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## Unraveling the Architecture and Structural Dynamics of Pathogens by High-Resolution *in vitro* Atomic Force Microscopy

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Progress in structural biology very much depends upon the development of new high-resolution techniques and tools. Despite decades of study of viruses, bacteria and bacterial spores and their pressing importance in human medicine and biodefense, many of their structural properties are poorly understood. Thus, characterization and understanding of the architecture of protein surface and internal structures of pathogens is critical to elucidating mechanisms of disease, immune response, physicochemical properties, environmental resistance and development of countermeasures against bioterrorist agents. Furthermore, even though complete genome sequences are available for various pathogens, the structure-function relationships are not understood. Because of their lack of symmetry and heterogeneity, large human pathogens are often refractory to X-ray crystallographic analysis or reconstruction by cryo-electron microscopy (cryo-EM). An alternative high-resolution method to examine native structure of pathogens is atomic force microscopy (AFM), which allows direct visualization of macromolecular assemblies at near-molecular resolution. The capability to image single pathogen surfaces at nanometer scale *in vitro* would profoundly impact mechanistic and structural studies of pathogenesis, immunobiology, specific cellular processes, environmental dynamics and biotransformation.

AFM provides a resolution sufficient not only to visualize the gross shape of virions but also enables visualization of the molecular details of surfaces of viruses. Thus we showed for the first time that by using *in vitro* AFM the structure of the virion could be visualized in fluid under physiological conditions to sufficiently high resolution, approaching  $\sim 20$  Å (Figure 1a). In these AFM images of T=3 viruses, which are remarkably consistent with the structures of the equivalent virions determined by X-ray diffraction analyses, individual protein capsomeres on a virion were clearly resolved [1]. Furthermore, we demonstrated [2] that viruses of different, but closely related virus families could be discriminated by AFM (Figures 1a,b) on the basis of differences in capsid structure.

We tested [3] the potential of AFM for molecular characterization (Figures 1c,d) of the pathogenic properties of one of the most widespread human viruses, Herpes Simplex Virus-1 (HSV-1). This work also demonstrated for the first time that the internal topography of virions could be revealed by AFM via chemical and enzymatic dissection. We visualized [3] (a) the intact, enveloped virus, (b) the underlying capsid with associated tegument proteins along with fragments of the membrane, (c)

the capsomer components of the capsid, and their surface arrangement after treatment of virions with detergent, (d) extrusion of viral DNA upon treatment of intact HSV-1 with detergent and protease. It was demonstrated that the resolution of AFM is comparable to that of cryo-EM single images. Thus, as seen in Figures 1c, d not only the structure of the capsid was clearly seen, but also substructures of individual capsomers (holes in the middle consistent with the structure of the capsid determined by EM) and small  $\sim 30$  Å protein clusters (triplexes), which link adjacent capsomers were visualized.

The work on individual virus particles, *in vitro*, culminated in the study of vaccinia virus [4]. Vaccinia virus, the basis for the smallpox vaccine, is a laboratory model for what many consider to be one of today's most significant bioterrorist threats. Vaccinia virus is among the largest and most complex human viruses. We have directly visualized both hydrated and dehydrated samples of intact intracellular mature vaccinia (IMV) virions. The vaccinia virions were subsequently treated individually, or with series of chemicals and/or enzymes intended to degrade the particles from outside to inside, and ultimately to reveal the internal structure of the virus. Consecutive subviral structures including core envelopes, viral cores, nucleoprotein filaments, as well as viral DNA were visualized (Figure 2). Our observations from the chemical and enzymatic dissection are consistent with a novel structural model of the vaccinia virion based on the hierarchy of observed substructures [2,4]. The double stranded genomic DNA is encapsidated within a segmented protein sheath, forming an extended  $\sim 16$  nm diameter "filament" with helical surface topography (Figure 2c). This filament is enclosed within a 30 - 40 nm diameter "tubule" which also shows helical topography. The ellipsoidal virion core apparently arises from a folded, condensed arrangement of the 30 - 40 nm tubules surrounded by a heavily protein-studded shell enshrouded by membranes heavily studded by proteins. Here it was demonstrated for the first time that AFM visualization of intact virions and internal structures makes possible the intriguing modeling of a complex architecture of a large human virus.

We have recently utilized AFM to directly visualize, for the first time, species-specific high-resolution native structures of bacterial endospores including the exosporium and crystalline layers of the spore coat (Figure 3) of four *Bacillus* species in their natural environments, namely air and fluid [5]. Strikingly different species-dependent structures of the spore coat seen in Figure 3 appear to be a consequence of species-specific nucleation and crystallization mechanisms that regulate the assembly of the outer spore coat [5]. These observations suggest that the spore coat architecture and topology are genetically and environmentally determined, and that AFM analysis could be used to reconstruct the environmental conditions that were present during spore formation.

The direct visualization of the environmental response of individual *B. atrophaeus* spores revealed that upon dehydration, spores dimensions decreased by  $\sim 12\%$  [5], followed by a nearly complete recovery in size upon rehydration (Figure 4). The observed decrease in the size of bacterial spores and concomitant change in spore coat surface morphology following dehydration are due to the contraction of the internal spore core and/or cortex [5]. These studies establish that the dormant spore is a dynamic physical structure and provide an experimental platform for investigating spore structural dynamics, germination and response to decontamination regimes.

We have demonstrated that AFM can address spatially explicit pathogen surface protein interactions and their structural consequences at near-molecular resolution. These studies establish AFM as a

powerful new tool capable of revealing pathogen architecture, structural dynamics and variability at nanometer-to-micrometer scales. [6]

### References

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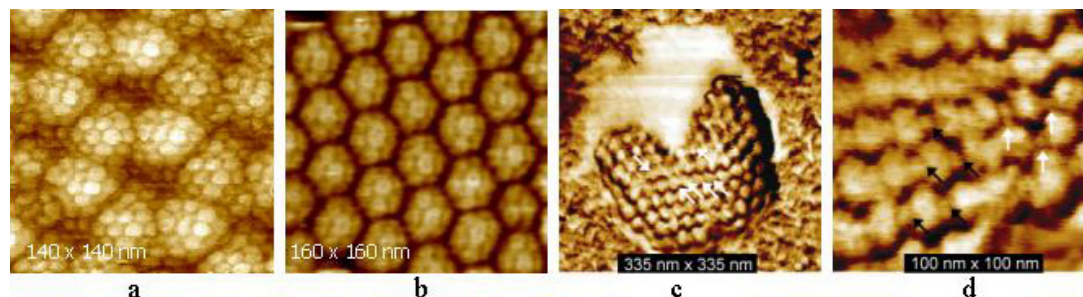


Fig. 1. In (a) – (b) Turnip Yellow Mosaic Virus (TYMV) and Brome Mosaic Virus (BMV) particles (different virus families: Tymovirus versus Bromovirus) clearly display capsomere structure. In BMV(b) the capsomeres are wide and have a crown-like structure with  $\sim 25 \text{ \AA}$  holes at the centers, those for TYMV (a) are close and dense. In (c) - (d) the HSV-1 capsid. Capsomeres with visible hollow channels in their center and triplexes are indicated with arrows in (c) and (d) respectively.

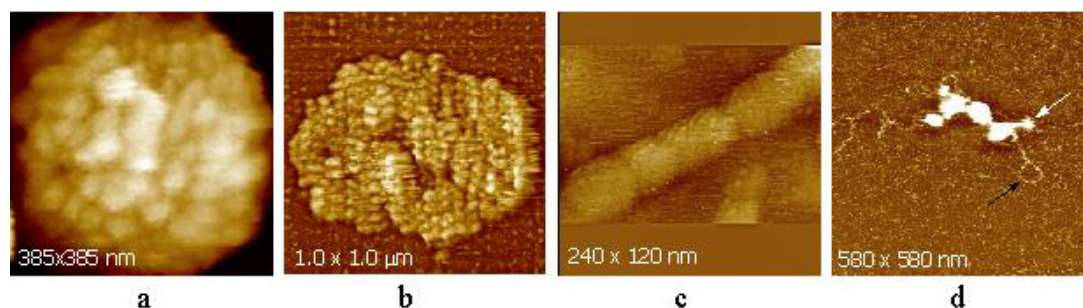


Fig. 2. In (a) Intracellular Mature Vaccinia (IMV virion). Dissection of IMV with detergent and/or enzyme revealed consecutive subviral structures. In (b) intact core envelope. In (c) 16 nm linearly segmented nucleoprotein filament. In. (d) double helical DNA strands formed upon extended treatment of IMV with protease. 16 nm tubular segments associated with DNA strands are indicated with white arrow. A ds-DNA strand is indicated with a black arrow.



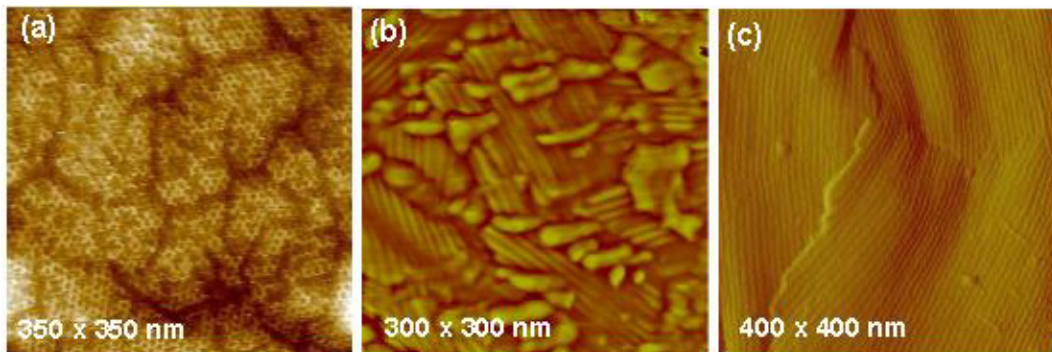


Fig. 3. Species-specific crystalline structures of the *Bacillus* spore coat: (a) *B. thuringiensis*: honeycomb structure (b) *B. cereus*: multi-domain rodlet structure and (c) *B. atrophaeus*: rodlet structure.

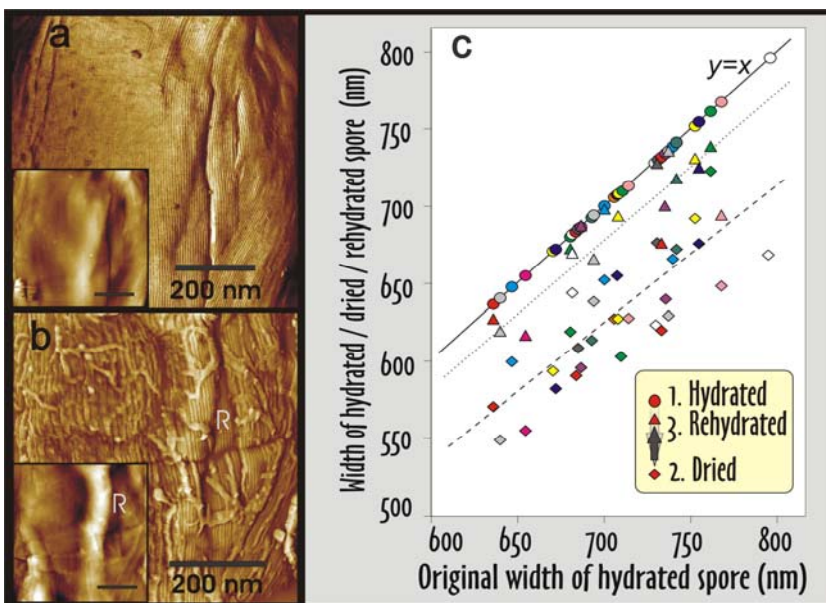


Fig. 4. The effects of changing the *B. atrophaeus* spore environment from aqueous to dehydrated state. (a) Phase image and height image (inset) of a *B. atrophaeus* spore in water, showing several shallow wrinkles. (b) The same spore after drying, showing a 60 nm high ridge (indicated with R). (c) Width change of 35 individual *B. atrophaeus* spores. Depicted is the individual measured spore width for a set of dehydrated (24 hrs) (diamonds, dashed trendline) and rehydrated (2 hours) spores (triangles, dotted trend line), as a function of the spore size of the originally hydrated spore. For ease of comparison, the original hydrated spore width is (redundantly) depicted as circles, which by definition lie on the solid  $y=x$  line. Adapted from [5].