Published in final edited form as:

J Phys Chem B. 2017 March 02; 121(8): 1843–1853. doi:10.1021/acs.jpcb.6b10131.

Probing the Link among Genomic Cargo, Contact Mechanics, and Nanoindentation in Recombinant Adeno-Associated Virus 2

Cheng Zeng[†], Sven Moller-Tank[‡], Aravind Asokan^{†,§,||}, and Bogdan Dragnea^{*,†}

[†] Department of Chemistry, Indiana University, Bloomington, Indiana 47405, United States

[‡] Gene Therapy Center, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, United States

[§] Department of Genetics, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, United States

^{||} Department of Biochemistry & Biophysics, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, United States

Abstract

Recombinant adeno-associated virus (AAV) is a promising gene therapy vector. To make progress in this direction, the relationship between the characteristics of the genomic cargo and the capsid stability must be understood in detail. The goal of this study is to determine the role of the packaged vector genome in the response of AAV particles to mechanical compression and adhesion to a substrate. Specifically, we used atomic force microscopy to compare the mechanical properties of empty AAV serotype 2 (AAV2) capsids and AAV2 vectors packaging single-stranded DNA or self-complementary DNA. We found that all species underwent partial deformation upon adsorption from buffer on an atomically flat graphite surface. Upon adsorption, a preferred orientation toward the twofold symmetry axis on the capsid, relative to the substrate, was observed. The magnitude of the bias depended on the cargo type, indicating that the interfacial properties may be influenced by cargo. All particles showed a significant relative strain before rupture. Different from interfacial interactions, which were clearly cargo-dependent, the elastic response to directional stress was largely dominated by the capsid properties. Nevertheless, small differences between particles laden with different cargo were measurable; scAAV vectors were the most resilient to external compression. We also show how elastic constant and rupture force data sets can be analyzed according a multivariate conditional probability approach to determine the genome content on the basis of a database of mechanical properties acquired from nanoindentation assays. Implications for understanding how recombinant AAV capsid–genome interactions can affect vector stability and effectiveness of gene therapy applications are discussed.

*Corresponding Author dragnea@indiana.edu.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jpcb.6b10131.

More information on AAV characterization, orientation determination, hydrophobicity map, nanoindentation results, and statistical model (PDF)

The authors declare no competing financial interest.

Graphical Abstract

INTRODUCTION

Viruses are ubiquitous biological nanoparticles that have been recently engineered for new technological roles, such as nanoreactors and delivery vectors.^{1–5} Understanding the relationship between genome content and capsid stability is not only vital for applications of virus-based nanomaterials, but also for fundamental insights into viruses as biological machines. Adeno-associated virus (AAV) is a small (25 nm) virus packaging a ~4.7 kb single-stranded DNA (ssDNA). The AAV capsid consists of 60 protein subunits arranged with $T=1$ icosahedral symmetry. The coding regions of wild-type genomes are flanked by inverted terminal hairpin repeats (ITRs) essential for packaging.⁶ Recombinant AAV is one of the most promising vectors currently being evaluated in gene therapy clinical trials.^{7–10} There are many serotypes of AAV, displaying diverse tissue tropisms. AAV has no known pathogenicity and requires a helper virus for replication and infection. Also, genome sequences between the flanking ITRs can be substituted without disrupting genome packaging, enabling a great deal of flexibility in transgene content. Moreover, AAV can infect nondividing cells and mediate long-term gene expression.^{6,11–14}

Although AAV has many desirable properties for gene therapy applications, there are several aspects that could still improve the AAV platform by vector design and development. For instance, one hurdle for the original AAV vectors was the rate-limiting step of the second-strand synthesis preceding transgene expression.¹⁵ The use of AAV vectors packaging an engineered self-complementary DNA (scDNA) genome with a mutant 3' ITR has made it possible to bypass the slow second-strand synthesis, which led to a significant enhancement in transduction efficiency.¹⁶ Thermal stability studies of scAAV vectors showed that the packaged scDNA genome is likely to be partially base-paired.¹² Because scDNA has a different persistence length from that of ssDNA, it may create stresses in the capsid, which could destabilize the virus in certain situations, but make it more stable in others.¹⁷ However, the relationship between vector stability and the state of the packaged genomic cargo remains unclear. This relationship plays a potential role in cellular barriers to AAV transduction, which are associated with the different stages between the cell membrane and

the nucleus, where AAV vectors must ultimately deliver their genomes for a successful transduction. Most of these stages involve extended interfacial interactions. Thus, the virion first adsorbs to the surface of the cell, where it binds via multiple receptors. The multivalent binding process triggers membrane invagination and endocytosis. Endosomal trafficking to the interface-rich Golgi network and/or the endoplasmic reticulum follows. After escape into the cytoplasm, intact AAV particles are imported into the nucleus through the confines of the nuclear pore complex.

Anisotropic confinement interactions raise the question of particle response at compression, whereas interfacial binding raises questions pertaining to contact mechanics,¹⁸ that is, the result of the balancing act between adhesion, which tends to increase the contact area, and the elastic deformation of the virus shell, which resists it. Therefore, the central focus of the current study was to determine the influence that different genomic cargos (or the lack of them) may have on the mechanochemical properties of the AAV vector. Specifically, it is not known for AAV in what measure the genomic cargo will affect vector compressibility and contact mechanics.

For other viruses, atomic force microscopy (AFM) indentation has successfully explored the issue of elastic response at compression.^{17,19–24} Contact mechanics was discussed so far only theoretically on continuum models.²⁵

AFM is a single-particle approach to mechanical probing and in situ imaging, which can acquire nanometer-scale spatial resolution in liquid.^{17,19,26,27} AFM studies of AAV have explored the specificity of interaction between different serotype virions and surface-immobilized heparan sulfate.²⁸ Nonspecific interfacial interactions with substrates have not been systematically studied. However, even nonspecific interactions may play a role in entry: because of the anisotropic surface properties, they may preorient the particle. Moreover, because of adhesion, morphological changes may occur, which are worth considering when constructing a mechanistic view for entry or translocation. For instance, as we shall see, a certain degree of reduction in particle height (h) may be expected upon substrate adsorption due to virion deformation caused by adhesive interactions with the substrate.²⁹ The height distribution of adsorbed particles contains information about the balance between cohesive and adhesive interactions at work and, of practical importance, on the resilience of viral particles to mechanochemical disturbance.

For an accurate measurement of the particle height distribution, a flat, chemically homogeneous substrate must be used. Two common AFM substrates—mica and highly oriented pyrolytic graphite (HOPG)—qualify, as they are atomically flat and chemically homogeneous. However, AAV was observed to have a poor surface affinity for polar freshly cleaved mica. As a consequence, the work was carried out on nonpolar HOPG.

Main results include observations of a strong orientational bias at adsorption on HOPG and a substrate-induced mechanical deformation of variable extent for all species (ssAAV, scAAV, and empty AAV). We also report on compressive deformation characteristics by AFM nanoindentation. In particular, the effective elastic constant for AAV (k_v) was measured, which is the most widely used mechanical property of viral particles and has been previously

shown to be a good indicator of genome content^{19,24,30} and structural anisotropy.^{17,31} Moreover, upon an applied force exceeding the linear elastic response limit, the capsid undergoes a nonlinear deformation and rupture event. The magnitude of the critical force (F_r) and the indentation depth (D_r) at rupture are useful indicators of capsid strength.^{23,32,33}

We found that AAV capsids are overall very stiff (relative to other small icosahedral viruses), regardless of the genome content. However, among the three types of particles investigated, scAAV was found to rupture at the deepest indentation. Finally, we show how the extraction of multiple parameters (height, rupture force, elastic constant), which can be cross-analyzed utilizing a multivariate conditional probability approach and a database of mechanical properties acquired from nanoindentation assays, could lead to a determination of genome type on a single-particle basis.

MATERIALS AND METHODS

Chemicals and Reagents.

Phosphate-buffered saline (PBS; 0.1 M sodium phosphate, 0.15 M sodium chloride, pH 7.2) was prepared from premixed packs (Thermo Scientific, Rockford, IL).

Virus Production and Purification.

All viruses were produced using the triple-plasmid transfection method involving the plasmids pXX6–80 encoding Ad helper genes, the pXR2 plasmid encoding AAV2 rep and cap genes, and different packaging constructs. Specifically, the virus samples include: (1) ssAAV vectors packaging an ssCMV-promoter-driven GFP genome (3.3 kb, 3.32 nM), (2) scAAV with an scCMV-promoter-driven GFP genome (4.5 kb total length, containing two 2.1 kb sc strands, 15.78 nM), and (3) empty AAV shells (1.66 nM). Recombinant AAV vectors were purified by iodixanol gradient ultracentrifugation followed by buffer exchange. Empty virion shells were further purified from iodixanol fractions following buffer exchange and sucrose gradient ultracentrifugation described earlier.³⁴

Transmission Electron Microscopy (TEM) of Viral Particles.

Carbon-coated copper TEM grids (Ted Pella, Redding, CA) were first glow-discharged for 15 s. Viral particles in 1× PBS were adsorbed onto the grids for 2 min and then blotted with filter paper. The grids were stained with 2% uranyl acetate for 30 s. After drying, images were taken with a JEOL 1010 TEM. Sizes of particles were measured with ImageJ to generate size histograms.

AFM of Viral Particles.

All AFM experiments were conducted with a Cypher AFM (Asylum Research, Santa Barbara, CA) in liquid at room temperature. A droplet of 50 μ L AAV particles in phosphate-buffered saline (PBS) was first deposited on a freshly cleaved HOPG (ZYG quality, NT-MDT, Moscow, Russia) substrate and incubated for 15 min. Two types of tips were used: (1) BioLever mini tips (Olympus, Tokyo, Japan) with a tip radius of 9 nm and a nominal spring constant of 0.09 N/m; (2) RC800PSA tips (Olympus, Tokyo, Japan) with a tip radius of 20 nm and a nominal spring constant of 0.39 N/m. A droplet of 50 μ L PBS was always added to

prewet the tips. The spring constant of the cantilever, k_{cant} , was calibrated in each individual experiment according to the thermal oscillation method.³⁵

AFM images were acquired in alternating current (dynamic) mode. Capsomeric resolution was achieved by BioLever mini tips with a typical imaging force of ~100 pN. A Gaussian filter and a max filter were applied in sequence to some images to better resolve capsomers. Orientation of particles were subsequently determined from images clearly showing capsomer features at higher than 4 nm lateral resolution. The particle height was measured by cross-sectioning image height data in IgorPro (Wavemetrics, Inc.).

In nanoindentation experiments, viral particles were first imaged for precise localization and then indented in the center by the RC800PSA tip with a loading rate of 2 $\mu\text{m/s}$. Only data from intact particles of nominal height were included in this analysis. An effective spring constant, k_{eff} , was extracted from the slope of the force—distance curve. The spring constant of the virion, k_v , was calculated by considering the virus and the cantilever as two springs in series as follows

$$k_v = \frac{k_{\text{eff}} * k_{\text{cant}}}{k_{\text{cant}} - k_{\text{eff}}} \quad (1)$$

Rupture force (F_r) and depth (D_r) were measured directly between the onset of strain and the first drastic drop in force response in the force—indentation curves.³⁶

Orientation Determination.

High-resolution AFM topography images were acquired as described above. A molecular model was created in Chimera with a resolution of 5 Å and surface-colored by distance to the center of particle.³⁷ The reference orientation was set with twofold symmetry axes parallel to the xyz reference system with Oz normal to the substrate. To resolve orientation relative to the reference in each single AFM image, the crystal structure was manually rotated to visually match the Chimera model with the greatest number of capsomers possible. Once a satisfactory match was achieved, the final orientation of the crystal structure was recorded. It is important to note that all final orientations were reduced by symmetry operations to the smallest asymmetric triangle enclosed by a fivefold and two neighboring threefold sites. The final orientations were plotted in a scatter plot with respect to spatial coordinates to demonstrate orientation distributions.

Multivariate Conditional Probability.

T-tests were run between all species to determine statistical confidence levels for differences between distributions of rupture force and elastic constants. A complete list of results can be found in Table S3.

Multivariate (2d) histograms of rupture force against spring constant were fitted with 2d Gaussian functions in the form

$$A e^{-1/2(1 - \cos^2)\left((x - x_0/\sigma_x)^2 + (y - y_0/\sigma_y)^2 - 2 \cos (x - x_0)(y - y_0)/\sigma_x \sigma_y\right)} \quad (2)$$

where x and y are variables, A is a normalization coefficient, x_0 and y_0 determine peak positions, σ_x and σ_y represent variance, and $\cos$ is the cosine of the rotation angle of the Gaussian axes with respect to the reference system. In practice, spring constant and rupture force were used as x and y variables. Fitting parameters are summarized in Table S4. Three probability distribution functions built on data were named f , g , and h , representing ssAAV, scAAV, and empty AAV, respectively. The mixed distribution function, j , was defined as the numerical average of f , g , and h .

RESULTS

Cargo-Dependent Surface-Induced Deformation.

The homogeneity of particles before AFM was checked by negative-stain TEM for all samples (Figure S1A–C). The majority of viral particle projections appeared round with the same diameter, with only a small fraction of broken or irregular particles. The TEM-measured diameter histograms (Figure S1D–F) show one dominating peak centered at ~26 nm, regardless of the genome content; 92.7% ($N = 578$) of ssAAV particles, 94.3% ($N = 438$) of scAAV particles, and 93.6% ($N = 580$) of empty AAV particles had the same apparent diameter. This suggested that all three samples are monodisperse in size, in solution, and remained so after dehydration during the TEM sample preparation. Sample purity has also been confirmed by silver-stained PAGE (Figure S2).

By contrast, AFM height measurements acquired from particles adsorbed on HOPG surface in 1× PBS buffer (Figure 1) indicated a distinct behavior among different particle species. AFM topographic maps of virions displayed a range of heights, indicating the existence of flattened particles (Figure 1A–D).

After analyzing at least 100 particles for each AAV type, height histograms were constructed (Figure 1E–G). Although intact capsids are known to be stable in solution under the reported buffer conditions, a multimodal height distribution with discrete peaks was observed for all species. Each height peak was associated with a single, well-defined geometry (Figure 2).

A peak centered near 23 nm can always be observed, in good agreement with the expected diameter of 25 nm for full capsids as revealed from the crystal structure of AAV2.³⁸ Other peaks between 10 and 23 nm are evenly distributed in the height histograms with a spacing of ~6 nm between the nearest peaks. Structures with height lower than 10 nm also exist and can be imaged with ~3 nm resolution (Figure S3). Their average height was determined to be 7.01 ± 0.14 nm ($N = 6$). This value is close to the thickness of one protein layer, which is ~6.3 nm. Thus, the structures correspond to segments of AAV shell adsorbed onto the substrate.³⁸

The 2 nm difference in nominal height with respect to the expected diameter is unlikely to come from the AFM imaging force (<0.1 nN), considering that capsid effective stiffness is

more than 1 nN/nm, as we will show later. More likely, this reduction in height is induced by strong adhesive interactions at the contact area between the particle and the AFM substrate.

The evenly spaced multipeak structure suggests that each peak corresponds to particle states, which are lacking the same capsomers. Thus, particles of measured heights between 10 and 23 nm correspond to shell sectors or caps. It is not clear whether these caps are sitting on a flattened base or if the base is missing altogether.

There are two possibilities concerning the origin of these subnominal height particles: (a) the initial solution contains “abnormal”, for example, incomplete, particles, which have higher affinity for the HOPG substrate; (b) the solution contains intact particles, which are distorted by interactions with the substrate during the adsorption and the formation of a contact area.

The first hypothesis can be discarded because iodixanol gradient is an established protocol for the ultracentrifugation-based purification of full particles from empty particles. Particles isolated through this method have been shown to possess a higher purity and infectivity compared to those produced by cesium-based methods.³⁹ Vector purity was routinely reported at 99%.⁴⁰ Negative-stain TEM sample preparation could result in the preferential elimination of partial or malformed particles, leading to a more homogeneous distribution of intact particles. Although cryo-EM does not suffer from the same type of artifact, it gives the same result of a homogeneous narrow size distribution. Moreover, recently, charge-detection mass spectrometry of similar samples has confirmed a negligible amount of “abnormal” particles in solution.⁴¹ Therefore, the second hypothesis of a partial flattening at the contact area upon adsorption is the more likely outcome. As there is preservation of the AAV morphology for the rest of the capsid, which is not in contact with the surface, a top-down projection image, such as that forming in TEM, will not capture flattening. This is likely why TEM data in Figure S1 show a narrow, single-peak distribution.

Interestingly, the fraction of affected adsorbed particles was found to be cargo-dependent. As the observed differences occurred on a nonpolar surface, they raise the issue of potentially different initial interactions of these AAV species with cellular membranes. To quantify the observed differences, we considered that particles exceeding 20 nm in height are the least morphologically affected by adhesion. This ensures that all particles within the main peak are less different in height than a protein layer. The percentage of unaffected particles obtained from AFM was compared to that for TEM experiments (Figure 1H, Table S1). The highest percentage of unaffected particles was observed for ssAAV, followed by scAAV, and empty AAV. Thus, although deformation is a general feature observed upon adsorption on HOPG, the integrity of ssAAV capsids is the least affected by it. This finding is important because it suggests that the genomic cargo, despite being isolated from direct interactions with the substrate surface, does play a role in adhesion. At this point, the mechanism by which this happens remains unclear and warrants further investigation; however, we will note that the allosteric regulation of interactions at the assembly interface in viruses is a common theme.⁴² Thus, the influence of the DNA cargo on virus–substrate adhesion interactions may occur: (i) via the allosteric action on chemical moieties at the outer interface, which are involved in adhesion forces, (ii) via an influence on cohesive

capsomeric interactions, or (iii) directly via an internal structure effect, which resists substrate-induced deformation. Specifically, scAAV has extended duplex regions in its structure—a difference that could influence particle stability at adsorption, by adding tensional integrity.

Empty AAV capsids appear to be the most prone to surface-induced deformation/disassembly. Assuming that the surface chemistry of the three types of particles is similar, as both scAAV and ssAAV are less affected by substrate adsorption than empty AAV, the results suggest that the nucleic acid exerts a stabilizing influence. The type of nucleic acid seems to make a difference: ssAAV is the most resilient to surface-induced deformation, with scAAV having intermediate resilience between ssAAV and empty capsids. We note that recent composition analysis by charge-detection mass spectroscopy revealed that scAAV vector preparations appear to contain particles with partially truncated genomes averaging at half the genome length.⁴¹ This could explain why scAAV is in between ssAAV and empty AAV in terms of the effect of surface-induced deformation.

We also note that possibilities (ii) and (iii) can be tested by nanoindentation. Thus, to further delve into the origin of these differences, we performed nanoindentation measurements.

Orientation Bias.

The structural symmetry of the shell is reflected in the anisotropic mechanical and chemical surface properties. Bias in orientation upon nonspecific adsorption is a manifestation of this anisotropy and can play a role in the stiffness measurement by AFM indentation.⁴³

Moreover, from a biophysical point of view, anisotropic interactions at the plasma membrane may position the virus for subsequent multivalent binding to receptors. For instance, for AAV, anisotropic fluctuations around the mean orientation may provide clues of the mode of surface-induced disassembly. Thus, high-resolution AFM imaging corroborated with crystal structure models were used to determine orientation distributions from individual particles. We found that these distributions were mainly determined by capsid surface properties; however, certain subtler features may be cargo-dependent.

Previous reports on the adsorption of viral particles categorized orientation by the nearest symmetry site, that is, twofold, threefold, or fivefold.^{17,19} Such coarse-grained classification helped with initial demonstrations of the influence of structural anisotropy on the mechanical response. However, the possibility of local deformation by surface adhesion and the consequent biased distribution of possible orientations was not considered. Prior orientation/nanoindentation studies of other viruses employed silica glass substrates. Silica glass surfaces are inherently rough and chemically inhomogeneous with respect to HOPG. Thus, the distribution of observed orientations on glass is expected to show heterogeneous broadening with respect to atomically flat and chemically homogeneous HOPG substrates.

To interpret orientation bias experiments, we assumed that the contact between the capsid and substrate is equivalent to the orientation imaged at the opposite, tip–virus interface. To determine the orientation of a particle from an AFM image (Figure 3A), the orientation obtained by AFM (Figure 3B) was matched visually by rotating a reference orientation

(Figure 3C) in Chimera. It would have been more objective and more effective to have an automated algorithm to determine the particle orientation, especially for larger data sets, yet the manual approach demonstrated satisfactory albeit qualitative one-to-one capsomer matches between AFM images and crystal structure. The capsomers in Figure 3 were numbered to emphasize the one-to-one capsomer match, which allowed for the first time an analysis of the distribution of orientations. Quantification was done according to two polar angles (Figure S4) and represented relative to the smallest asymmetric unit.³⁸

The entire orientation data set for ssAAV, scAAV, and empty AAV adsorbed on the HOPG surface is provided in Figure 4A. There is clear clustering around the twofold axis. The center of mass (mean position) of final orientations and the standard deviation about the mean were plotted in Figure 4B. On average, the particles display an orientation bias toward a twofold site, regardless of the genome content. Also, no significant differences are observed on the average of fluctuations around the mean as quantified by the standard deviation. However, inspection of orientations for the three species (separately plotted in Figure S5 for clarity) indicates that a subtle difference in spread between empty AAV and DNA-laden AAV may exist, with empty AAV orientations occupying mostly dihedral edges. Thus, anisotropic adsorption bias originates in capsid properties, the most likely candidates of which are morphology and hydrophobicity; however, subtle effects arising from the genomic cargo may also play a role.

To tentatively offer an explanation for the observed bias, we first note that the surface of AAV2 has a distinctive morphology with threefold-proximal protrusions (red protruding clusters, Figure 4D,E).³⁸ Such protrusions will likely form initial contacts with the substrate and work collectively in tripod-like structures to support and stabilize the entire capsid on the substrate.

At the same time, a hydrophobicity map created in Chimera³⁷ (Figure S6) shows regions near the twofold and threefold sites being dominantly hydrophobic, whereas some regions near the fivefold site are hydrophilic. Thus, although the areas near the twofold and threefold sites protrude more and have a significantly smaller initial contact area than the fivefold proximal area, they are favored by hydrophobic interactions. Morphology alone could not provide a complete explanation to the orientation bias observed. Instead, we propose a chemical argument by which an increase in the hydrophobic interaction strength associated with the vicinal areas of twofold symmetry axes could be accomplished by structural distortion.

Nanoindentation Analysis.

Measurements of the elastic constant (Figure 5A,D) and rupture force (Figure 5B,C,E,F) were obtained by AFM nanoindentation. Data are summarized in Table S2. When compared with other simple icosahedral viruses,^{22,30,43–45} overall, all AAV particles are remarkably stiff (over 1 N/m) and require a high force to rupture (over 4 nN), regardless of the genome content. Interestingly, when compared with minute virus of mice (MVM), which belongs to the same family of viruses as AAV, we found that the stiffness values of DNA-filled capsids are similar.^{17,46} We also found that surface-adsorbed AAV shows mostly twofold and threefold orientations. For MVM, those two orientations yielded similar stiffness values of

1.4 and 0.8 N/m, respectively. Yet the stiffness of AAV does not show an obvious correlation with the genome content as that of MVM.^{46,47} This is likely due to the diversity among particles. Distribution breadths in Figure 5 are the narrowest for empty capsids. Therefore, the genome does exert an influence on the mechanical parameters measured by nanoindentation and is responsible for the observed heterogeneous broadening.

The second reason for the lack of an obvious shift in properties with genome content is that nanoindentation mainly probes intraparticle constitutive forces, which are very strong to begin with, as the empty capsid data show. As a consequence, similar elastic constants and rupture thresholds were observed for all AAV species. As the instrumental width obtained from measuring a single particle repeatedly is at least an order of magnitude narrower, we can neglect the role of homogeneous broadening.

Elastic behavior of virus capsid under normal compression at small deformations is often modeled according the thin-shell approximation method.^{19,27,43,48} Because shell thickness is non-negligible with respect to particle radius in AAV, the thin-shell approximation will tend to overestimate the elastic modulus. However, it is still informative to compare the elastic parameters found for AAV with respect to other viruses, which were treated in a similar way. Thus, Young's modulus of the AAV capsid material could be calculated as

$$E = \alpha \frac{k_v R}{h^2} \quad 3$$

where R is the outer radius, h is the wall thickness, α is a geometry-dependent coefficient, and k_v is the spring constant.²⁷ The mean spring constant for empty AAV capsid is 1.62 N/m, as determined by AFM nanoindentation. The radius and protein thickness are well characterized by structural studies using cryo-EM. Setting $R=14$ nm, $h=5.6$ nm, and $\alpha=1$, we get

$$E(\text{empty AAV}) = 0.72 \text{ GPa}$$

Such values align well with previously reported ones for other virus capsid proteins.⁴³ Therefore, apparent stiffness is a geometric effect coming from AAV being a small virus.

In the light of findings of cargo-dependent surface-induced deformation (Figure 1), the relative insensitivity of rupture forces and elastic constant mean values to the cargo type (Figure 5) is intriguing. It suggests that intraparticle cohesive forces, which are mainly probed by nanoindentation, are less affected by the presence of genome than interfacial adhesive forces, which are probed by height distribution measurements. We do not have a molecular mechanistic explanation for this; however, the findings highlight the necessity of further scrutiny of adhesion properties of this virus.

Despite the small differences in the median values of mechanical parameters relative to the breadths of distributions, an analysis of correlations between stiffness and rupture thresholds helps to highlight a cargo-dependence that is observable in the AFM nanoindentation data but otherwise buried in the broad distributions of the simple histograms of Figure 5.

Bivariate histograms of rupture force (F_r) and elastic constant (k_v) are presented in Figure 6. Uncorrelated maps are expected to show symmetry about a line parallel to one of the axes. If there is correlation between rupture force and stiffness, the map is expected to have an oblique orientation relative to the axes.

It is important to note that the elastic constant is a parameter measured from the entire elastic deformation regime at lower indentation values with respect to the rupture force, which is measured at relatively large indentations. This implies that the elastic constant probes the particle more locally, and presumably the mechanical response is mainly coming from capsid structural changes in the vicinity of the tip–virus contact area. In contrast, both genome and capsid structures are affected by compression near rupture at every spatial scale. We see from Figure 6 that correlations are conditioned by the presence of genome: weaker for ssDNA, stronger for scDNA. Empty AAV provides a control or baseline, showing little correlation between the two parameters when the genome is absent. Therefore, the genome is involved in promoting correlations across the entire spatial scale range of the particle.

How does an essentially surface-localized phenomenon correlate with a global one? We propose that DNA modulates constitutive capsid interactions responsible for the early elastic response in AAV. The heterogeneous nature of this modulation obscures differences between the three types of particles when only mean values of parameters are compared, but the multivariate analysis in Figure 6 reveals it. Because the steeper the slope of F_r versus k_v , the larger the minimal indentation that is expected to lead to particle breakdown, a significant difference is observed in terms of rupture depth, that is, the average indentation at which rupture occurs, D_r . Thus, we found that scAAV ruptures at 24.5% of the original height on average, whereas this value is 19.5% for ssAAV and 18.5% for empty AAV. scAAV is mechanically more stable and can resist a deeper indentation.

Multivariate Conditional Probability To Determine the Type of Genome Content.

The above bivariate analysis inspired us to consider a potential correlative approach for distinguishing between different types of genome content from measurements on a single-particle level. To this end, normalized probability distributions $P(k_v, F_r)$ were built for each pure sample on the basis of data in Figure 6.

According to Bayes theorem,⁴⁹ the probability of the genomic species encapsulated in a certain particle can be predicted by determining the rupture force and the elastic constant for that particle

$$P(\text{GC}|k_v, F_r) = \frac{P(k_v, F_r|\text{GC}) * P(\text{GC})}{P(k_v, F_r)} \quad 4$$

where GC stands for genome content (ssDNA, scDNA, or empty). A value of 1/3 was used for $P(\text{GC})$ (prior probability), assuming an even mixture of all particles. The probability of finding any particle with certain k_v and F_r , that is, $P(k_v, F_r)$, was achieved by a numerical average of all probabilities for finding an identical particle in each pure sample.

$P(GC|k_v, F_r)$ was plotted with respect to k_v and F_r (Figure S8). The most probable genome content can be found for each set of k_v and F_r values. For instance, a particle with a spring constant of 1.5 N/m and a rupture force of 7 nN has 98.1% probability of being scAAV.

The differences between ssAAV and empty AAV in spring constant and rupture force are less resolved, making it difficult to distinguish single ssAAV particles from empty capsids. However, it is important to note that indentation experiments were done exclusively on intact particles; thus, the parameters discussed here are only a subset of the height histograms, which also potentially contain genome-specific features. On an ensemble level, height histograms are quite distinct. While particle height (h) was not included in the Bayesian analysis sketched above, an extension to include h is possible.

DISCUSSION

The physical properties of AAV capsids significantly affect their biology and the effectiveness of AAV vectors for gene therapy.^{12,50,51} While the capsid affinity to glycan receptors influences tissue tropism, rigidity, elasticity, and structural integrity of the capsid are integral to capsid formation, packaging, trafficking, and uncoating. To understand the latter aspects, we analyzed the morphological and mechanical properties of model AAV gene-delivery vectors in liquid using AFM imaging and nanoindentation for three types of recombinant AAV particles: empty AAV capsids, scDNA AAV, and ssDNA AAV. Heterogeneous mixtures of either two or all three of these particles are present in clinical-grade preparations of AAV. While we found that, to a certain extent, the HOPG substrate induces deformation and possibly partial disassembly of all types of AAV, ssAAV was significantly more resilient to distortion by interfacial interactions with the substrate. Upon substrate adsorption, a strong orientational bias was observed. We propose this orientational bias to be predominantly a result of the hydrophobic interaction with the substrate, a potentially important aspect for better understanding endocytosis and virion–membrane interactions. Substrate-induced deformation is the result of balancing of cohesive forces responsible for elasticity and interfacial forces responsible for adhesion. The final state result of this balancing act was found to be dependent on genome content. To probe whether cohesive forces are responsible for genome dependence of surface-induced deformation, we performed nano-indentation experiments on the least surface affected particles (undeformed).

The nanoindentation assays showed that, in general, AAV capsids are resistant to compression, with mechanical properties dominated by the constituent interactions of the capsid. Correlations with genome content are small and could be established only through quantitative statistical analysis. Thus, the presence of scDNA helps the capsid to be more resilient against deep deformation. The issue of mechanical or mechanochemical stability arises at several stages of the virus life cycle when perturbations of the mechanical character are likely to occur. For instance, upon binding to the cell membrane, AAV particles are engulfed by the cell through receptor-mediated endocytosis.^{15,52} A purely mechanical model described earlier pointed out the possibility of elastic particles to deform during this process and deformability to be an integral part for signaling and interacting with the membrane during cellular uptake.⁵³ As an example of mechanochemical transformation occurring in

certain cases, global conformational changes in the virus are triggered by multivalent receptor binding previous cellular uptake.⁵⁴

An instance of particular interest for gene delivery concerns viral particles being imported into the nucleus. Could this process represent a rate-limiting step? If yes, then the pathway to optimization will require a better understanding of capsid mechanics.¹⁵ The mechanistic specifics of AAV entering the nucleus have not been completely elucidated.⁵⁵ However, because the nuclear pore is gated by biopolymers, it is likely that AAV needs to deform under compression to pass through the steric barrier.^{56–59} As nuclear entry is believed to be an inefficient and rate-limiting step, scAAV vectors may offer an advantage, as they can survive greater deformation upon translocation through the nuclear pore complex, which would lead to a more efficient transgene expression. Another interesting observation is that empty AAV particles appear to be most prone to surface-induced deformation/disassembly. Earlier reports stating that empty AAV particles do not enter the nucleus on the basis of immunofluorescence analysis⁶⁰ make it tempting to speculate that empty AAV shells may readily rupture upon contact with nuclear pores and compression.

CONCLUSIONS

Mechanical properties of three types of AAV vectors were compared: ssAAV vectors packaging an ssCMV-promoter-driven GFP genome (3.3 kb), scAAV with an scCMV-promoter-driven GFP genome (4.5 kb total length, containing two 2.1 kb sc strands), and empty AAV shells. All types of particles are stiff with apparent average elastic constants exceeding 1 N/m. Adhesive interactions with a flat nonpolar surface are strong and lead to significant orientational bias at adsorption. Incidentally, this is beneficial for avoiding anisotropy broadening in the measurement of elastic constants and rupture forces. The magnitude of adhesive interactions is modulated by the genome content, while cohesive interactions are less sensitive to it. Broad spreads around the mean of mechanical parameters, especially in genome-filled particles, suggest a significant particle diversity. Nevertheless, an approach based on multiplexed Bayesian analysis was described, which could facilitate identification of the type of genome through simultaneous analysis of all mechanical response parameters. Overall, these initial studies provide new insight into the relationship among genome packaging, AAV capsid stability, and contact mechanics, through the use of AFM techniques. Expanding this approach will help in understanding the differences between various AAV serotypes in their interaction with host organelles and the physical phenomena that may play a role in the efficiency of transduction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

This work was supported by the U.S. Department of Energy, Office of Science, Basic Energy Sciences, under award DE-SC0010507 (to B.D., for work on virus-substrate interaction and nanoindentation), and the National Institutes for Health (to A.A., grants R01HL089221 and P01HL112761).

REFERENCES

- (1). Douglas T; Young M Viruses: Making friends with old foes. *Science* 2006, 312, 873–875. [PubMed: 16690856]
- (2). Sun J; DuFort C; Daniel M-C; Murali A; Chen C; Gopinath K; Stein B; De M; Rotello VM; Holzenburg A; et al. Core-controlled polymorphism in virus-like particles. *Proc. Natl. Acad. Sci USA* 2007, 104, 1354–1359. [PubMed: 17227841]
- (3). Yildiz I; Shukla S; Steinmetz NF Applications of viral nanoparticles in medicine. *Curr. Opin. Biotechnol* 2011, 22, 901–908. [PubMed: 21592772]
- (4). Jordan PC; Patterson DP; Saboda KN; Edwards EJ; Miettinen HM; Basu G; Thielges MC; Douglas T Self-assembling biomolecular catalysts for hydrogen production. *Nat. Chem.* 2015, 8, 179–185. [PubMed: 26791902]
- (5). Miller RA; Stephanopoulos N; McFarland JM; Rosko AS; Geissler PL; Francis MB Impact of assembly state on the defect tolerance of TMV-based light harvesting arrays. *J. Am. Chem. Soc.* 2010, 132, 6068–6074. [PubMed: 20392093]
- (6). Samulski RJ; Muzyczka N AAV-mediated gene therapy for research and therapeutic purposes. *Annu. Rev. Virol.* 2014, 1, 427–451. [PubMed: 26958729]
- (7). Gao G; Alvira MR; Somanathan S; Lu Y; Vandenberghe LH; Rux JJ; Calcedo R; Sanmiguel J; Abbas Z; Wilson JM Adeno-associated viruses undergo substantial evolution in primates during natural infections. *Proc. Natl. Acad. Sci. U.S.A.* 2003, 100, 6081–6086. [PubMed: 12716974]
- (8). Gao G; Vandenberghe LH; Alvira MR; Lu Y; Calcedo R; Zhou X; Wilson JM Clades of adeno-associated viruses are widely disseminated in human tissues. *J. Virol.* 2004, 78, 6381–6388. [PubMed: 15163731]
- (9). Hastie E; Samulski RJ Adeno-associated virus at 50: A golden anniversary of discovery, research, and gene therapy success—a personal perspective. *Hum. Gene Ther.* 2015, 26, 257–265. [PubMed: 25807962]
- (10). Flotte T R Birth of a new therapeutic platform: 47 years of adeno-associated virus biology from virus discovery to licensed gene therapy. *Mol. Ther.* 2013, 21, 1976–1981. [PubMed: 24201212]
- (11). Mingozzi F; High KA Therapeutic in vivo gene transfer for genetic disease using AAV: Progress and challenges. *Nat. Rev. Genet.* 2011, 12, 341–355. [PubMed: 21499295]
- (12). Horowitz ED; Rahman KS; Bower BD; Dismuke DJ; Falvo MR; Griffith JD; Harvey SC; Asokan A Biophysical and ultrastructural characterization of adeno-associated virus capsid uncoating and genome release. *J. Virol.* 2013, 87, 2994–3002. [PubMed: 23269804]
- (13). Daya S; Berns KI Gene therapy using adeno-associated virus vectors. *Clin. Microbiol. Rev.* 2008, 21, 583–593. [PubMed: 18854481]
- (14). Vasileva A; Jessberger R Precise hit: Adeno-associated virus in gene targeting. *Nat. Rev. Microbiol.* 2005, 3, 837–847. [PubMed: 16261169]
- (15). Nonnenmacher M; Weber T Intracellular transport of recombinant adeno-associated virus vectors. *Gene Ther.* 2012, 19, 649–658. [PubMed: 22357511]
- (16). McCarty DM Self-complementary AAV vectors; advances and applications. *Mol. Ther.* 2008, 16, 1648–1656. [PubMed: 18682697]
- (17). Carrasco C; Carreira A; Schaap I; Serena P; Gomez-Herrero J; Mateu M; De Pablo P DNA-mediated anisotropic mechanical reinforcement of a virus. *Proc. Natl. Acad. Sci. U.S.A.* 2006, 103, 13706–13711. [PubMed: 16945903]
- (18). Maugis D Contact, Adhesion, and Rupture of Elastic Solids; Springer: Berlin, 1999.
- (19). Michel JP; Ivanovska IL; Gibbons MM; Klug WS; Knobler CM; Wuite GJL; Schmidt CF Nanoindentation studies of full and empty viral capsids and the effects of capsid protein mutations on elasticity and strength. *Proc. Natl. Acad. Sci. U.S.A.* 2006, 103, 6184–6189. [PubMed: 16606825]
- (20). Ivanovska I; Wuite G; Jonsson B; Evilevitch A Internal DNA pressure modifies stability of WT phage. *Proc. Natl. Acad. Sci. U.S.A.* 2007, 104, 9603–9608. [PubMed: 17535894]

- (21). Hernando-Perez M; Miranda R; Aznar M; Carrascosa JL; Schaap IA; Reguera D; de Pablo PJ Direct measurement of phage phi29 stiffness provides evidence of internal pressure. *Small* 2012, 8, 2366–2370. [PubMed: 22648860]
- (22). Ni P; Wang Z; Ma X; Das NC; Sokol P; Chiu W; Dragnea B; Hagan M; Kao CC An examination of the electrostatic interactions between the N-terminal tail of the brome mosaic virus coat protein and encapsidated RNAs. *J. Mol. Biol.* 2012, 419, 284–300. [PubMed: 22472420]
- (23). Snijder J; Uetrecht C; Rose R; Sanchez-Eugenio R; Marti G; Agirre J; Guerin D; Wuite G; Heck A; Roos W Probing the biophysical interplay between a viral genome and its capsid. *Nat. Chem.* 2013, 5, 502–509. [PubMed: 23695632]
- (24). Sae-Ueng U; Li D; Zuo X; Huffman JB; Homa FL; Rau D; Evilevitch A Solid-to- fluid DNA transition inside HSV-1 capsid close to the temperature of infection. *Nat. Chem. Biol.* 2014, 10, 861–867. [PubMed: 25195012]
- (25). Bousquet A; Dragnea B; Tayachi M; Temam R Towards the modeling of nanoindentation of virus shells: Do substrate adhesion and geometry matter? *Phys. D* 2016, 336, 28–38.
- (26). Hernando-Perez M; Lambert S; Nakatani-Webster E; Catalano C; De Pablo P Cementing proteins provide extra mechanical stabilization to viral cages. *Nat. Commun.* 2014, 5, No. 4520.
- (27). Ivanovska IL; De Pablo PJ; Ibarra B; Sgalari G; MacKintosh FC; Carrascosa JL; Schmidt CF; Wuite GJL Bacteriophage capsids: tough nanoshells with complex elastic properties. *Proc. Natl. Acad. Sci. U.S.A.* 2004, 101, 7600–7605. [PubMed: 15133147]
- (28). Negishi A; Chen J; McCarty DM; Samulski RJ; Liu J; Superfine R Analysis of the interaction between adeno-associated virus and heparan sulfate using atomic force microscopy. *Glycobiology* 2004, 14, 969–977. [PubMed: 15215232]
- (29). Knez M; Sumser M; Bittner A; Wege C; Jeske H; Hoffmann D; Kuhnke K; Kern K Binding the tobacco mosaic virus to inorganic surfaces. *Langmuir* 2004, 20, 441–447. [PubMed: 15743089]
- (30). Vaughan R; Tragesser B; Ni P; Ma X; Dragnea B; Kao CC The tripartite virions of the brome mosaic virus have distinct physical properties that affect the timing of the infection process. *J. Virol.* 2014, 88, 6483–6491. [PubMed: 24672042]
- (31). Hernando-Perez M; Pascual E; Aznar M; Ionel A; Caston JR; Luque A; Carrascosa JL; Reguera D; de Pablo PJ The interplay between mechanics and stability of viral cages. *Nanoscale* 2014, 6, 2702–2709. [PubMed: 24452242]
- (32). Buenemann M; Lenz P Mechanical limits of viral capsids. *Proc. Natl. Acad. Sci. U.S.A.* 2007, 104, 9925–9930. [PubMed: 17545309]
- (33). Klug WS; Bruinsma RF; Michel J-P; Knobler CM; Ivanovska IL; Schmidt CF; Wuite GJ Failure of viral shells. *Phys. Rev. Lett.* 2006, 97, No. 228101.
- (34). Pulicherla N; Asokan A Peptide affinity reagents for AAV capsid recognition and purification. *Gene Ther.* 2011, 18, 1020–1024. [PubMed: 21490687]
- (35). Proksch R; Schaffer TE; Callahan R; Viani M; Cleveland J Finite optical spot size and position corrections in thermal spring constant calibration. *Nanotechnology* 2004, 15, 1344.
- (36). Roos WH How to Perform a Nanoindentation Experiment on a Virus In *Methods in Molecular Biology*; Humana Press, 2011; pp 251–264.
- (37). Pettersen EF; Goddard TD; Huang CC; Couch GS; Greenblatt DM; Meng EC; Ferrin TE UCSF Chimera-a visualization system for exploratory research and analysis. *J. Comput. Chem.* 2004, 25, 1605–1612. [PubMed: 15264254]
- (38). Xie Q; Bu W; Bhatia S; Hare J; Somasundaram T; Azzi A; Chapman MS The atomic structure of adeno-associated virus (AAV-2), a vector for human gene therapy. *Proc. Natl. Acad. Sci. U.S.A.* 2002, 99, 10405–10410. [PubMed: 12136130]
- (39). Strobel B; Miller FD; Rist W; Lamla T Comparative Analysis of Cesium Chloride-and Iodixanol-Based Purification of Recombinant Adeno-Associated Viral Vectors for Preclinical Applications. *Hum. Gene Ther. Methods* 2015, 26, 147–157. [PubMed: 26222983]
- (40). Zolotukhin S; Byrne BJ; Mason E; Zolotukhin I; Potter M; Chesnut K; Summerford C; Samulski R J.; Muzyczka, N. Recombinant adeno-associated virus purification using novel methods improves infectious titer and yield. *Gene Ther.* 1999, 6, 973–985. [PubMed: 10455399]

- (41). Pierson EE; Keifer DZ; Asokan A; Jarrold MF Resolving adeno-associated viral particle diversity with charge detection mass spectrometry. *Anal. Chem.* 2016, 88, 6718–6725. [PubMed: 27310298]
- (42). Zlotnick A; Mukhopadhyay S Virus assembly, allostery and antivirals. *Trends Microbiol.* 2011, 19, 14–23. [PubMed: 21163649]
- (43). Roos W; Bruinsma R; Wuite G Physical virology. *Nat. Phys.* 2010, 6, 733–743.
- (44). Wilts BD; Schaap IA; Schmidt CF Swelling and softening of the cowpea chlorotic mottle virus in response to pH shifts. *Biophys. J.* 2015, 108, 2541–2549. [PubMed: 25992732]
- (45). Hernando-Perez M; Zeng C; Delalande L; Tsvetkova IB; Bousquet A; Tayachi-Pigeonnat M; Temam R; Dragnea B Nanoindentation of isometric viruses on deterministically corrugated substrates. *J. Phys. Chem. B* 2016, 120, 340–347. [PubMed: 26674071]
- (46). Carrasco C; Castellanos M; de Pablo PJ; Mateu MG Manipulation of the mechanical properties of a virus by protein engineering. *Proc. Natl. Acad. Sci. U.S.A.* 2008, 105, 4150–4155. [PubMed: 18334651]
- (47). Castellanos M; Carrillo PJ; Mateu MG Quantitatively probing propensity for structural transitions in engineered virus nanoparticles by single-molecule mechanical analysis. *Nanoscale* 2015, 7, 5654–5664. [PubMed: 25744136]
- (48). Baclayon M; Shoemaker GK; Uetrecht C; Crawford SE; Estes MK; Prasad BV; Heck AJ; Wuite GJ; Roos WH Prestress strengthens the shell of Norwalk virus nanoparticles. *Nano Lett.* 2011, 11, 4865–4869. [PubMed: 21967663]
- (49). Bayes M; Price M An Essay towards Solving a Problem in the Doctrine of Chances. By the Late Rev. Mr. Bayes, F.R.S. Communicated by Mr. Price, in a Letter to John Canton, A.M.F.R.S. *Philos. Trans. R. Soc. London* 1763, 370–418.
- (50). Rayaprolu V; Kruse S; Kant R; Venkatakrishnan B; Movahed N; Brooke D; Lins B; Bennett A; Potter T; McKenna R; et al. Comparative analysis of adeno-associated virus capsid stability and dynamics. *J. Virol.* 2013, 87, 13150–13160. [PubMed: 24067976]
- (51). Grieger JC; Samulski RJ Packaging capacity of adeno-associated virus serotypes: impact of larger genomes on infectivity and postentry steps. *J. Virol.* 2005, 79, 9933–9944. [PubMed: 16014954]
- (52). Seisenberger G; Ried MU; Endress T; Buning H; Hallek M; Brauchle C Real-time single-molecule imaging of the infection pathway of an adeno-associated virus. *Science* 2001, 294, 1929–1932. [PubMed: 11729319]
- (53). Yi X; Shi X; Gao H Cellular uptake of elastic nanoparticles. *Phys. Rev. Lett.* 2011, 107, No. 098101.
- (54). Levy HC; Bowman VD; Govindasamy L; McKenna R; Nash K; Warrington K; Chen W; Muzyczka N; Yan X; Baker TS; et al. Heparin binding induces conformational changes in Adeno-associated virus serotype 2. *J. Struct. Biol.* 2009, 165, 146–156. [PubMed: 19121398]
- (55). Kobiler O; Drayman N; Butin-Israeli V; Oppenheim A Virus strategies for passing the nuclear envelope barrier. *Nucleus* 2012, 3, 526–539. [PubMed: 22929056]
- (56). Ma J; Goryaynov A; Sarma A; Yang W Self-regulated viscous channel in the nuclear pore complex. *Proc. Natl. Acad. Sci. U.S.A.* 2012, 109, 7326–7331. [PubMed: 22529346]
- (57). Hoelz A; Debler EW; Blobel G The structure of the nuclear pore complex. *Annu. Rev. Biochem.* 2011, 80, 613–643. [PubMed: 21495847]
- (58). Lim RY; Fahrenkrog B; Koser J; Schwarz-Herion K; Deng J; Aeby U Nanomechanical basis of selective gating by the nuclear pore complex. *Science* 2007, 318, 640–643. [PubMed: 17916694]
- (59). Frey S; Richter RP; Gorlich D FG-rich repeats of nuclear pore proteins form a three-dimensional meshwork with hydrogel-like properties. *Science* 2006, 314, 815–817. [PubMed: 17082456]
- (60). Johnson JS; Samulski RJ Enhancement of adeno-associated virus infection by mobilizing capsids into and out of the nucleolus. *J. Virol.* 2009, 83, 2632–2644. [PubMed: 19109385]

Figure 1.

Particle height AFM measurements reveal deformation upon adsorption. (A–C) Representative AFM images of ssAAV, scAAV, and empty AAV, respectively (scale bar: 200 nm). (D) Illustration of particle height measurement by AFM (inset: cross section, scale bar: 100 nm); color scales are the same for all images in A–D. (E–G) Height histograms of ssAAV, scAAV, and empty AAV, respectively; particles less than 10 nm were not included. (H) Comparison of percentage of intact particles between TEM and AFM results.

Figure 2.

Top: AFM topographic maps of particles representative for each of the peaks in the height histograms in Figure 1. Bottom: molecular models of AAV particles having the same orientation.

Figure 3.

Example of orientation determination for a single AFM image. (A) Representative AFM image (scale bar 16 nm); (B) crystal structure in Chimera orientated to match the structure in (A); and (C) reference orientation with a twofold site in the center. Crystal structures were colored according to the distance from particle center, with blue indicating a larger distance than green.

Figure 4.

Particle orientation distributions from AAV adsorbed on HOPG. (A) Scatter plot of orientations relative to the symmetry axes represented as red symbols. (B) Center of mass locations for orientation distributions of the three AAV species. Error bars represent standard deviations; dashed lines represent boundaries of the asymmetric unit. (C) Final orientations relative to the icosahedron surface. (D) Orientations with respect to molecular model colored by distance from particle center (redder appearance means farther from the center). (E) Zoomed-in central portion of (D).

Figure 5.

AFM nanoindentation results. Histograms of (A) spring constant, (B) rupture force, and (C) indentation depth at rupture event for ssAAV (blue), scAAV (green), and empty AAV (yellow), and quartile plots of (D) spring constant, (E) rupture force, and (F) indentation depth at rupture event. The difference in spring constant between scAAV and the other two species is marginal, statistically speaking (* means $p < 0.05$). However, statistically significant differences exist for rupture depth, which is larger for scAAV than for ssAAV (***) means $p < 0.001$) and empty AAV (** means $p < 0.01$).

Figure 6.

Maps of correlated mechanical properties: bivariate histograms of rupture force (F_r) against spring constant (k_v) for ssAAV, scAAV, and empty AAV (contour lines are separated by one count).