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A NEGATIVE STAIN FOR ELECTRON MICROSCOPIC TOMOGRAPHY

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Abstract

Negative staining can provide detailed, two-dimensional images of biological structures, but combining tomography with negative staining can provide three-dimensional images. Basic requirements for a negative stain for tomography are that the density and atomic number of the stain are optimal, and that the stain is not degraded with the intensive electron dose needed to collect a full set of tomographic images. A commercially available, tungsten-based stain, methylamine tungstate, appears to satisfy these prerequisites. Tomograms derived from multiple projections of EM images of the same structure yielded detailed images of single proteins on the surface of influenza A virus. Comparison of these images with published results from other methods served to evaluate this negative stain tomography. Images of surface renderings of the virus are a good fit to images derived from cryomicroscopy, as well as to the shapes of crystallized surface proteins. Thus, negative stain tomography provides realistic and detailed images of individual molecules in their normal setting on the surface of influenza A virus.

Keywords

Tomography; Influenza Virus; Negative Staining; Back Projection; Imaging Molecules

INTRODUCTION

Negative staining is a pivotal technique for visualizing the averaged structure of protein molecules, counting among its successes the organization of acetylcholine receptors (Stroud, 1983) and structural proteins in ribosomes (Wabl, 1974), the structure and flexibility of myosin molecules (Burgess *et al.*, 1997; Burgess, White *et al.*, 2004), and changes in the organization of influenza hemagglutinin with pH (Ruigrok *et al.*, 1986). Averaging images of negative stained CaMKII has provided images at a molecular level comparable to those from cryomicroscopy (Kolodziej *et al.*, 2000). Averaged images of negative stained bacterial flagellar filaments show individual α -helices (Cohen-Krausz & Trachtenberg, 2003).

Detailed structure has been less forthcoming when negative staining has been applied to complex structures not amenable to averaging (Burgess, Sakakibara *et al.*, 2004) or viruses (Ruigrok *et al.*, 1984 & 1985). In particular, overlap of small components of complex structures, piled layer upon layer, obscures their structure. However, it has been shown that application of single axis tomography to paramyxovirus negatively stained with

phosphotungstic acid reveals individual spikes on the surface of the virus (Mast *et al.*, 2009). This stain has been used to make three dimensional reconstructions of single synthase molecules (Kolodziej *et al.*, 1997),

Application of tomography to negatively stained material encounters the limitation that the large electron doses needed to collect a dual axis tomographic series can degrade the negative stain (Hosoi *et al.*, 1981), inducing aggregation, (Unwin, 1974). Furthermore, heavy-metal stains, when thick enough to envelop larger structures, can form layers so dense they compromise visualization of the finest specimen details (Chen *et al.*, 2008). Here, we report that methylamine tungstate (NanoW) has an electron scattering cross section and resistance to electron irradiation that make it suitable for dual axis tomography of Influenza A virus.

MATERIALS AND METHODS

Sample preparation

A commercial suspension of egg-grown influenza virus A/Japan/305/57, H2N2 (Charles River; total protein 2mg/ml) was centrifuged at 20,000 g in order to eliminate virus aggregates, and the supernatant (protein >0.6 mg/ml) was used directly. Freshly glow-discharged, hexagonal grids coated with Formvar were floated for 10 min on 10 μ l of virus solution before wicking off the excess. Fiduciary markers were added by exposing grids for 10 min to goat anti-rabbit antibody linked to 5 nm gold (Ted Pella Inc., Redding, CA). Finally, the samples were negatively stained for one minute by floating grids on a 10 μ l-drop of 2.0 % methyl-amine tungstate at pH 6.8, as provided (NanoW, Nanoprobes Incorporated, Yaphank, NY, USA; available at: www.nanoprobes.com/Inf2018.html).

Electron Microscopy Tomography

Images for tomography were collected at 300 kV at 1 μ m under focus in a Tecnai TF30 transmission electron microscope, at pixel sizes of 0.37 or 0.3 nm, at each 2° of tilt $\pm 72^\circ$ (at least 66° in instances where the area was vignetted) in orthogonal X and Y axes. A single image was acquired with 5×10^3 e⁻/nm². Up to 144 images contributed to each tomogram. Since the samples have been continuously illuminated during the experiment, the resulting total electron dose was in the range of 2×10^6 e⁻/nm², including the total two-axis acquisition time as well as time spent for automated focusing and tilting of the specimen. When images at zero tilt taken before collecting the tilt series were compared with images at zero tilt from the series no beam damage detected (Supporting Material S1, A&B). In contrast images of influenza virus negatively stained with uranyl acetate and phosphotungstic acid displayed rearrangement of the stain after collecting less than half of the images for a tomographic series (Supporting Material S1, C&D).

Images from each tilt axis were aligned separately to sub-pixel accuracy using IMOD (Kremer *et al.*, 1996) with the 5-nm gold particles serving as fiduciary markers. The two tilt series were then combined into a tomogram at an accuracy of 0.35 pixels, the upper limit for the acceptable residual warping error in IMOD. Virtual sections, one voxel thick, were derived from the tomograms. The resulting stacks of virtual sections manifested the high signal to noise ratio expected from combining raw images (Chen *et al.*, 2008). Small (5 nm) gold particles, corresponding to ~ 100 pixels in the micrographs, served as fiduciary markers. ImageJ (<http://rsbweb.nih.gov/ij/>) and EM3D (<http://em3d.stanford.edu/>), were used to display virtual images for evaluation and measurement. Segmentation was performed by thresholding with AMIRA (<http://www.visageimaging.com/solutions/amira.php>). The signal to noise ratio (S/N) in the data sets collected at 0.3 nm pixels (Fig. 2) was improved by binning by two and applying a spatial filter of 0.6 pixel FWHM (ImageJ).

The data sets collected as 0.37 nm pixels were used to generate tomograms of the same voxel dimensions and segmented after spatial filtering without further binning. The data displayed were spatially filtered and modified only for brightness and contrast; pairs of pictures in the supporting material were modified in parallel.

RESULTS

A total of 47 influenza viral particles were imaged and analyzed in five two-axis tomograms. Spikes on the surfaces of virus are apparent even in full field views, but finer details as well as the presence of different types of spike became evident in virtual sections from tomograms (Fig. 1). Some negatively stained influenza virus particles break and avulse their contents and are penetrated by stain, the latter a finding previously interpreted to indicate that stains can penetrate the outer membrane of the virus (Booy *et al.*, 1985). Regardless of whether a virus was broken or intact it manifested at least two populations of spikes, a more plentiful type that was branched, shorter and more uniform along its length than a second, rarer, type distinguished by being longer, and ending in a large head on a narrow stalk (Fig. 1C, left inset). Grazing virtual sections affording *en face* views of spikes show that the distal ends of the shorter types typically have triangular shapes (Fig. 1C, right inset). The average ratio of the number of short spikes to the longer, lollipop-shaped spikes was one to eight measured at the equators of viruses where resolution is optimal (Fig. 1). Spikes near the substrate, trapped under virus particles, appeared shorter by 4 ± 2 nm, and were more difficult to examine in detail.

The smaller, more common type of spike was 12.5 ± 1.5 (n=45) nm tall and 3.5×4.5 nm at its thickest point, and typically appeared to end as three branches (Fig. 2). In some instances, one of the branches extended to one side further than the others, making it 1.5 ± 0.5 nm (n=6) longer (not illustrated). A narrow stalk 2.0 ± 1.0 nm long joined the body of the molecule to the outer membrane of the virus (Fig. 2). Images as clear as the example shown were rare because the smaller type of molecule was often entangled with its neighbors, resulting in aggregates of four or more branches.

The second, less frequent type of lollipop-shaped spike was taller, 16.2 ± 1.3 (n=9) nm, with a head $9.5 \times 7.5 \times 7.7$ nm³ (Fig. 3). The base of the head appeared to be connected to the stalk by several small branches (Fig. 3B), and a multi-lobed foot typically connected the base of the larger type of spike to the outer membrane of the virus. This type of spike was typically arrayed in groups of 2–4 molecules interspersed among the smaller type of spikes.

DISCUSSION

The present findings open the way to using negative stain tomography to realize fine molecular detail in individual viral spikes imaged near focus. While breaks in the viral envelop incident to negative stains do not appear to affect the distributions or structure of individual spikes, negative staining itself can distort proteins (Heuser *et al.*, 1979). Indeed, the structure of influenza A virus depends on the species of negative stain used (Ruigrok *et al.*, 1985), raising the issue of how real are the structures revealed by negative stain tomography. For instance, influenza A virus manifests much less pleiomorphism when negative stained with uranyl acetate than with phosphotungstic acid (Ruigrok *et al.*, 1985).

The numbers and distribution of spikes on the surface of the virus can be compared with results with other techniques. Cryomicroscopy of influenza virus (Harris *et al.*, 2006), which is expected to present a realistic picture of the numbers and distribution of spikes, provides sufficient detail after averaging, to compare with the distributions and sizes of the two types of spikes seen in negative stain tomography. The ratio of the small type to the large type of

spike in samples from the equator of the negatively stained virus is eight to one. For comparison, counts of spikes on two influenza A viruses viewed by cryomicroscopy yielded ratios of eight to one for one virus and six to one for the other (Harris *et al.*, 2006).

The more frequent, shorter spike seen with cryomicroscopy is proposed to represent hemagglutinin, and the longer spike to represent neuraminidase (Harris *et al.*, 2006). Because both proteins have been sequenced and their atomic structures resolved (Xu *et al.*, 2008; Gamblin *et al.*, 2004), they can serve as a further benchmark for the structures of two types of spikes derived from tomography of negatively stained virus. In the hemagglutinin molecule, as expected from its atomic structure (<http://www.rcsb.org>), three branches lie above a body thicker at the branch point. Both cryomicroscopy and negative stain tomography show that the spike corresponding to hemagglutinin is joined to the virus membrane by a stalk. The height of this molecule by cryomicroscopy is ~12 nm, while the stalk is 1.5 nm long (Harris *et al.*, 2006). Measurements of the shorter type of spike by negatively stain tomography yield a height of 12.5 ± 1.5 nm and a stalk of 2 ± 1.0 nm.

The branches of the hemagglutinin after negative stain often appear more spread out and variable in configuration than expected from the atomic structures of crystallized molecules. Indeed, the appreciation of the variability between individual molecules is a potential strength of negative stain tomography, though it remains possible that changes occur during the staining. This variability could also depend on capturing disulfide bridges in their open state, since the pH of the negative stain is buffered just below neutral pH where both open and closed bridges exist (Cremlyn, 1996). Hemagglutinin has only one single disulfide bridge linking the two polypeptide chains in each monomer (Gamblin *et al.*, 2004; Wilson *et al.*, 1981; Ruigrok *et al.*, 1986), so opening this bridge would allow monomers to remain in an extended form, while crystallization, which stabilizes disulfide bridges, might leave hemagglutinin in a closed state. The results with negative stain, if real, suggest a degree of flexibility in the configuration of the hemagglutinin which might enhance its binding to its target.

The longer type of spike corresponds to neuraminidase, which by atomic structure shows a branching stalk 7.6 nm long supporting a larger head consisting of four lobes, giving the molecule a total length of 16 nm (Colman *et al.*, 1983; Varghese *et al.*, 1983; Xu *et al.*, 2008). These dimensions compare well to 16.2 ± 1.3 nm for the height and 8.5 ± 1.3 nm for the stalk measured from negative stain tomography, which also shows a four-lobed head.

CONCLUSIONS

Here, we report that methylamine tungstate (NanoW) has a density and resistance to electron irradiation that makes it suitable for dual axis tomography of Influenza A virus. The structure of individual spikes on the surfaces of the virus are consistent with their distribution and atomic structure from cryomicroscopy and X-ray crystallography. Negative stain tomography thus opens the door to examining conformations of individual protein molecules, and provides a useful complementary tool for elucidating structures that cannot be spatially averaged with low-dose cryo-electron tomograms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Comparison of a wide field image (A) with a virtual section (B, calculated from the corresponding tomogram) of influenza virus negative stained with Nano-W. The virtual section, 0.6 nm thick, eliminates overlap between structures on the surface of the virus so individual spikes are seen in detail. Also apparent only in virtual sections are breaks in the walls of virus (arrowheads) that account for penetration of stain into some virus but, the spikes do not appear to be disrupted on damaged virus. Scale bar A & B: 20 nm. C. Detailed images of spikes in a virtual section 0.3 nm thick, showing sectors of three intact viruses. Spikes are of two types (area above arrow) distinguished by the greater height and presence of a head on the less common type. Inset: Grazing virtual section showing that spikes are on the surface of the virus.

face have roughly triangular shapes. Image shown in inverted contrast. Scale bar: 10 nm. Gold particles are fiduciary markers.

Fig. 2.

A. Series of virtual serial sections 0.3 nm thick showing a smaller, shorter type of spike. The arms of these spikes are often intertwined with those of neighbors, but this one stood alone.

B. When each virtual section through the spike is segmented (see Methods), a three-dimensional surface image can be rendered (front, side and top views) showing that the peripheral end of this type of spike is comprised of three arms. Scale bar: 10 nm.

Fig. 3.
 A. Series of virtual serial sections 0.37 nm thick through a taller, less frequent type of spike. This type of spike has a thin stalk capped by a head. B. When each virtual section through the spike is segmented, a three-dimensional surface rendering (front, side and top views) shows that the peripheral end of this type of spike has a large head divided into several lobes. Scale bar: 10 nm.