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Mapping in vitro local material properties of intact and disrupted virions at high resolution using multi-harmonic atomic force microscopy†

Alexander Cartagena,‡a Mercedes Hernando-Pérez,‡b José L. Carrascosa,‡ Pedro J. de Pablo*‡b and Arvind Raman*a

Understanding the relationships between viral material properties (stiffness, strength, charge density, adhesion, hydration, viscosity, etc.), structure (protein sub-units, genome, surface receptors, appendages), and functions (self-assembly, stability, disassembly, infection) is of significant importance in physical virology and nanomedicine. Conventional Atomic Force Microscopy (AFM) methods have measured a single physical property such as the stiffness of the entire virus from nano-indentation at a few points which severely limits the study of structure–property–function relationships. We present an in vitro dynamic AFM technique operating in the intermittent contact regime which synthesizes anharmonic Lorentz-force excited AFM cantilevers to map quantitatively at nanometer resolution the local electro-mechanical force gradient, adhesion, and hydration layer viscosity within individual φ29 virions. Furthermore, the changes in material properties over the entire φ29 virion provoked by the local disruption of its shell are studied, providing evidence of bacteriophage depressurization. The technique significantly generalizes recent multi-harmonic theory (A. Raman, et al., Nat. Nanotechnol., 2011, 6, 809–814) and enables high-resolution in vitro quantitative mapping of multiple material properties within weakly bonded viruses and nanoparticles with complex structure that otherwise cannot be observed using standard AFM techniques.

However, key to unraveling the structure–property–function relationships of viruses is the ability to quantitatively map the spatial variation of multiple local material properties within an individual viral particle under physiological conditions – a challenging goal for conventional AFM methods.

Viruses are relatively stiff and often icosahedral particles. As a result isolated viruses only bind weakly to substrates over small contact areas and can be easily removed by the lateral forces applied to the particle if the AFM tip is in continuous contact with the particle while scanning.16 Intermittent contact can be achieved through the pointwise acquisition of static force–distance curves or through its variants.17,18 However at present these methods require great care to reduce forces at the edges of weakly bonded isolated particles and have rarely been used to map properties within individual viruses.19 Conventional dynamic AFM methods, amplitude and frequency modulation (AM-AFM and FM-AFM), use resonant cantilevers to intermittently and gently tap the virus while mapping material property contrast within a virus.20,21 Nevertheless only one channel for material property contrast is available, phase lag φ1 in AM-AFM22 or dissipation channel in FM-AFM,20 which provides scarce information to quantify material properties.

Our proposed technique (Fig. 1) in vitro multi-harmonic AFM exploits two important facts. On one hand the nonlinearity of the combined electrostatic/elastic/hydration forces $F_{\text{nl}}$ between

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Introduction

Viruses are striking examples of macromolecular nanomachines which carry out complex functions with minimalistic structure.1 Understanding the relationships between viral material properties (stiffness, strength, charge density, adhesion, hydration, viscosity, etc.), structure (protein sub-units, genome, surface receptors, appendages), and functions (self-assembly, stability, disassembly, infection) is of significant importance in physical virology and nanomedicine,2–4 for designing nano-containers9 and synthetic virus-like particles,10 and for the templated assembly of nanomaterials.11–13 Standard AFM techniques based on nano-indentation have measured single physical property (stiffness) of individual viruses at few points, but they lack spatial and temporal resolution.5,14,15

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† Electronic supplementary information (ESI) available: In detail theory of multi-harmonic AFM imaging in intermittent contact with sample, method validation of robustness, and additional viruses. See DOI: 10.1039/c3nr34088k
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the tip and the virus ensures that the in vitro cantilever vibration spectrum features strong anharmonicities of the excitation frequency $\omega$ especially at $0\omega$, $1\omega$, and $2\omega$. Thus the amplitudes and phases ($A_0$, $\phi_1$, $A_2$, $\phi_2$) at these frequencies boost by four-fold the available independent channels for material contrast in AM-AFM. On the other hand Lorentz-force excited AFM cantilevers in liquids have a well-defined and calibratable transfer function that can accurately relate the amplitude and phase of these cantilever harmonics to the harmonics of the tip–sample interaction forces.

Here we have studied Bacillus subtilis bacteriophage $\phi$29 virions deposited on freshly cleaved HOPG surfaces in physiological buffer solution (see Materials and methods). The relative simplicity of phage $\phi$29 has placed this virus in the spotlight of research, providing insights into the strategies used by tailed viruses. $\phi$29 is endowed with striking mechanical properties which have been related to its functionality for genome storage and translocation to the host.

Materials and methods

Virus description

Bacteriophage $\phi$29 is assembled in a prolate icosahedral capsid (54 × 42 nm) with a 1.6 nm thickness shell wall. The shell is made of 235 gp8 protein subunits arranged in a $T = 3$, $Q = 5$ lattice with 11 pentameric plus 20 hexameric morphological units forming icosahedral end caps, and 10 hexameric units forming the cylindrical equatorial region. The hexamers and pentamers are built by the interaction of trimers of the shell protein, each trimer building the basic triangle of the icosahedral face. The neck/tail and connector complex is 44 nm long, consists of four proteins (gp10, 11, 12 and 9), and it is held in a unique five-fold vertex of the head. Additional components of the head are the fibers made of protein gp8.5, which are dispensable for virus infectivity. The molecule of 19.8 kbp ds-DNA (6.3 μm long) is highly packed inside of the viral particle.

Sample preparation

Bacteriophage $\phi$29 virions were stored in TMS buffer (10 mM MgCl$_2$, 50 mM Tris and 100 mM NaCl, 7.8 pH). A single drop of 20 μl stock solution of viral particles was deposited on freshly cleaved HOPG (ZYA quality NTMDT). After 30 minutes the sample was washed at least 5 times with the same buffer solution. The resulting Debeye length ($\lambda_0$) is ~2 to 4 nm as measured from static force–distance curves on the virus (see ESI†).

AFM imaging

In the experiments the samples were imaged in tapping mode AFM and the cantilever excited at the natural frequency. Experimental measurements of $\phi$29 virions were made by using a commercial MFP-3D-BioAFM system (Asylum Research, Santa Barbara, CA, USA), and using extremely soft microcantilevers BL-TR-400PB (Olympus, Tokio, Japan) with a nominal spring constant of 0.09 N m$^{-1}$, $Q = 1.8$, $\omega_n = 8.4$ kHz (in liquid) and tip radius of 50 nm that were excited using Lorentz force excitation ($i$Drive™ at $A_{free} \sim 4$ nm ($A_1/A_{free} = 0.8–0.85$). Lorentz force excitation directly applies an oscillating excitation force to the microcantilever allowing for clean phase-distortion free resonance peaks. The AFM probe is directly driven near its resonant frequency of its first frequency flexural mode and then engaged to the sample.

The $\phi$29 virions deposited on HOPG in buffer solution are mounted on a MFP-3D-BioAFM for dynamic AFM imaging and material properties measurements. A custom-made cooling system was mounted inside the AFM enclosure to cool down the sample and AFM system to approximately 18 °C. This ensures viral capsid stability and reduces the system drift for more stable and higher resolution imaging. After the laser is properly aligned on the cantilever, the deflection sensitivity of the cantilever was calibrated by recording a static force–distance curve on freshly cleaved mica in the buffer solution to avoid tip contamination. The cantilever spring constant and quality factor of the first flexural mode was calibrated by using the thermal noise method in liquid. The excitation frequency was chosen from the peak of the tuning curve, where the phase lag becomes 90°.

For all dynamic AFM images presented in this letter, the signal acquisition and processing of multi-harmonic data were achieved using a second built-in lock-in amplifier in the MFP-3D-BioAFM controller. Unfiltered data coming from the photodiode output are then filtered using a low-pass filter with a cut-off frequency in the range of ~500 Hz. This low-pass filtered output data go into the second lock-in amplifier where the frequency to track has been set to the second harmonic (second integer multiple of the drive frequency) with a cut-off frequency of 1.5 kHz. The 0th harmonic data are acquired by low-pass filtering the photodiode output below 500 Hz.

For the nanoindentation experiments an image of the virus is taken in the tapping mode in order to check the integrity of the virus and locate the center of the shell. Then, the AFM tip is moved on the top of the particle and a set of steps of 5–10 force–distance curves are acquired. After each nanoindentation assay a new image is recorded to check the particle integrity.
dynamic and static approach force–distance curves recorded on the system was done using an average speed around 50 nm s⁻¹ with a trigger force set around 0.8–1 nN. In the performed dynamic approach curves, the observables \((A_0, A_1, A_2, \phi_1, \phi_2)\) were recorded for each curve.

**Data processing**

The recorded topography and multi-harmonic observable images were rendered and processed using WSxM⁵ and IGOR Pro 6.2 (WaveMetrics, Lake Oswego, OR) software for optimal presentation. All computations and data processing are performed using MATLAB (The Mathworks). For each set of multi-harmonic observable \((A_0, A_1, \phi_1, A_2, \phi_2)\) maps, the local effective stiffness \((k_{\text{eff}})\), effective viscosity \((\eta_{\text{eff}})\) and adhesive force \((F_{\text{ad}})\) were estimated simultaneously with nonlinear least-squared fits to the conservative and dissipative Fourier components of the interaction forces \(F_{\text{ts,s,DISS}}^{1}, F_{\text{ts,s,CONS}}^{1}, F_{\text{ts,s,CONS}}^{2}, \) and \(F_{\text{ts,s,DISS}}^{2}\). The equations used for this are presented in the ESI.

**Results**

Fig. 2b shows that when resonantly excited soft AFM cantilevers approach the \(\phi 29\) virion, the multi-harmonic observables \(A_0, A_1, \) and \(A_2\) and their corresponding phases (not shown) are clearly detected with high signal to noise ratio. In order to link these observables to local material properties it is important to understand which interaction forces are sensed by the oscillating tip as it scans the sample. By noting the values of \(A_0\) and \(Z\) corresponding to a typical amplitude reduction of 80–85% used for imaging, we determine (Fig. 2c) that the tip only penetrates the regime of low-force interactions with the virus (forces \(\sim <200\) pN) which are characterized by an effective repulsive force gradient \(k_{\text{eff}}\). This repulsive gradient can be regarded as an effective value combining the elastic stiffness of the hydrated virus \(k_{\text{el}}\) with a force gradient \(k_{\text{DLVO}}\) due to electrostatic and van der Waals forces between the gold coated tip and the charged capsomers in the buffer solution (Debye length \(\sim 2\) to 4 nm) i.e. \(1/k_{\text{eff}} = 1/k_{\text{DLVO}} + 1/k_{\text{el}}\). We have determined (see ESI†) that under our buffer conditions the dominant contributors to \(k_{\text{eff}}\) are \(k_{\text{DLVO}}\), which is dominated by electrostatic interactions on the virus, followed by \(k_{\text{el}}\). Thus, \(k_{\text{eff}}\) can be regarded as the repulsive electro-mechanical force gradient sensed by the oscillating tip while scanning. Another physical property characterizing these interactions is the tip–sample adhesion force \(F_{\text{ad}}\) which depends among other factors on the local van der Waals forces and local chemical forces.

![Fig. 2](image-url)
(hydrophobicity/hydrophilicity) which reflect the local chemical composition of the surface. To account for energy dissipation within the hydration layers surrounding the virus, an effective viscosity $\eta_{\text{eff}}$ can be introduced, representing (a) the intrinsic viscosity of a local volume of the virion, which includes both hydrated capsomeres\textsuperscript{26-28} and sub-surface DNA, and (b) the energy transfer to higher cantilever eigenmodes.\textsuperscript{21} Thus a tip–sample force model $F_{\text{ts}}$ describing the interactions between the AFM tip and virus particles as well as HOPG (Fig. 2d) is:

$$F_{\text{ts}} = -F_{\text{ad}} - k_{\text{eff}}(d) - \eta_{\text{eff}} \dot{d}, \text{ when } d < 0, \dot{d} = 0, \text{ when } d > 0$$

where the tip–sample gap is $d$ and its time derivative is $\dot{d}$. Our interest therefore is to quantitatively map these material properties: $F_{\text{ad}}$, $k_{\text{eff}}$ and $\eta_{\text{eff}}$, within individual virions using the multi-harmonic observables.

To do so we simultaneously map the four additional observable channels $A_0$, $\Phi_1$, $A_2$, $\Phi_2$ images into quantitative maps of local material properties, we have developed a perturbation theory for multi-harmonic AFM (see ESI)\textsuperscript{†} which applies for the case of directly excited cantilevers (magnetic, Lorentz force, or photo-thermal excitation\textsuperscript{29}) where a well-defined transfer function exists.\textsuperscript{28} As we have shown the oscillating tip only intermittently taps on the viral particle, quite unlike multi-harmonic methods on live cells\textsuperscript{30} where the tip is continuously pressed against the cell. To quantify material properties using the multi-harmonic observables in this nonlinear regime requires a significant generalization of multi-harmonic AFM theory which is presented here.

If $Z$ is the Z-piezo displacement in an AFM and $g(t)$ is the tip displacement, then tip–sample gap is $d(t) = Z + g(t)$. $F_{\text{ts}}$, the tip–sample-interaction force consists of a conservative (only dependent on $d(t)$) and a dissipative component $F_{\text{ts,CONS}}$, $F_{\text{ts,DISS}}$, respectively, so that $F_{\text{ts}} = F_{\text{ts,CONS}}(Z + \dot{q}) + F_{\text{ts,DISS}}(Z + \dot{q}, \dot{q})$. The theory assumes, as seen in experiments, that the tip oscillations are dominated by the 0\textsuperscript{th}, 1\textsuperscript{st}, and 2\textsuperscript{nd} harmonics and that $F_{\text{ts}}$ is periodic in $\theta = \omega t - \phi_1$, so that

$$g(t) = A_0 + A_1 \sin(\theta) + A_2 \sin(2\theta + 2\phi_1 - \phi_2)$$

$$F_{\text{ts}} = F_{\text{ts,0}} + \sum_{n=1}^{\infty} F_{\text{ts,DISS}} \cos(n\theta) + \sum_{n=1}^{\infty} F_{\text{ts,CONS}} \sin(n\theta)$$

The multi-harmonic observables $A_0$, $A_1$, $\phi_1$, $A_2$, $\phi_2$ can be converted quantitatively without further assumptions using simple expressions into $F_{\text{ts,0}}$, $F_{\text{ts,DISS}}$, $F_{\text{ts,CONS}}$, $F_{\text{ts,CONS}}$, and $F_{\text{ts,DISS}}$ as shown in the ESI,\textsuperscript{†} and mapped over the virus as shown in Fig. 3f-j. Striking patterns in material property heterogeneities now emerge when the experimental observables are rendered in terms of the force harmonics, not only within the viral shell and

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**Fig. 3** Multi-harmonic observables maps. (a) Topography image acquired of a φ29 virion along with the multi-harmonic observable maps (unfiltered) of (b) $A_0$, (c) $\phi_1$, (d) $A_2$, and (e) $\phi_2$. These maps are converted using analytical formulae (ESI) based on the transfer function of Lorentz force excited cantilevers into quantitative harmonics of the conservative and dissipative components of the interaction forces (pN), (f) $F_{\text{ts,0}}$, (g) $F_{\text{ts,CONS}}$, (h) $F_{\text{ts,DISS}}$, (i) $F_{\text{ts,CONS}}$, and (j) $F_{\text{ts,DISS}}$. Scale bar, 40 nm (size; 200 × 200 nm, pixels; 256 × 256).
between the shell, collar, and tail but also within the collar and tail.

The quantitative measurement of a few harmonics of the interaction forces $F_{ts}$, $F_{ts,DISS}$, $F_{ts,CONS}$, and $F_{ts,DISS}$ cannot be sufficient to fully reconstruct the interaction force for which many more harmonics are needed. However since the interaction force only has a small number of unknown material property parameters, $F_{ad}$, $k_{eff}$, and $\eta_{eff}$ it is possible to quantify them from only a few force harmonics. We determine exact analytical expressions (see ESI†) that relate the measured force harmonics $F_{ts}$, $F_{ts,DISS}$, $F_{ts,CONS}$, and $F_{ts,DISS}$ to the material properties $F_{ad}$, $k_{eff}$, and $\eta_{eff}$. Using these expressions, a nonlinear least-squared fit is performed pointwise on the maps to find those values of the material properties that best fit the five measured force harmonics (Materials and methods).

Fig. 4a–d show the quantitative material property maps on a single φ29. Fig. 4e and f provide the cryo-electron microscopy reconstruction of the φ29 virion, which compare fairly well with the AFM images after taking into account the inherent tip–sample geometrical dilation. Important intra-viral property contrasts, often uncorrelated to each other and to the topography, can be discerned from the maps over the capsomers, collar and tail knob protein domains. For example, the cavities formed by the appendage structure around the connector (Fig. 4f, green outlined) are clearly observed as dark spots of reduced $k_{eff}$ (Fig. 4a–c), reflecting the local reduction in these tip–sample properties over the cavities, while $\eta_{eff}$ remains relatively unchanged. From a line profile along the longitudinal axis of the virion (Fig. 4g) we observe that $k_{eff}$ is larger on the capsid and tail but smaller on the collar while $\eta_{eff}$ and $F_{ad}$ are relatively constant. The extracted properties from the multi-harmonic method compare excellently (within 10%) with the values obtained from static force–distance curves in the virus (Fig. 4h–i). Phase contrast has been observed in these regions before but the present work unambiguously relates the observed contrast to quantitative local material properties.

Our in vitro multi-harmonic dynamic AFM method can be applied to gain insights into the local physical properties of the φ29 virion after disruption. Indeed, AFM induced dismantling of viral particles has been shown to be a powerful approach to understand virus assembly/disassembly, revealing the discrete mechanical building blocks of φ29 prohead32 and the intermediate kinetic assembly structures of minute virus of mice. However, the material properties characterization of partially dismantled viral particles has not progressed beyond single indentation assays, providing a limited understanding of the disrupted virus mechanics. Thus, our experiments consist of

Fig. 4 Local material property maps within a φ29 virion and comparison with quasi-static $F$–$Z$ curves. Using the analytical expressions derived in the ESI† (a) the local electro-mechanical force gradient $k_{eff}$ (N m$^{-1}$), (b) the intrinsic viscosity $\eta_{eff}$ (N s m$^{-1}$), and (c) the adhesion force $F_{ad}$ (pN) can be mapped over the virion (unfiltered data). In (d) the mean force map $F_{0,ts}$ is also shown as a reference. (e) Cryo-EM reconstruction model of the φ29 virion and (f) a zoom-in image outlining the separation of the collar appendages which are well-correlated with the dark spots seen in the same region on the quantitative material property maps. (g) Line profiles of the topology and local property maps along the longitudinal axis of the virion showing many important contrasts in $k_{eff}$ on different regions, but $\eta_{eff}$ and $F_{ad}$ remain constant. Comparison of histograms corresponding to the area in maps marked by a cross in topography insets and quasi-static $F$–$Z$ curve (green and orange lines) for (h) the force gradient $k_{eff}$ and (i) adhesion force $F_{ad}$ (pN). Values of material property maps are within 10% of those from static $F$–$Z$ curves. Scale bar AFM images, 29 nm (size; 145 × 145 nm, pixels; 256 × 256).
partially disrupting φ29 virions by performing a nanoindentation where the maximum force is slightly higher than the breakage force (Materials and methods). The multi-harmonic images before and after partial disruption were acquired and processed as described earlier (Fig. 5) demonstrating a wealth of new features within the virion. Profiles along the evidently damaged viral shell zone (lines #1) before (black) and after damage (red) shown in the insets of Fig. 5a demonstrate the expected decrease in height due to the removed capsomers. However, topographical profiles (lines #2) before (black) and after damage (red), obtained along shell regions which remain undamaged, show an intriguing decrease of height that does not correspond to affected trimers.

**Discussion**

An in-depth analysis of these results is provided in Fig. 6, where in (a) the topography of the partially disrupted φ29 virion is shown with a superposed schematic representation of the icosahedral network in which the protein subunits are arranged in trimers which, in turn, build the morphologically distinct hexamers and pentamers of the intact virus. In addition, in Fig. 6c–f the difference in the topography and material properties $F_{\text{ad}}$, $k_{\text{eff}}$, and $\eta_{\text{eff}}$ between the intact and disrupted virion are also shown. As can be observed in Fig. 6a the nanoindentation clearly eliminates trimer 1 and either removes or brings down trimer 2, but does not apparently further affect the remaining shell. Fig. 6c not only demonstrates the location of the directly affected shell area (trimers 1, 2, and 3), but also a homogeneous height decrease on the remaining intact shell (Fig. 6b, black), which corresponds to a volume loss of about 25%. This volume loss cannot be attributed only to the lost capsomers, and thus must partly arise from the loss of part of the packed DNA. This conclusion is consistent with prior results of internal pressurization of phage φ29, suggesting that in Fig. 6a the virion internal pressure has been relaxed probably because the genome has been ejected. A detailed inspection of Fig. 6b–f provides further information. For instance, $k_{\text{eff}}$ and $F_{\text{ad}}$ decrease at removed trimers 1 and 2, while remaining nearly constant all over the intact virion shell, including trimer 3, thus indicating that the material of this shell region is the same as those of the intact trimers. As a consequence, we can infer that trimer 3 has not been removed but pressed down. Secondly, $k_{\text{eff}}$ decreases by ~10% over all the intact shell area, as indicated by its profile (green, Fig. 6b) along the white dotted line (Fig. 6c) map. Strikingly, the effective viscosity map ($\eta_{\text{eff}}$) (Fig. 6f) displays salient features which are not correlated with $k_{\text{eff}}$ and $F_{\text{ad}}$ maps (Fig. 6b, red and green curves respectively). For example, Fig. 6f clearly shows that $\eta_{\text{eff}}$ decreases significantly at affected trimers 1, 2, and 3 as expected, but there are areas of the unbroken virion shell (trimer 4) that also exhibit a significant decrease in $\eta_{\text{eff}}$ (Fig. 6b, blue). The energy lost to higher cantilever eigenmodes is mediated by the local force gradient $k_{\text{eff}}$ which varies little across the trimer 4. 

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**Fig. 5** AFM-induced partial disruption of the φ29 virion. (a) Topography image of an intact and disrupted φ29 virion along with the corresponding material property maps: (b) the mean force map $F_{\text{m}}$ (pN), (c) adhesion force $F_{\text{ad}}$ (pN), (d) force gradient $k_{\text{eff}}$ (N m$^{-1}$), and (e) the intrinsic viscosity $\eta_{\text{eff}}$ (N s m$^{-1}$). In the second column the virion has been disrupted at a point by applying a point force of ~800 pN. It is clear that the local fracture of the virus shell compromises their structure integrity in intriguing ways, generating variation in topography and local properties in areas that are well removed from the damage. The insets in are
including the repulsive electro-mechanical force gradient, intrinsic viscosity and adhesion at nanometer resolution within weakly bonded viruses. Our maps on partially disrupted viruses demonstrate that material properties of viruses exhibit local variations which are often uncorrelated to each other and the topography. Simultaneous mapping of multiple physical quantities reveal that the properties do not just depend globally on the superficial viral shell, but also on local subsurface structures such as DNA. Our experimental results provide new evidence of $\phi$29 pressurization showing that DNA can escape from the virion not only through the tail, but also through cracks or punctures of the viral shell. Thus, our experiments help to understand bacteriophages like pressurized vessels which can release DNA through any fracture present on the viral shell. This method opens an unprecedented window to study structure–property–function relationships of viruses, nanocontainers and nanoparticles in liquid environments, which are increasingly important not only for nanomedicine and physical virology, but also to emerging applications of viruses in materials science and nanotechnology.

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Notes and references

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