Organization of influenza A virus envelope at neutral and low pH

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Fusion of the influenza A H1N1 virus envelope with the endosomal membrane at low pH allows the intracellular delivery of the viral genome and plays an essential role in the infection process. Low pH induces an irreversible modification of the virus envelope, which has so far resisted 3D structural analysis, partly due to the virus pleiomorphy. This study showed that atomic force microscopy (AFM) in physiological buffer could be used to image the structural details of the virus envelope, both at neutral pH and after a low-pH treatment. At low and intermediate magnification, AFM of control virions confirmed both the pleiomorphy and the existence of zones devoid of glycoprotein spikes at the virus surface, as established by electron microscopy (EM). At higher magnification, the unique vertical resolution of the AFM in 3D topography demonstrated the lateral heterogeneity in spike distribution and strongly suggested that, at least locally, the spikes can be organized in an irregular honeycomb pattern. The surface honeycomb pattern was more easily detected due to an increase in spike height following low-pH treatment at low temperature, which probably prevented disruption of the organization. This enhanced contrast associated with low-pH treatment emphasized differences in the glycoprotein distribution between virions. It was concluded that, together with EM approaches, AFM may help to establish a correlation between surface structure and influenza virus infectivity/pathogenicity.

INTRODUCTION

Responsible for the major pandemics of respiratory illness during the 20th century, influenza A virus is a negative-stranded RNA virus surrounded by a lipid-bilayer envelope containing trimeric haemagglutinin (HA) and neuraminidase (NA), the major glycoproteins, forming ‘spikes’ that protrude from the membrane, and the M2 ion channel. Influenza A virus contains a matrix protein (M1), and the prevailing model of virus assembly (Schmitt & Lamb, 2005) suggests that M1 underlies the lipid envelope, built from plasma membrane microdomains enriched in cholesterol and sphingolipids (Scheiffele et al., 1999; Takeda et al., 2003). M1 interacts with the cytoplasmic tails of the HA, NA and M2 proteins and also with the ribonucleoprotein (RNP) structures making up the viral core (Barman et al., 2004; Schmitt & Lamb, 2005). The structure of the complete virus has been studied by electron microscopy (EM), originally using negatively stained samples (Hoyle et al., 1961; Nermut, 1972; Ruigrok et al., 1984) and more recently using vitrified aqueous virus suspensions (Booy et al., 1985; Fujiyoshi et al., 1994). The results have shown that influenza virus particles vary in size and shape. Virions freshly isolated from infected humans or animals are often filamentous, whereas viruses adapted to high yields in eggs most frequently exhibit a spherical morphology, although all influenza viruses are pleiomorphic (Wrigley, 1979). Control of virus shape depends on interactions of the cytoplasmic tails of HA and NA with M1 (Jin et al., 1997).

Virus pleiomorphy has precluded the use of 3D reconstruction methods that rely on averaging many identical electron micrographs. Consequently, knowledge of the detailed structural organization of the virus surface remains limited. Recently, influenza virus was characterized by cryo-electron tomography (cryo-Tom) (Harris et al., 2006) and Zernike phase-contrast cryo-transmission electron microscopy (cryo-TEM) (Yamaguchi et al., 2008). Although individual glycoprotein spikes were difficult to
resolve, it was suggested from cryo-Tom that the distribution of HA and NA over the surface was not entirely random, with local clusters of NA. In contrast, Zernike phase-contrast cryo-TEM could not distinguish between NA and HA. These results were obtained at neutral pH, and neither cryo-Tom nor Zernike phase-contrast cryo-TEM was applied to characterize the low-pH structure of influenza virus. Fusion of virus envelope with endosomal membranes ensuring the entry of RNPs into the host cell is induced by acidic pH (pH 5.5) and is associated with conformational changes in HA (Godley et al., 1992; Hernandez et al., 1996; Maeda et al., 1981; White et al., 1982).

Because of its capacity to image the surface of biological structures under physiological conditions with a subnanometre vertical resolution (Engel et al., 1997), atomic force microscopy (AFM) was originally applied to the study of virus–eukaryotic cell interactions (Haberle et al., 1992). Since then, AFM has provided structures of viruses consistent with models derived by EM and X-ray crystallography (Kuznetsov et al., 2001) and has been applied to studies of the morphology of enveloped viruses such as human immunodeficiency virus (Kuznetsov et al., 2003), Moloney murine leukemia virus (Kol et al., 2006; Kuznetsov et al., 2004) and severe acute respiratory syndrome coronavirus (Lin et al., 2005). Here, we report 3D high-resolution imaging by AFM of influenza virus A H1N1 in buffer, both under control conditions and after incubation at low pH, at a low temperature.

**RESULTS AND DISCUSSION**

**Low-magnification AFM imaging of influenza virus in buffer**

Low-magnification AFM imaging was first used to determine, in buffer, the shape and size of egg-grown influenza virus (H1N1/New Caledonia) control samples. Diluted samples were required to allow the characterization of single-virion gross morphological properties. Preliminary experiments established that pre-treatment of the negatively charged surface at a neutral pH by Ni²⁺ allowed the adsorption of the virus onto mica. However, interactions with the support were weak and required, for imaging, the oscillating ‘tapping’ mode, which markedly reduces the friction forces during scanning. With a few exceptions, images were obtained in buffer for samples that were fixed with glutaraldehyde, which increased the sample stiffness, and washed in PBS. The flexibility of the spikes (the virus glycoproteins projecting from the bilayer surface), the softness of the virus envelope and frequent contamination of the AFM tip during scanning explain this choice of experimental procedure. In contrast, using polyllysine-coated mica or positively charged supported lipid bilayers as a substrate resulted in flatter, larger viruses that were often severely damaged (data not shown). When observed using different EM techniques, influenza viruses are pleomorphic, with sizes ranging from small spherical particles of less than 100 nm in diameter to filamentous particles up to several micrometres in length (Booy et al., 1985; Laver, 1973; Wrigley, 1979). Thus, the large variation in size and shape described from negative-staining experiments (Hoyle et al., 1961; Nermut, 1972; Wrigley et al., 1986) has also been reported using Zernike phase-contrast cryo-TEM (Booy et al., 1985; Fujiyoshi et al., 1994; Yamaguchi et al., 2008) and cryo-EM-Tom (Harris et al., 2006). Low-magnification AFM imaging confirmed this variability between virions for samples constantly maintained under liquid. In accordance with EM studies, virus particles could be grouped into small (<150 nm diameter) spherical particles, larger spherical particles, and elongated or rod-shaped particles (Fig. 1a) (Fujiyoshi et al., 1994; Wrigley et al., 1986). In our experiments, the apparent diameter of the spherical viruses varied by a factor of 4, from ~75 to 290 nm. The height of most ‘spherical’ viruses adsorbed on mica was close to but slightly smaller than their apparent diameter, with axial ratios of ≤1.3 (Fig. 1b), a difference that can mostly be accounted for by the AFM tip convolution (Engel et al., 1997). The apparent diameter of elongated viruses was between ~90 and 140 nm and their length was up to ~400 nm. Virus aggregates were also

![Fig. 1. Low-magnification AFM imaging of influenza virus. (a) Height image of control diluted influenza virus sample on mica, using tapping mode imaging under PBS buffer. Bar, 1 μm; z scale, 500 nm. Black arrows indicate aggregated material, whilst white arrows show examples of virus pleiomorphism. (b) Virtual cross-section within the zone marked by the frame. The corresponding vertical scale was reduced to enhance the contrast.](image-url)
present in most samples (Fig. 1a, black arrows). These data reinforce the view that AFM provides a potent complementary tool for rapid characterization of virus particles (Ferreira et al., 2008; Kuznetsov et al., 2001). At this imaging scale, the morphology of low-pH-treated samples did not differ from that of controls (not shown). Because of the limited virus–substrate interactions on mica, the use of diluted samples, required for optimizing pleiomorphy detection, was not adapted for the reproducible imaging of the virus surface details. More concentrated virus solutions, leaving only small areas of the substrate uncovered, were therefore used for higher resolution AFM imaging.

**Envelope heterogeneity of single virions**

By decreasing the scan size to 1 μm, we were able to obtain a more detailed view of the virus morphology using AFM imaging of control samples maintained at neutral pH throughout the experiment. Fig. 2(a) shows a height (topography) image of closely packed viruses examined in buffer. The corresponding amplitude image, i.e. AFM error signal image, which often provided sharper details but at the expense of quantitative height measurements (Putman et al., 1992), is presented in Fig. 2(c). Both height and amplitude AFM images demonstrated that, in contrast with the smooth and flat mica substrate (Fig. 2, asterisk), the virus surface essentially showed a grainy appearance. Besides the overall roughness, deformations of the envelope protruding locally with ‘plaque’ zones presenting linear and angular borders (see examples marked by black outlines in Fig. 2a and c) were another frequently observed feature. The grainy aspect of the virions was due to the presence of small structures protruding from the surface, which should correspond to HA and NA glycoproteins spikes. These structures appeared as isolated globular particles of 10.3 ± 2.5 nm (mean ± SD, n=68) in apparent diameter (characteristic globular particles indicated by green arrowheads were more easily discerned using the electronic zoom, as shown in Fig. 2e), alignments of globular particles (Fig. 2, black arrows) or crown-like structures (black arrowheads in Fig. 2a, c and e). At this intermediate magnification, the topography and amplitude images demonstrated a large variation in the roughness between virions, which was apparently linked to the

![Fig. 2. Envelope heterogeneity of single virions: intermediate magnification AFM imaging of control and low-pH influenza viruses. (a, c) Height (a) and corresponding amplitude image (c) of control H1N1 influenza virus. (b, d) Height (b) and corresponding amplitude image (d) of virions pre-treated at pH 5 before dialysis at neutral pH (see Methods). (e, f) Electronic zooms of a single virion marked by a black rectangle in (a) and (b), respectively. Examples of ‘plaque’ regions with linear and/or angular contours are identified by a black outline in (a)–(d). White arrowheads indicate smooth envelope regions that are probably devoid of spikes. Black arrows and arrowheads indicate aligned glycoprotein spikes and ‘crown-like’ structures, respectively. Examples of structures identified as single spikes are shown by green arrowheads. Asterisks indicate uncovered substrate. Bar, 250 nm.]
relative amount of single globular particles and crown-like structures detected by the AFM tip on the accessible part of each virus. As expected from scans at intermediate magnification, details of the surface structure were difficult to extract from control samples. In particular, at neutral pH, some organization could be detected on one virion but not on the next (Fig. 3a). As shown by the black dotted outline, the surface topography evoked the presence of a local honeycomb pattern made of ‘cells’ showing irregular shapes and sizes. At neutral pH, details of the honeycomb pattern could only be imaged with a reasonable resolution in a few cases (Fig. 3c). The images strongly suggested that the irregular honeycomb pattern was built from the crown-like structures identified at lower magnification (Fig. 3c, outline). The apparent diameter of the crown central depression was 11.8 ± 1.6 nm (n=44). Trying to decrease the scan size further generally blurred the outline of the honeycomb unit cells. It is worth noting that there was satisfactory correspondence between the images recorded simultaneously in the trace and retrace directions of the AFM tip for these detailed images (see Supplementary Fig. S1, available in JGV Online), ensuring the validity of the reported data (Muller et al., 1999).

The estimated diameter of the single globular particles detected was slightly larger than the ~7 nm (Gamblin et al., 2004; Wilson et al., 1981) and ~9 nm (Russell et al., 2006; Varghese et al., 1983) corresponding to the HA homotrimer and the NA tetramer, respectively, established with crystallography. It should be remembered, however, that although AFM lateral resolution is close to 1 nm for 2D crystals of membrane proteins (Fechner et al., 2009), tip convolution decreases this resolution for curved and irregular surfaces. For such cases, the geometry of the probe tip restricts access to the topmost fraction of spherical or cylindrical structures such as the HA and NA spikes, whose apparent diameter will be enlarged (Engel et al., 1997; Kuznetsov et al., 2001). In contrast, the vertical resolution of 0.1–0.2 nm (Engel et al., 1997) when the biological sample is in a buffer solution remains higher than that of EM techniques. Accordingly, detection of spikes on the influenza A virus surface indicated a surprisingly good resolution taking into account its curvature, the finite size of the tip and the flexibility of these elongated proteins. The mean distance between the closest protruding globular neighbours, estimated from height images, was 19.4 ± 5.2 nm, centre to centre. This

Fig. 3. High-magnification AFM images of control and low-pH-treated influenza viruses. 
(a, c, e) Height images of control virions; (e) is an enlargement of the zone marked by a rectangle in (c) in which black circles 10 nm in diameter were superimposed on the AFM topography. 
(b, d) Height images of low-pH-treated virions. 
(i, f) Effect of tip convolution and blurring on visualization of spheres deposited on a flat surface. Spheres, 10 units in diameter, localized at each vertex of regular hexagons (i) of 12.5 units side length were used as a schematic representation of the spikes (Apostolov & Flewett, 1969). In (ii), a few spheres were withdrawn to model an irregular honeycomb pattern. In (iii) and (iv), images are shown that are expected from (i) and (ii), respectively, with an AFM tip radius of 10 units including a 15% noise signal and after blurring linked to the movement of spheres and/or their deformation by the tip. Symbols are as described in Fig. 2. Bars, 100 nm (a, b); 50 nm (c, d).
value was nearly twice as large as that reported in studies using Zernike phase-contrast cryo-TEM and cryo-Tom (Harris et al., 2006; Yamaguchi et al., 2008). However, the limited apparent height of protrusions rendered their detection difficult and possibly incomplete. For instance, spikes were generally present, but were more difficult to visualize in ‘plaque’ regions. Precise measurement of globular spike spacing was also made difficult by the presence of the crown-like structures, which might correspond to a hexagonal and/or pentagonal spike organization, first described in the 1960s (Archetti et al., 1967; Nermut & Frank, 1971) but later challenged (Wrigley et al., 1986). The height difference between adjacent structures determines the accuracy of single spike detection by AFM. Fig. 3(e) is a zoom of the zone outlined by the rectangle in Fig. 3(c). Superimposing disks of a diameter corresponding to a single spike onto the height image suggested that most crown structures could correspond to a hexagonal or pentagonal arrangement of spikes. Moreover, spikes of comparable height positioned sufficiently close will appear to form a single structure. A simple tip convolution model assuming an AFM tip radius comparable to the diameter of the spikes (Fig. 3f, i and iii) indicated that both the shape and size of the crown-like structures visualized on the control virions might be accounted for by the existence of a hexagonal spike organization with a spike density similar to that reported in the literature (Harris et al., 2006). The same limiting factors probably explain why AFM did not resolve HA from NA. As suggested by the images presented in Fig. 2(e) and Fig. 3(a), the protrusion height of spikes either isolated or forming alignments, and of the honeycomb unit cell walls, was comparable, varying from ~0.8 to 3.4 nm (1.68 ± 0.67 nm, n=31) (Fig. 4a). Accordingly, height measurements did not distinguish a homogeneous population of NA spikes protruding by ~2 nm from the surrounding HA as suggested by cryo-Tom (Harris et al., 2006). Most EM section images also do not allow us to discriminate between HA and NA spikes (Booy et al., 1985), which have been reported to protrude from membrane lipids by ~10–14 nm (Doms & Helenius, 1986; Shangguan et al., 1998; Yamaguchi et al., 2008). As reported in a cryo-Tom study (Harris et al., 2006), zones of envelopes, probably devoid of spikes, that appeared to be as smooth as uncovered substrate were frequently observed in control virions (Fig. 2a, c, white arrowheads). They allowed an estimate of spike height extending from the border of the naked patches (11.6 ± 1.9 nm, n=21), which fell in the range of the EM values. Added to the local honeycomb patterns, the presence of bare patches of variable size indicated that the distribution of glycoprotein spikes throughout the viral envelope, which might depend on the viral strain or culture conditions used (Fujiyoshi et al., 1994; Shangguan et al., 1998; Yamaguchi et al., 2008), is far from homogeneous.

Under the AFM tip, the virion surface frequently appeared to be deformed, with protruding zones showing angular and/or linear sides. These ‘plaque’ regions, easily detected in amplitude images, often occupied a large part of the virus top, but could be as small as ~50 nm. Estimates of the envelope height deformation at the plaque borders gave values varying between 4.5 and 42 nm (14.5 ± 8.6 nm, n=119). Influenza virus angular envelope shapes were also observed previously in negatively stained (Lovas & Takatsy, 1965; Nermut, 1972; Nermut & Frank, 1971) and in cryo-TEM and cryo-Tom (Harris et al., 2006; Ruigrok et al., 1992) samples, indicating that envelope deformation exists even in the absence of a solid substrate. In the absence of a 2D crystal organization of glycoprotein spikes at the envelope surface, the plaque aspect must result from an anisotropic distribution of submembranous and/or core constituents. Because they have been shown to play a determinant role in virus morphology (Jin et al., 1997; Schmitt & Lamb, 2005), interactions between matrix protein M1, the virus lipid membrane, glycoprotein cytoplasmic tails and RNPs are probably involved in this surface deformation, although the effect of proteins from the host cell (Shaw et al., 2008) cannot be excluded. Virions with discontinuous, and even without, an M1 matrix layer have been observed by conventional staining techniques, cryo-TEM, cryo-Tom and Zernike phase-contrast cryo-TEM (Fujiyoshi et al., 1994; Harris et al., 2006; Nermut, 1972; Yamaguchi et al., 2008). M1 makes a lattice below (Ruigrok et al., 2000) or inserted into (Fujiyoshi et al., 1994) the envelope inner leaflet. Localized interactions between M1 lattice pieces and RNPs, which show linear and

![Fig. 4. Relative height of influenza virus protruding spikes. The relative apparent height and lateral distance of protruding spikes in control (a) and low-pH-treated (b) viruses were measured by virtual sections on low-pass-filtered height images. Blue (M1) and red (M2) arrowheads mark the external diameter of crown structures. Green arrowheads (M3) indicate the position of the top of the crown. A turquoise arrowhead (M4) indicates the tip-accessible lower level of the crown central depression.](http://vir.sgmjournals.org)
angular structures (Lovas & Takatsy, 1965; Noda et al., 2006; Wrigley et al., 1986), could largely explain our topographic observations and the variations in the apparent height of deformations. Future experiments will be required to establish this hypothesis, in line with the current view of the complexity in the architecture of enveloped viruses (Grunewald & Cyrklaff, 2006), which also suggests a local heterogeneity of mechanical properties in individual influenza virus particles.

Envelope architecture after low-pH treatment

Low-pH conditions induced irreversible conformational changes in HA, exposing the HA2 N-terminal fusion peptide, which inserts into the endosomal membrane leading to fusion (Gruenke et al., 2002; Skehel & Wiley, 2000). In our experiments, we followed the low-pH procedure developed by Ruigrok et al. (1984), but with low-temperature incubation, before returning to a neutral buffer for adsorption onto mica. Accordingly, all of the pH effects reported here corresponded to irreversible effects. Because the treatment was carried out in the absence of a target membrane, it probably resulted in an extended but fusion-inactivated form of HA (Hernandez et al., 1996; Shangguan et al., 1998). When imaged at low magnification, viruses resembled controls except for a much more grainy appearance following low-pH treatment (not shown). As shown in Fig. 2, keeping the same 1 μm scan range and AFM imaging conditions used in neutral pH controls, this grainier aspect was due to an increased resolution of glycoprotein spikes, in both height (Fig. 2b, f) and amplitude (Fig. 2d) images. Globular spikes (Fig. 2, green arrowheads) of a diameter similar to controls (10.1 ± 2.1 nm, centre-to-centre spacing 16.2 ± 4.4 nm, n = 90), that could form rows of particles at the surface of some viruses (Fig. 2, black arrows), again co-existed with crown structures (Fig. 2, black arrowheads) that appeared to be like the ‘cells’ of an irregular honeycomb pattern. Enhanced resolution was also applied to structures that were only faintly discernible in the controls, such as spikes in the ‘plaque’ regions (Fig. 2b, d, black outlines). Naked patches (Fig. 2, white arrowheads) were larger and more frequent after low-pH treatment than in controls, an observation that could be explained by the duration of the treatment (Shangguan et al., 1998). The apparent maximal height of the glycoprotein spikes of 13.8 ± 2.1 nm (n = 35), estimated at the border of patches, was slightly higher than that of controls. All of the low-pH-treated samples were characterized by a higher contrast of surface structures (Fig. 3b, d), explained by an increase of ~2 nm in the protrusion height of spikes, which protruded by ~2–6 nm from their environment (3.95 ± 1.44 nm, n = 87) (Fig. 4b) and which probably contributed to lowering the apparent distance between spikes. This value was comparable to EM observations reporting an increase of 1–2 nm in the spike lengths on virus at low pH (Doms & Helenius, 1986; Ruigrok et al., 1986), but remained significantly less than the 5 nm estimated from light scattering (Campbell et al., 2004). Because the length of the NA ectodomain is not affected by pH (Ruigrok et al., 1986), the increased contrast of the walls lining the honeycomb pattern cells could be attributed essentially to the change in HA properties. Indeed, as discussed above for control virions, the striking ‘fence’ appearance of the honeycomb cell units resulted from both the HA flexibility under the tip plus the tip convolution effect on close enough ‘poles’. As shown in Fig. 3(d), the honeycomb organization, with some ‘cells’ outlined by thin black lines, characterized by a central depression lined by spikes too close to be individualized, was highly irregular and strongly differed from the regular hexagonal lattice that has been described for influenza C virus (Archetti et al., 1967). Moreover, besides variations in the spike organization at the surface of a single virus, the surface of neighbouring viruses within the same sample often presented differences in the organization and size of the honeycomb pattern. Thus, the topography of three adjacent viruses showed crown structures that could correspond to hexagons and/or pentagons (Fig. 5, lower virus), honeycombs made of large cell units containing a central spike (Fig. 5, middle virus), and the co-existence of rows and uncompleted honeycomb pattern with central spikes in some of the unit cells (Fig. 5, upper virus).

As mentioned earlier for the controls, the validity of the imaging was attested by the good correspondence between the trace and retrace AFM images. Moreover, similar structures were essentially imaged following successive scans of the same zone (not shown). Thus, the particular topography for each influenza virus surface was not

Fig. 5. Variability in the local organization of spikes at the virus surface. Height image of three adjacent virions treated at acidic pH before returning to neutral pH buffer. Open arrowheads indicate the honeycomb ‘cells’ possessing a central spike. Symbols are as described in Fig. 2. Bar, 100 nm.
attributable to an AFM tip-induced reorganization of membrane glycoproteins. In some cases, the organization of spikes could even adopt a strip configuration, as shown for the virus located in the upper right corner of Fig. 6(a, b). Spikes were arranged in linear structures that seemed to radiate from a circular zone, 68 nm in diameter, surrounded by a border ~9 nm in diameter (black arrows in Fig. 6c, d). The images also strongly suggested the presence of a series of spike alignments of similar width disposed in an orthogonal direction. A similar organization with spikes forming strips, probably made by local arrays of NA, was also observed in the left adjacent virus. These results established that, following low-pH treatment at low temperature, spikes are not disposed at random, but form irregular polygonal arrangements. Because of the large variability between honeycomb units in low-pH virions, estimation of their size was carried out by regrouping them into two classes. The first corresponded to ‘crown-like structure’ cell units made of a wall surrounding a central depression. Large irregular structures in which the length-to-width axial ratio was >1.5, assimilated to rectangles, constituted the second. Measurements were carried out on 249 individual cells from 42 low-pH-treated virions imaged from three different samples. For the first class, which accounted for 87% of the total, the apparent diameter of the central depression was 11.1 ± 2.3 nm, whilst the crown external diameter was 26.2 ± 4.0 nm (n=217). The second class had mean dimensions of 37.1 ± 5.3 × 20.8 ± 4.8 nm with a central zone that contained a central spike in 21 of the 32 cells. Unfortunately, the limited contrast in control virions did not allow us to ascertain whether, as strongly suggested by high-resolution images, the size and shape of the honeycomb unit cells were modified by low-pH treatment. The tip convolution model suggested that the missing spikes in the hexagonal/pentagonal distribution would lead to topographies closer to those observed (Fig. 3f, panels 2 and 4). Such rearrangement could participate in the increased binding of virus to cell surface at low pH and low temperature (Matlin et al., 1981). HA and NA cytoplasmic tails bind to M1 (Schmitt & Lamb, 2005), which self-assembles to form a network of elongated ribbons (Nermut & Frank, 1971; Ruigrok et al., 2000; Wrigley, 1979). This suggests that submembranous organization of M1 may play an important role in the surface distribution and organization of the spikes. It is worth noting that, after acidification of influenza viruses and liposomes, small blister-like protrusions of 10–20 nm in diameter, which often form polygonal arrangements, are found on the fractured convex face of the liposomal membrane (Kanaseki et al., 1997).

All of the images presented so far were obtained in buffer using samples that were fixed with glutaraldehyde, which increases the sample stiffness, and washed in PBS. The flexibility of the spikes, the softness of the virus envelope and the frequent contamination of the AFM tip during scanning explain our choice of this experimental procedure. An example of AFM images of native, unfixed samples in the same buffer is illustrated by Fig. 7. Clearly, topographical

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**Fig. 6.** Example of the orthogonal arrangement of spikes in the envelope of influenza virus treated at low pH. (a, b) Height (a) and amplitude (b) images of virions treated at pH 5.0. (c, d) Zooms corresponding to the upper right corner regions of (a) and (b), respectively. Symbols are as described in Fig. 2. Bars, 200 nm (a); 50 nm (c).
images of control (Fig. 7a) and low-pH (Fig. 7b) viruses were of lower quality than those of fixed samples. Nevertheless, the grainy character of low-pH viruses could still be observed in the topographic images, and the low-pass filtering of the corresponding error signal (Fig. 7c) gave images that resembled those recorded from fixed samples.

In contrast with negative-staining EM data, which have generally reported the disappearance of the ‘ordered’ spike structure following low-pH treatment (Doms & Helenius, 1986; Ruigrok et al., 1984; see, however, Nermut & Frank, 1971), an irregular honeycomb pattern was still present in the samples examined by AFM. In EM experiments, samples were treated at room temperature (Ruigrok et al., 1992), 30 °C (Shangguan et al., 1998) or 37 °C (Doms & Helenius, 1986; Puri et al., 1990). In this range of temperatures, virions rapidly developed envelope discontinuities and blebs as a function of the low-pH incubation time (Shangguan et al., 1998). Our results strongly suggest that low-temperature incubation prevented disorganization of the spikes at the envelope surface. This would explain why incubating virus at low pH at 0 °C followed by warming to 37 °C does not impair their entry into cells (Matlin et al., 1981).

Conclusions

Negative staining, cryo-TEM and more recently cryo-Tom have provided valuable information on the influenza virus envelope organization. However, EM techniques, essentially based on section analysis, allow us to get few details of the inplane distribution of spikes. AFM imaging in buffer gives direct 3D access to the virus topography. Low- and intermediate-magnification AFM images confirmed the virus pleiomorphy and the lateral heterogeneity of glycoprotein distribution at the surface of virions established by EM. At high magnification, with its unique vertical resolution, the AFM tip detected subnanometre differences in the exposure of the top of spikes to the medium. The images obtained reinforced the view of a lateral heterogeneity in the distribution of spikes at the H1N1 influenza virus envelope surface and strongly suggested that spikes are organized in a honeycomb pattern, at least locally. By increasing the protrusion height of the spikes, low-pH treatment at a low temperature facilitated this pattern visualization, whose detailed structure varied among virions. When added to a cell culture at neutral pH, the majority of viruses do not bind to the cell surface, suggesting that binding to viral receptors depends on the lateral distribution of HA spikes. The fraction of bound virus increases up to ~55 % when the pH of the medium is lowered, but not of all the virions are internalized (Matlin et al., 1981). Moreover, after endocytosis, multiple HA trimers are required to initiate fusion according to a process that may involve cooperative interaction of adjacent trimers (Danieli et al., 1996; Floyd et al., 2008; Lee et al., 2006). The local honeycomb organization revealed by AFM would favour the ability of spikes to interact laterally for membrane fusion. Taken together, these observations

Fig. 7. Imaging of influenza virus without glutaraldehyde fixation. Control viruses maintained at neutral pH (a) and viruses treated at pH 5.0 for 24 h at a low temperature (b) were imaged in PBS-Ni without fixation. (a, b) Height images. Bar, 200 nm; z vertical colour scale: 190 nm (a); 80 nm (b). (c) 3D amplitude (error signal) imaging of low-pH virus (scan, 750×750 nm; vertical scale, 15 nm). White arrows indicate the characteristic crown-like structures.
strongly suggest that AFM access to the detailed in-plane distribution of spikes at the envelope surface will help to establish, in association with EM approaches, a correlation between surface structure and influenza virus infectivity/pathogenicity.

**METHODS**

**Viruses.** An egg-grown influenza virus preparation, influenza virus A/New Caledonia/20/99 (H1N1), was obtained from allantoic fluid centrifuged on a sucrose gradient. The viral protein concentration, determined using a Micro BCA Protein Assay Reagent kit (Pierce), was 2 mg ml⁻¹ in PBS at pH 7.5 after overnight dialysis. The preparation was divided into two dialysis bags (Pierce) with a 10 kDa cut-off. The first was further dialysed in 100 mM phosphate buffer at pH 5 for 24 h (Ruigrok et al., 1984) and both preparations were then dialysed for 24 h in PBS at pH 7.5. During all treatments, the temperature was maintained at 4 °C. For safety purposes, the two viral preparations were inactivated by β-propiolactone (β-PL) treatment (Goldstein & Tausaro, 1970; Ruigrok et al., 1984). The addition of sodium citrate (33 mM) and sodium phosphate (100 mM) at pH 7.5 allowed us pH stability control after the addition of 0.1% (v/v) β-PL with gentle stirring incubation overnight at 4 °C. Both preparations were again dialysed for 24 h at 4 °C in PBS at pH 7.5. The viral protein concentration was checked before further experiments. Samples were stored at 4 °C with 0.01% (w/v) sodium azide and used within 2 months (Booy et al., 1985; Nermut & Frank, 1971).

**AFM.** Freshly cleaved mica (0.5 inch diameter; JBG-Metafix) was incubated for 30 min in PBS containing 2.5 mM NiCl₂ (PBS-Ni) at pH 7.4. Excess buffer was removed and 10 µl virus was suspended in PBS/PBS-Ni (1 : 1, v/v) (~ 2.5 µg virus protein) was deposited on the mica placed in a humid chamber at 4 °C for 2 h. Samples were washed with cold buffer and, unless otherwise specified, fixed with glutaraldehyde (2.5% in PBS for 30 min) (Kuznetsov & McPherson, 2006; Kuznetsov et al., 2005). After three washes in PBS-Ni, samples were kept in the same medium and assessed by AFM.

AFM was performed using the oscillating ‘tapping’ mode (Zhong et al., 1993). In this mode, the oscillating tip touches the surface once each cycle, thus minimizing the friction forces present in the contact region of the influenza hemagglutinin abolishes membrane fusion activity. *Appl Microbiol* 19, 290–294.


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