Imaging of viruses by atomic force microscopy


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Introduction

Two techniques that have proven particularly useful for elucidating the detailed structures of viruses have been electron microscopy (EM) and X-ray crystallography. These techniques have led to our current descriptions of icosahedral and helical viruses and the delineation of capsomere arrangements and protein subunit distributions on their surfaces (Horne & Wildy, 1961; Caspar & Klug, 1962). For this, EM relied principally on heavy metal staining or shadowing of virus particles dried in a vacuum (Finch & Holmes, 1967; Horne & Wildy, 1963). More recently, however, cryogenic EM has permitted more detailed analysis of virus particles and, in many cases, even their interior structure for about the last ten years. Its great advantages over other imaging methods are that it can be carried out in a fluid environment, including physiological medium, and that it does not disturb the specimen from its natural state. Furthermore, it spans a range of dimensions from a nanometre up to a hundred microns, which is only marginally accessible by other techniques.

AFM technology

Fig. 1 is a schematic diagram of an atomic force microscope for imaging in fluids. The principles that define it are rather simple, surprisingly so given its acuity, but the instrument depends on associated advanced technologies for its application. In practice, an exceedingly sharp probe is brought into atomic contact with the surface of the sample object, in vacuum, air or liquid, and it is then scanned in a systematic manner over the surface of the object. The stylus, similar to a very sharp phonograph needle with a tip radius of about 5 to 40 nm, extends from the end of a short cantilever, typically 100 to 250 µm in length. To minimize the force between the tip and sample in ‘contact’ mode, the cantilever has a low spring constant of less than 1 N/m.

Scanning using contact mode is accomplished by translating the stylus, which is under piezoelectric control, along a series of raster lines. As the probe tip moves over the surface, it interacts through aggregate atomic forces with topological features on the surface. As a consequence of these interactions, the probe is deflected. Cantilever displacements are amplified by corresponding deflections of a laser beam reflected into a split photodiode from the upper surface of the probe. Photoelectric circuitry then converts the deflections into height information. The resulting data, recorded as a digital image, can then be presented in a number of different visual formats.

AFM may be operated in either ‘height’ mode or ‘deflection’ mode. In height mode, the sample surface is maintained at a constant distance from the tip of the probe by the piezoelectric positioner below by a feedback mechanism. The cantilever deflection in this case is very small, tending to zero. In deflection mode, the sample is stationary and cantilever deflection data are collected directly.

Microfabricated cantilevers exert a force on the substrate surface and, as one might anticipate, the resolution of the technique depends on the degree of force employed. The greater the force between the probe and the surface, the more sensitive the probe is to surface variations. On the other hand, the greater the force, the more the probe will disturb the surface. Sample damage thus becomes an issue.

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Fig. 1. A schematic diagram of an atomic force microscope used for imaging viruses in liquids. AFM fluid cells, sealed by O rings, are probed by a silicon nitride, ultra-sharp stylus, which moves in a raster manner over the surface of specified objects. A reflected laser beam amplifies and reports deflections of the stylus, attached to a cantilever, to a split photodiode.

Problems arising in imaging in contact mode from unfavourable probe–surface interactions, particularly lateral force, have been overcome to some extent by the development of what are known as tapping mode instruments (Hansma et al., 1994). In ‘tapping’ mode, the probe tip is not in continuous contact with the surface (referred to as contact mode), but oscillates rapidly up and down as it is scanned over the surface, essentially tapping its way gently, as with a blind man’s cane, but firmly and rapidly, and sensing the height of features it encounters.

In tapping mode, the feedback mechanism adjusts, through the piezoelectric positioner, the vertical height of the sample surface in order to keep the amplitude of the freely oscillating probe constant. Tapping mode minimizes the contact between the cantilever tip and the sample surface and it greatly reduces lateral forces. As with contact mode, cantilevers made of silicon nitride, which have a low spring constant, are used for tapping mode operation in liquids. For some specimens, it is preferable to examine virus samples dried in air. For tapping mode imaging of air-dried samples, a stiffer cantilever made of silicon is used, which has a high spring constant of about 50 N/m.

This tapping mode approach has proven to be a significant blessing to biological researchers, as it has allowed the characterization of samples that would otherwise be too soft or too fragile to withstand contact mode examination. Operating in a liquid environment presents some complications due to fluid dynamics, but these are not severe. A constraint that sometimes presents obstacles is that the specimen under study must be fixed to, or made to adhere firmly to, the substrate surface of the fluid cell, which may be glass, cleaved mica, plastic or any other hard material. To achieve this, it may be necessary to treat the substrate with various reagents in order to induce better adhesion of samples. If this condition is not met, the specimen will move due to interaction with the probe and no useful information will be gathered.

AFM can be applied to scan fields ranging in size from less than 20 nm up to about 150 µm and with a spatial resolution on soft biological materials, in the best of cases, of about 2 to 3 nm, with a height resolution as great as 0.5 nm. Its application extends over the range lying between individual macromolecules, which are accessible by X-ray crystallography, macromolecular assemblies, amenable to EM, and living cells, which can be seen using light microscopy (Allen et al., 1997; Bustamante & Keller, 1995). Because visualization is carried out in a fluid environment, specimens suffer no dehydration, as is generally the case with EM, and they require no fixing or staining. Indeed, specimens can be observed over long periods as long as they stay relatively immobilized. For the most part, specimens seem to be oblivious to the presence of the probe tip.

The measurement of particle size and the dimensions of features of individual particles must be treated with some care. Lateral sizes of individual particles adsorbed onto mica appear considerably larger than might be expected because the image obtained is the convolution of the AFM tip shape with that of the particle. That is, the tip is not infinitely sharp and it has finite width. Thus, the curved surface immediately adjacent to the absolute tip causes vertical displacement of the cantilever and, therefore, gives rise to edges in the image before, as well as after, the absolute tip encounters the object. This does not, however, affect the total vertical displacement of the cantilever. As a consequence, single objects visualized by AFM appear broader than their true dimensions but yield an accurate and precise vertical dimension. For roughly spherical particles, such as icosahedral viruses, although their diameter appears greater than in fact the case, the vertical height of the particles gives a remarkably accurate value for their true diameter.
Many viruses with dimensions ranging from 17 to 150 nm whose capsid sizes were accurately established by EM, light scattering or X-ray crystallography have now been examined. Images of these particles consistently showed them to have lateral dimensions of about 2.5 times their actual diameter, which serves as a reasonable rule of thumb. In all cases, however, heights measured by AFM were accurate to within a few per cent. Therefore, precise free particle diameters must be based on vertical measurements.

In the case of virus particle crystals, the situation is different. With virus crystals, each particle is embedded in a lattice composed of similar members. As the AFM tip passes over the surface, the tip never approaches the ‘bottom’ of a single virion (i.e. a surface equivalent to the flat mica substrate) before it encounters a neighbouring virion. Thus, the height of the particle in crystalline form does not give a valid measure of its true diameter. However, it is a simple matter to measure the centre-to-centre distances of the virus particles in the lattice and, because the virions are, in general, closely packed, these distances do yield precise diameters of better than a few per cent. Fourier transformation of the lattice arrays (Kuznetsov et al., 1997) can improve these values.

More distinct images of virus particles can generally be obtained from virus crystals, rather than from single, free particles adsorbed onto mica or glass. This is not due to any averaging process or Fourier filtering, but seems to be an inherent property of the technique. Presumably, the immobilization of particles and their physical stability is responsible for the quality of the crystal’s images. When crystals cannot be obtained, slowly drying the virus particles onto the mica substrate may induce them to form aggregates and clusters, often ordered into closely packed, two-dimensional para-

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**Fig. 2.** (a) STMV from solution has been adsorbed onto mica and visualized as a field of 17 nm diameter particles. (b) Surface of a cubic STMV crystal. (c, d) Small areas on the surface of an orthorhombic STMV crystal, where only the barest indications of capsid substructure are visible.
crystals. These sometimes serve nearly as well as crystals. In these semi-ordered arrays, the centre-to-centre distance of particles also provides a reasonably accurate estimate of diameters.

**Examples of AFM images of viruses**

Fig. 2(a) is an image obtained when the smallest of the spherical viruses, satellite tobacco mosaic virus (STMV), is simply adsorbed onto fresh cleaved mica and imaged in the fluid cell under buffer. The field is filled with more or less spherical particles, each of 17 nm diameter based on height measurements. These correlate exactly to those known from X-ray diffraction analysis (Larson et al., 1998; Ban et al., 1995) and quasi-elastic light scattering studies (Malkin et al., 1993). In Fig. 2(b), swollen STMV virions are visualized in cubic STMV crystals and, in Fig. 2(c), normal particles in the orthorhombic crystal lattice (Koszelak et al., 1989) can be seen. In both of these lattices, individual virus particles are readily discriminated and a hexagonal outline, characteristic of icosahedrons in projection, is evident. Centre-to-centre distances of particles in the two lattices are 18 and 17 nm, respectively, in agreement with data from X-ray crystallography. Fig. 2(d) is a high-magnification AFM image of STMV in the orthorhombic lattice. While some suggestions of substructure are evident on the 17 nm particle surfaces, no recognizable detail of the icosahedral architecture is present.

A $T = 3$ plant virus, brome mosaic virus (BMV), is seen in Fig. 3(a, b) as individual virus particles adsorbed onto mica (Bancroft & Horne, 1977). Virions adhere well to the substrate...
Fig. 4. TYMV particles embedded in the surface of a virus crystal exhibit an icosahedral array of capsomeres; at higher magnification, some even present pentagonal and hexagonal shapes.

Fig. 5. (a, b) Individual virions of CaMV on a mica substrate. Even at this magnification, some capsid substructure is seen. (c, d) At higher magnification, the capsomeres on the virus surface are apparent.
and are stable to repeated scanning. The measured heights, and therefore the diameters of the BMV virions, are 28 nm, consistent with data from X-ray crystallography (Lucas et al., 2001), but the lateral diameter in the AFM images is about 70 nm, or about 2.5 times the correct value. Even on free virus particles, substructure is evident and capsomeres consisting of either five or six protein capsid subunits can be seen. Further inspection indicates the presence of a depression or hole at the centres of the capsomeres. BMV particles incorporated into the lattice of an orthorhombic form of their crystals can be seen in Fig. 3(c). Here, the 28 nm centre-to-centre distances agree with height estimates and with data from other techniques. The capsomeres on the virion surfaces are again well-resolved and the depressions at their centres visible.

If BMV native particles of $T = 3$ are exposed to $\text{CaCl}_2$, they dissociate into capsid protein. The protein can then, upon removal of the $\text{CaCl}_2$, be induced to reassociate into $T = 1$ empty capsids (Yamazaki & Kaesberg, 1963; Zhao et al., 1995; Cuillel et al., 1981). Fig. 3(d) is an AFM image of the surface lattice of their tetragonal crystals (Lucas et al., 2001). Heights and inter-particle distances are in agreement with the 18 to 19 nm diameters deduced from X-ray crystallography. Unlike STMV, large, pentameric capsomeres on $T = 3$ BMV protrude from the surfaces of the smaller $T = 1$ particles (Fig. 3a–c).

Fig. 4 shows a small area on the surface of a turnip yellow mosaic virus (TYMV) crystal (Malkin et al., 1999; Hirth & Givord, 1988). Groups of six crystallographically related $T = 3$ particles of 28 nm diameter gather around large, open channels with widths that are roughly equal to a virion diameter, presumably filled with solvent. Even at this resolution, each of the virions appears as a roughly spherical ‘cluster of grapes’. In many cases, individual grapes or substructures in the clusters can be seen to exhibit hexagonal or pentagonal outlines characteristic of icosahedral capsomeres. The capsomeres are well-resolved from one another on the top surfaces of the virions and have dimensions consistent with those obtained from X-ray crystallography, about 5 to 6 nm (Canady et al., 1996).

Comparison of the capsomeres of TYMV and the overall architecture of the virion with that of BMV, also $T = 3$ but from another virus family (Tymovirus versus Bromovirus), shows them to be strikingly different. Whereas the capsomeres for BMV are wide and have crown-like structures with depressions at their centres, those for TYMV are closed and dense. Hence, it is possible, using AFM, to discriminate the two virus families on the basis of capsid structure.

Fig. 5(a) shows a larger, spherical plant virus, cauliflower mosaic virus (CaMV), adsorbed onto mica. Height measurements suggest its diameter to be 48 nm, though its width in the AFM images is about 100 nm. X-ray crystallography indicates it to be 52 nm (Gong et al., 1990). With AFM, even at a relatively low magnification, quilting on the virion surface suggests the emergence of substructure. At higher magnifications, the underlying architecture begins to develop and individual, large capsomeric units become visible on the virion surface (Fig. 5b–d). These have centre-to-centre distances of 10 to 12 nm and are organized into hexameric patterns.

As virus particles become larger, their properties become more mechanically fragile. Particles are more easily distorted from spherical form and, in some cases, breakage or rupture of the particles is seen. AFM may be useful in assessing the damage caused by various procedures. As seen in Fig. 6(a), DNA can be seen leaking from CaMV virions and spreading onto the substrate. In Fig. 6(b), long strands of RNA still wrapped with subunits of protein can be seen extending from the ends of tobacco mosaic virus (TMV). In addition, it is evident that most TMV particles in sample preparations are...
less than full-length and therefore unlikely to be infectious. In Fig. 6(c), circular RNA molecules originating from ruptured virions of Moloney murine leukaemia virus (MoMLV) can be seen (Lowey, 1985).

The largest virus to be investigated by AFM is the insect virus tipula iridescent virus (TIV). It is known from EM to have a diameter of about 120 nm and a complicated capsid architecture enveloped by a membrane (Williams, 1998). If the virus is dried from water onto mica, it forms regular hexagonal arrays that have some degree of crystalline order. An array of air-dried TIV is shown in Fig. 7. Though not properly crystalline, the constraints introduced by association into an array make it possible to measure centre-to-centre distances that agree well with height measurements and dimensions determined by other methods. Although the capsid structure is not clear on these dried virions, probably because of the membranes, the virus particles do exhibit distinctive polygonal forms with large triangular faces.

Herpesvirus is an enveloped virus of about 100 nm with a lipoprotein membrane surrounding a protein capsid (Kaper, 1975; Roizman & Batterson, 1985; Wagner & Hewlett, 1999a, b). Enveloped viruses adsorb well to a mica surface, but the protein capsid substructure is veiled by the membrane and obscured. If the membrane is removed by detergent, the protein capsids frequently fail to adhere. In Fig. 8(a), the protein capsid can be seen through the membrane of an
enveloped herpesvirus virion. Despite the membrane, some substructure does emerge beneath the shroud as ordered units on the capsid surface. Herpesvirus treated with detergent and dried onto a mica substrate is seen in Fig. 8(b). Here, the capsid structure is clearly evident and even depressions are visible at the centres of some capsomeres.

Viruses do not need to be icosahedral to be studied by AFM. The classic rod-shaped TMV is, for example, seen in Fig. 9. Infectious virions are known from EM and X-ray diffraction (Bloomer & Butler, 1986) to have lengths of about 300 nm, cylindrical diameters of 18 nm and a helical pitch of 23 nm. Many of the virus particles in Fig. 9 are not full-length, but are fragments of TMV. At the same time, anomalously long TMV particles, several times the normal length, can also be seen in abundance, including some particles in Fig. 9. Height measurements of these segments consistently give a value of 18 nm, which corresponds well to the known cylindrical diameter.

Apparent widths in the AFM images, however, are again about 2.5 times that value, or about 45 nm. The repeat along the helical axis cannot be resolved unambiguously with AFM, though there are fleeting and suggestive periodicities in some images.

Some preliminary conclusions

AFM provides a useful approach for evaluating the dimensions, general architecture, capsomere arrangement and, in some cases, even the detailed capsid structure of viruses. Because it can be applied under physiological conditions, it captures the particles in a natural state without the distortions imposed by drying, fixing or staining. Even on individual, isolated virus particles, in the best of cases, capsomere structure can be resolved and underlying motifs identified. At this time, the method does not allow delineation of the protein subunit...
distribution within the capsomeres. Images suggest that viruses can be better visualized when restrained as a member of an ordered array, e.g. a crystal, but some advantage is gained even when the particles are only members of semi-ordered masses, e.g. a two-dimensional aggregate or a paracrystalline array.

Heights of single particles on mica and centre-to-centre distances of particles in ordered arrays are the most reliable measures of a virion’s dimensions. If these are used, shapes and dimensions obtained by AFM agree well, to within a few per cent, with results based on X-ray structure determination. The size of the virus particle does not appear to be a limitation in terms of resolving capsid detail. That is, 17 nm virus capsids, as seen here for BMV $T = 1$ particles, and 28 nm viruses, such as TYMV, yield as much detail as 50 or 100 nm viruses. The favourable increase in virion curvature with increase in diameter is probably compensated for negatively by increasing virion deformity. Even membrane-encapsidated virions yield some information regarding the core or protein capsid substructure.

Unrelated $T = 3$ plant viruses, such as TYMV and BMV, can be discriminated from one another on the basis of shape, size and capsomere structure, even with current technology. Resolution of very closely related viruses, such as cowpea chlorotic mottle virus and BMV, would probably not be possible at this time. Nonetheless, AFM can give some useful information regarding the presence or distribution of different viruses in a sample. As AFM technology progresses and better, more acute tips are developed (Woolley et al., 2000), the technique will become increasingly powerful and may begin to challenge and supplement low-resolution X-ray crystallography.

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