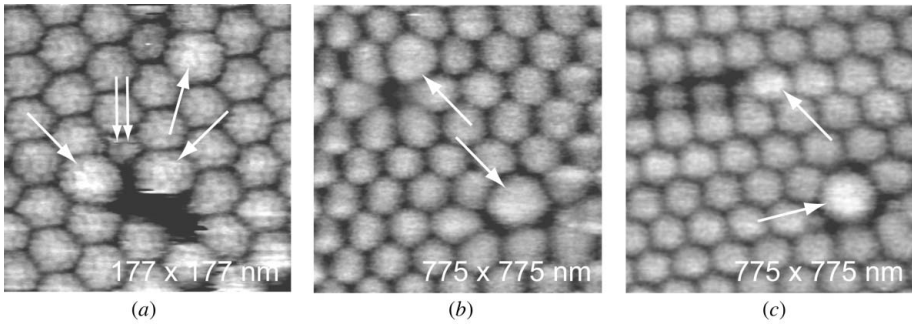


Figure 2
In (*a*) the incorporation of anomalously large virions (single arrows) and an aberrant small particle (double arrows) produce a local defect in a CMV crystal. In (*b*) two defect producing, oversize virions in a BMV crystal and in (*c*) anomalously large virions are incorporated into the lattice of a CMV crystal producing two distinctive defects in the packing.

One of the most common sources of the absences and localized defects are virions of irregular size that are nonetheless incorporated into the crystal lattice. These anomalous particles may be either smaller, or more commonly larger than the general population. Indeed, we have observed in a number of investigations that purified virus preparations often are rank with individuals of odd size. Presumably, these are aberrant particles arising from assembly errors and include virions of higher or lower triangulation (T) numbers, multishelled virions, virions lacking nucleic acid, or malformed, non-icosahedral particles. Fig. 2 provides examples of the incorporation of such anomalous virions.

The remarkable thing is that such virions are incorporated into the lattice at all, as the contacts with neighbors, because of particle size mismatch, must be quite different than for other virions in the crystal. Yet, they appear to be firmly embedded and stable in the lattice. Also noteworthy, is that when such overweight or underweight particles enter the crystals, the disturbance to the lattice is surprisingly limited and localized. Generally, the orientations and positions of only a few virions in the immediate neighborhood are affected, and only a single point defect or absence results. But these events are quite common.

Similar in the effects they produce to aberrant size virions is the incorporation of damaged virions. An example of this is shown for a hexagonal crystal form of turnip yellow mosaic virus (TYMV) in Fig. 3. Here, a misformed particle introduces a localized lesion in the lattice, again restricted to a small volume of the crystal and disturbing only a few neighbors.

Fig. 4 illustrates a truly unusual example of impurity incorporation. Panicum mosaic virus (PMV), which infects barley and

grasses, has a T = 3 virion of about 300 Å diameter. PMV infection, however, is often accompanied by coinfection with a small satellite virus (SPMV) which is completely dependent on PMV for its replication (Ban & McPherson, 1995). Preparations of PMV, even after purification, still contain significant amounts of the T = 1 SPMV, the satellite virus having a diameter of only 170 Å, and *vice versa*. As can be seen in Fig. 4, the smaller satellite virus fits nicely into the interstices between particles of the T = 3 virus in its monoclinic (pseudo cubic) crystal. Because of the exactness of fit, the random incorporation of the satellite virus between the larger particles of the PMV master virus causes no visible dislocations or defects.

An even more dramatic, and certainly more disruptive example of crystallization from a mixed virus preparation is shown in Fig. 5. Here, a preparation of satellite tobacco mosaic virus (STMV), a T = 1 icosahedral particle of diameter 170 Å, is heavily contaminated by its helical, rod-shaped helper virus, tobacco mosaic virus (TMV).

In spite of the extensive presence of the long (1000 Å) rod-shaped TMV, the satellite virus proceeds to crystallize in its orthorhombic form. TMV are arbitrarily incorporated into the crystals, a remarkable phenomenon, but with substantial disruption of the order of the underlying lattice. Thus, even crystallization fails to completely remove the TMV, though, as the STMV is successively, recrystallized, the amount of TMV is substantially reduced. With recrystallization, the defect density declines as well, and the diffraction properties of the STMV crystals improve.

Fig. 6(*a*) shows a better studied, and previously described (Malkin *et al.*, 1996*b*, 1997) case, and this is the incorporation of microcrystals into a growing crystal. This has been seen many times in simple protein crystals like canavalin (McPherson *et al.*, 1996; Land *et al.*, 1995) and catalase (Malkin *et al.*, 1997), and is equally common in virus crystals such as the STMV crystals shown here. Incorporation of microcrystals in the size range of 1 µm has been recorded, as well as smaller crystals. Fig. 6(*b*) is an example of the incorporation of a small, misaligned two-dimensional island into the lattice of a T = 3 virus, cucumber mosaic virus (CMV). In most cases, incorporation of microcrystals produces more wide ranging and severe dislocations to the lattice such as planar defects.

A final example of impurity incorporation is presented in Fig. 7, and this too has been described previously (Kuznetsov *et al.*, 2001). Nonetheless, it deserves mention here as well. Small protein fibers, apparently degradation products of contaminating microorganisms present in mother liquors, accumulate over the course of storage of crystallization samples. These fibers, probably actin or a similar protein polymer, remain after microbes die and disintegrate. The fibers, because of their small size, are not removed by conventional microfiltration. As seen in Fig. 7, these fibers, which may be as much as several hundred nanometers length, sediment on developing surfaces of virus crystals, in this case crystals of STMV. An interesting feature of this process is that the fibers always lie in, and align themselves with the linear spaces between rows of virions in the underlying lattice. Thus, when they subsequently become incorporated into the crystal, they do so having a discrete set of crystallographic orientations. For the cubic STMV crystals, as in Fig. 7,

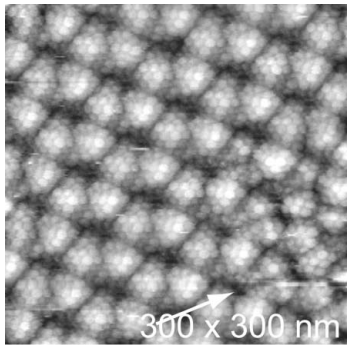


Figure 3
The incorporation of a damaged virion causes a local disruption in the lattice of a crystal of turnip yellow mosaic virus (TYMV).

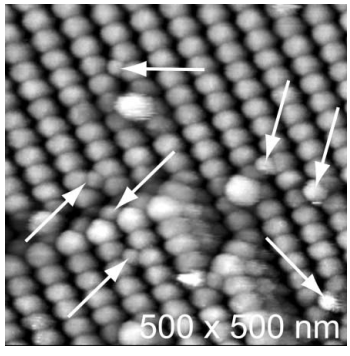


Figure 4
On the surface of a crystal of Panicum mosaic virus (PMV) the arrows indicate virions of satellite Panicum mosaic virus (SPMV) that are being incorporated between PMV particles making up the lattice. The PMV are T = 3 icosahedra, and have 300 nm diameter virions, while the SPMV are T = 1 icosahedra, with 17 nm diameter virions.

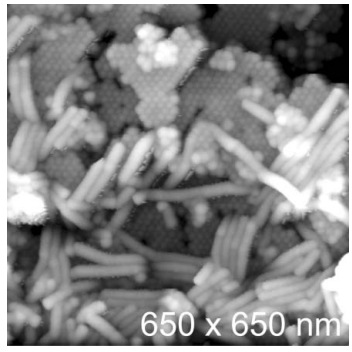
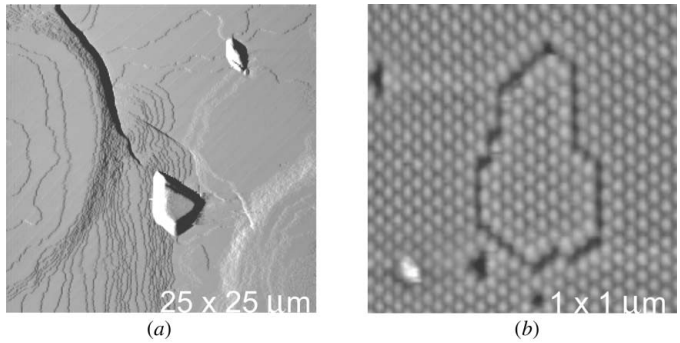


Figure 5
A crystal of STMV, whose ordered lattice is evident in the background, grows even in the presence of very large amounts of the helical tobacco mosaic virus (TMV) seen in the foreground. Many of these TMV particles will be incorporated as impurities into the STMV crystal.



(*a*) (*b*)

Figure 6
In (*a*) two microcrystals sedimenting on the surface of a growing STMV crystal are about to be swept into the crystal by a large macrostep approaching from the left. In (*b*) a small misaligned block of crystalline CMV particles is incorporated into the surface of a larger CMV crystal. Incorporation of small crystals, sometimes as large as a micrometre in dimension, into large, rapidly growing virus crystals is a common phenomenon.

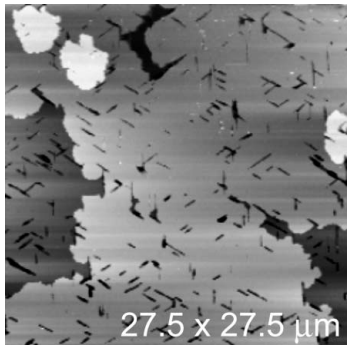


Figure 7
Fibers derived from microbes are seen extensively contaminating the surface of a growing STMV crystal. As seen here, the fibers are readily incorporated into the growing virus crystal where they produce linear defects along crystallographic directions. This is due to their lying in the furrows between virus particles making up the crystal lattice.

which grow by development of octahedral faces, these are the 111 and equivalent directions.

In addition to the examples presented here, and which are more or less unique to virus crystals, the other types of impurities common to protein crystals are also incorporated. These likely include clusters of virus particles, non-proteinaceous macromolecules, and inorganic impurities acquired from the environment.

4. Discussion

Unlike conventional crystals, macromolecular crystals permit the incorporation of a wide variety of impurities having sizes comparable to, or even much larger than the asymmetric units comprising the crystals. This is possible only because of the forgiving nature of macromolecular crystals which are able to sustain far higher defect densities, without long-range disruption of the lattice. Virus crystals, for reasons already set down, are even more accommodating.

In the examples presented here, a remarkable range of anomalous particles, coinfecting viruses, biological materials, and other sorts of contaminants are seen to be readily incorporated. This attests to the striking ability of the crystals to respond by local deformations in packing, formation of alternate networks of bonds between neighboring particles, and likely as well to the plasticity of the virions themselves which alter their shapes slightly to accommodate curious partners.

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