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AFM nanoindentation of protein shells, expanding the approach beyond viruses

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Perspective

AFM nanoindentation of protein shells, expanding the approach beyond viruses

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ABSTRACT

The archetypal protein nanoshell is the capsid that surrounds viral genomes. These capsids protect the viral RNA or DNA and function as transport vehicle for their nucleic acid. The material properties of a variety of viral capsids have been probed by Atomic Force Microscopy. In particular nanoindentation measurements revealed the complex mechanics of these shells and the intricate interplay of the capsid with its genomic content. Furthermore, effects of capsid protein mutations, capsid maturation and the effect of environmental changes have been probed. In addition, biological questions have been addressed by AFM nanoindentation of viruses and a direct link between mechanics and infectivity has been revealed. Recently, non-viral protein nanoshells have come under intense scrutiny and now the nanoindentation approach has been expanded to such particles as well. Both natural as well as engineered non-viral protein shells have been probed by this technique. Next to the material properties of viruses, therefore also the mechanics of encapsulins, carboxysomes, vault particles, lumazine synthase and artificial protein nanoshells is discussed here.

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1. Introduction

Viruses are ubiquitous in nature and species in all kingdoms of life are prone to viral infection. While the infectious pathway of a variety of viruses has been described, there remain a lot of open questions regarding this pathway. In order to better fight viral infections, and hence to make better use of viral nanoparticles in nanomedicine and nanotechnology, it is paramount to obtain a profound understanding of their structure and function. A variety of techniques is used to study viral structure including Electron Microscopy, X-ray Crystallography, Nuclear Magnetic Resonance,

Mass Spectrometry and Atomic Force Microscopy (AFM) [1–7]. All these techniques have their specific advantages and the strength of AFM is that it can probe structure, mechanics and dynamics of proteins and proteinaceous assemblies such as viruses under buffer conditions at the single particle level [8–11]. Soon after the invention of AFM, this technique was already used to study viruses [12] and the majority of the initial studies was focused on imaging the viruses, in liquid or in air [13]. The seminal paper of Ivanovska et al. [14] changed the landscape by reporting mechanical studies of viruses under near-to-physiological conditions. This study introduced the AFM nanoindentation approach to describe the elasticity and failure of viruses. In particular, in this work the mechanics of bacteriophage phi29 was probed and material parameters such as the Young's modulus were derived from the indentation stud-

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ies. Herewith the field of “Mechanical Virology” was opened, a research field that can be seen as a part of the much wider research efforts in Physical Virology. A variety of studies followed this first paper [15–18] and the focus of viral nanoindentation studies is now broadening from pre-dominantly physics oriented research on biomaterials to also answer biological questions on the viral life cycle. In addition more and more non-viral nanoshells have been discovered over the last decades and such particles have now come under scrutiny of AFM nanoindentation as well.

2. Brief experimental description of protein shell nanoindentation

The nanoindentation experiments start with imaging of the nanoparticles [13]. Subsequently the middle of the particle is located and the particle is next deformed [19]. Fig. 1a shows a schematic representation of such an indentation where the tip is located at the centre of the protein nanoshell. During the deformation the Force-Distance (FZ) curves are recorded and these curves provide information on the elasticity of the particles and the force at which irreversible deformation occurs. Many protein nanoshells show a clear breaking event, evidenced by a drop in force in the curves [20–22]. The irreversible deformation for other nanoparticles is more subtle and is more typical for plastic deformation. Here an abrupt decrease in force is absent in the FZ-curves, as for instance observed for some wild type and mutant viral capsids [23,24]. There are even conditions under which viral particles behave completely elastic and that they bounce back to their original size after large deformations [25]. Fig. 1b–d show example FZ-curves of respectively elastic deformation, plastic deformation and failure. An AFM image of what a herpes virus capsid looks like after failure is depicted in Fig. 1e.

3. Viral nanoindentation

3.1. Properties of proteinaceous viral structures

As mentioned in the introduction, phage phi29 was probed by viral nanoindentation and it turned out that the mechanics of this particle was rather complex [14]. It could withstand high forces and it was shown that the Young's modulus was comparable to that of hard plastics. Detailed studies on this prolate particle with dimensions $\sim 42 \times 54$ nm followed, focusing for instance on the internal pressure of the particles, using multivalent cations to screen DNA–DNA interactions [26]. In other approaches the (loading rate dependent) breaking behaviour of phi29 was scrutinized [27,28], and it was shown that there is an anisotropic built-in stress in these prolate bacteriophages [29]. Other phages that have been studied by AFM nanoindentation are for instance bacteriophage T7, lambda, HK97 and P22. For the ~ 60 nm sized phage T7 the buckling behaviour upon nanoindentation was shown to be reversible and independent of genomic content [30]. Interestingly, the similarly sized bacteriophage lambda was shown to have a genome that stabilizes its capsid in an intriguing manner. The high internal pressure of the tightly packed DNA inside phage lambda has a clear stabilizing effect, signified by an increased spring constant and breaking force, on the genome filled viral nanoshell as compared to empty shells [31]. Remarkably, mutants that only carry a fraction less DNA, show a mechanical behaviour that is similar to that of empty particles. Using an analytical model including DNA-hydrating water molecules the authors explain the observed behaviour. In a follow-up experiment the developed theory was further tested by using different concentrations of multivalent cations which effectively screen the interactions between the tightly packed DNA strands [32]. It was also shown how the gpD decoration protein of phage

lambda imparts a stabilizing effect on the viral nanoshell [33]. The mechanics of the size-wise and structurally very similar phage HK97 (protein data bank accession number (pdb): 3E8K for Prohead II and pdb: 1OHG for Head II) was also tested by nanoindentation. It turns out that, as later also reported for phage lambda, HK97 has found a method to stabilize its quasi- and icosahedral three-fold axes of symmetry [34]. However, HK97 does not add additional capsid proteins at these sites during maturation, but it triggers covalent crosslinking of the major capsid protein. This step occurs during maturation of the particle, simultaneously with DNA packaging. It seems that the particle needs to be flexible during self-assembly, to correct for possible mistakes in this process. If self-assembly was a more rigid process, the occurrence of non-functional, partial assemblies would probably be higher. When these partial assemblies are not flexible, in order for mistakes to be corrected, the energy stored in protein synthesis for these dead-end products would have been lost. Therefore, a flexible assembly pathway seems optimal. However, during and after DNA packaging high pressures by the tightly packed DNA will be exerted onto the capsid walls and a strong particle is needed. HK97 solved these possibly contradicting requirements by introducing covalent cross-linking of the shell during maturation [35], so that it can be weak and flexible during assembly and strong during and after DNA packaging. The AFM experiments revealed that indeed one sees this strong stabilizing effect for HK97 maturation [34]. At the same time it was also shown that HK97 found a more robust maturation pathway than phage lambda as it can deform much more before irreversible failure occurs. As for phage lambda, the mechanics of the similarly sized phage P22 (pdb: 3IYI) was also studied in the presence and absence of decorating proteins [36]. Here in particular it was striking to see how this protein was restoring the mechanics of pentonless particles (i.e. particles in which the pentons, which are rings of five capsid proteins occupying the vertices of the icosahedral shells, are removed). P22 and other viruses were additionally studied in more nanotechnology oriented approaches by studying their mechanical properties in the presence of non-endogenous cargo molecules [37,38]. This shows how AFM is not only well suited to better understand how viruses are built-up, but that it also has strong potential in the study of virus-like particles (VLPs) for potential uses in nanotechnology.

The AFM nanoindentation technique is not only applied to bacteriophages but also to eukaryotic cell infecting viruses. For instance a variety of human viral capsids have been studied. The human Herpes Simplex Virus (HSV) has an icosahedral shell of ~ 125 nm diameter that bears similarities to bacteriophage capsids. Its mechanical properties were studied revealing how both the empty as well as the genome filled capsids can withstand high forces before breakage and how DNA packaging is triggering a stabilization switch in this virus [39,40]. A detailed, combined experimental and modelling study on the mechanics of pentonless and intact HSV capsids attempted to test the predictions of continuum elastic theory that the vertices of icosahedral particles should be under a state of pre-stress [22]. It turned out that it was possible to test and confirm the prediction as indeed the five-fold disclinations of the capsids were under a state of pre-stress. The vertex properties of HSV were even scrutinized more as the influence of the vertex-associated proteins UL17 and UL25 on capsid stability was tested. Detailed knowledge on the orientation of the UL17/UL25 complex is known from reconstructions [41]. Using deletion mutants for either UL17 or for UL25 it was shown how both proteins are essential for stabilizing the icosahedral vertices of the HSV capsids [42]. Capsids of other human viruses that have been studied include those of the viruses Hepatitis B Virus (HBV) and Norovirus (also called Norwalk virus). The icosahedral HBV capsids can form in two different morphologies,

Fig. 1. AFM nanoindentation. a) Schematic of AFM nanoindentation approach with bend cantilever and a chosen tip radius a bit smaller than the nanocage radius. The particles are typically adhered onto glass cover slips, mica or Highly Oriented Pyrolytic Graphite (HOPG). A modified version of this figure was previously published in ref [86]. b) Three consecutive FZ curves on Hepatitis B Virus (HBV) capsids show linear deformation behaviour and fall on top of each other, indicating that at these relatively low indentation forces elastic deformation is taking place. The height before and after the indentation is the same. c) Indentation at higher force shows that the particle plastically deforms. This can be concluded from the non-linear, but continuous FZ 1 curves, the absence of a drop in the force in these curves and the decrease in the slope in the three consecutive FZ curves. The height after indentation is $\sim 10\%$ lower than before, but no obvious damage can be observed. Here indentation curves on two different particles (A and B) are shown. Panels b) and c) are modified and reprinted from ref. [23] with permission from Elsevier. d) First indentation curves on 5 different Herpes Virus capsids. A clear drop in the force is seen at a certain force. This indicates failure. e) Imaging the herpes particles after indentation shows a clear hole in the capsid. Panels d) and e) are modified from Ref. [22] with permission from the American Physical Society.

Fig. 2. Noro Virus. a) AFM image of the NVLP. b) Image of the CT303 mutant. c) Height profiles of the two particles along the lines in panels a) and b) indicating how the removal of the arch-like protrusions results in a drastic decrease in particle diameter. d) Three dimensional rendering of a NVLP particle reveals the depressions in the shell in between the arch-like protrusions. The inset at the bottom right shows a reconstruction of the NVLP where the arch-like domains are clearly visible on the surface. The experiments were performed in a buffer of 200 mM sodium phosphate, 500 mM sodium chloride at pH 6.2 and the particles were absorbed onto hydrophobic glass cover slips [19]. Modified and reprinted with permission from Ref. [24]. Copyright 2011, American Chemical Society.

T=3 consisting of 90 dimers and T=4 (pdb: 1QGT) consisting of 120 dimers. The diameter of the T=4 capsid is $\sim 32\text{--}36\text{ nm}$ and the T=3 capsid is $\sim 15\%$ smaller. Studies combining nanoindentation with finite element and molecular dynamics simulations have revealed how complementary these approaches are and that only by combining all of them one gets in-depth information on the viral structure [23,43,44]. Noro virus ($\sim 38\text{ nm}$ diameter, pdb: 1IHM) turned out to have very special material properties as it was shown how the particle is under a state of permanent, isotropic pre-stress [24]. Fig. 2 shows AFM images and height profiles of the Noro Virus Like Particle (NVLP) and the CT303 mutant ($\sim 27\text{ nm}$ diameter), which has a smooth surface. The arch-like protrusions that are present on the NVLP capsid outer surface are responsible for the recorded pre-stress, thereby significantly strengthening the particles. This feature could be helpful for the particles to survive under harsh conditions. Not only capsid structures influence viral properties, also the genome can be of influence as also shown for bacteriophage lambda. In this regard studies on Adeno Associated

Virus (AAV) are of interest. AAV is a $\sim 25\text{ nm}$ single stranded DNA virus and nanoindentation experiments indicated that the genomic cargo might influence capsid absorption to the graphite surface [45]. A human virus of which the mechanics is studied a lot by AFM is Adenovirus (pdb: 4CWU). This virus, with a pseudo-T=25 icosahedral capsid, has a diameter over 90 nm and encapsidates a $\sim 36\text{ kbp}$ double stranded DNA genome. For Adenovirus maturation [21] and disassembly [46–48] was studied in detail by AFM manipulation. The disassembly studies revealed on the one hand technological advances in our analysis techniques by introducing a detailed study of disassembly by imaging instead of nanoindentation [46] and by fluorescent studies of genome expansion following on shell rupture [48]. On the other hand, they provided direct biological insight into the viral infectious pathway by revealing how the secondary receptor integrin is responsible for specifically weakening the capsid vertices [47]. Thereby for the first time a direct link between mechanics and viral genome uncoating was established. Finally, adenovirus was used to develop a new model for

Fig. 3. BMV on corrugated surfaces. a) Overview image with a closed pack array of 100 nm diameter polystyrene beads with BMV particles adhered to it. b) Zoomed in image with one BMV particle at the interface of two beads and one on top of a bead. c) Image with two BMV particles at the interface of three beads and one on top of a bead. Scale bar in the three panels is 40 nm d) Schematic of forces on deformed BMV particle at the interface of the beads. The polystyrene beads are deposited on a graphite substrate and the virus-bead assemblies were imaged in liquid. Reprinted with permission from Ref. [59]. Copyright 2016, American Chemical Society.

indentation analysis combining Hertzian and elastic deformation components [49].

Next to capsids of human viruses, also capsids of for instance insect, avian, mice and plant viruses were studied by AFM nanoindentation. The ssRNA insect virus Triatoma Virus (TrV, pdb: 3NAP) is of particular interest as it has a potential usage as biopesticide to fight Chagas disease [50]. TrV is available upon purification as a mixed population of the genome containing virion and the empty capsid which both have a diameter of ~ 30 nm. By scrutinizing the pH dependent dynamic properties of TrV in a combined Mass Spectrometry (MS) and AFM approach, it was possible to elucidate the influence of the genome on viral stability [20]. It turned out that the viral RNA has a stabilizing effect at neutral pH, but a destabilizing effect at alkaline pH. It is suggested that the pH dependent properties are tuned for the particle staying stable in the acidic insect crop and to start priming the virus for uncoating in the more alkaline gut of the insect. Another example of how nucleic acids can influence the mechanical stability of viruses is shown for the dsRNA avian infectious bursal disease virus for which it was reported how viral ribonucleoproteins stabilize the capsid [51]. The ssDNA Minute Virus of Mice (MVM, diameter ~ 25 nm, pdb: 1MVM) has a particular way of being stabilized by its genome as it predominantly stabilizes the two-fold icosahedral axis of the capsid and not the five-fold axes [52,53]. In addition it was reported how weak interactions between the protein subunits influence the virus yield upon infection. Interpreting these results it was suggested that these weak interactions would promote successful viral assembly and therefore increase the yield during infection [54]. In addition to the direct role of the genome on the stability of some viral capsids, also calcium ions can influence the mechanics of viral capsids drastically as was shown for the ~ 33 nm sized Tomato Bushy Stunt Virus [55]. The mechanical properties of the plant virus Cowpea Chlorotic Mottle Virus (CCMV, diameter ~ 28 nm, pdb: 1CWP) were tested as function of loading rate [28], genomic content [56] and non-endogenous cargo [38]. Moreover, the dynamic indentation response was studied [57] as well as the intriguing pH dependent swelling behaviour and the associated softening [25,58]. Finally, a related virus with a similar size, Brome Mosaic Virus (BMV, pdb: 3J7L), was studied by nanoindentation. The dependence of the deformability and yield force on contact area was tested using corrugated surfaces (Fig. 3) [59]. It turned out that the spring constant is not affected by absorption geometry on the structured surface, but that the yield force is affected. Furthermore the influence of the tripartite genome on material properties of the virus was tested and a different mechanical behaviour was found for the three subsets of BMV virions [60].

3.2. Mechanics of enveloped viral particles

Next to the material properties of viral capsids, also the mechanics of viruses with an outer lipid bilayer leaflet has been probed. For instance the maturation of Murine Leukemia Virus (MLV, diameter

~ 100 nm) and Human Immunodeficiency Virus (HIV, diameter ~ 100 nm) was studied in detail [61,62]. For HIV it was found how the stiffness of the viral particle is a regulator for entry [62,63]. Herewith a direct link between the mechanics of a viral particle and its infectivity was revealed. Furthermore it was shown how reverse transcription of the viral genome is increasing the pressure inside the virus and initiates the disassembly of the particle after infection [64]. In order to understand the mechanics of enveloped viruses, it is essential to get a picture on the mechanics of lipid bilayers alone. Various AFM studies have been performed to probe the material properties of liposomes [65–67], including detailed studies incorporating a model based on Canham-Helfrich theory and probing the liposome deformation response for different cantilever tip radii [68,69]. For viral studies in particular, liposomes made up of Influenza virus envelope lipids were studied in a nanoindentation approach [70]. These results supported creating an overall view on the mechanics of influenza virus (diameter ~ 100 nm) and in follow-up studies the envelope protein dependent mechanical behaviour was studied as well as the viral uncoating process [71,72]. In the latter studies it was shown how uncoating proceeds in two mechanically different, and pH dependent, steps [72,73]. All in all it remains quite a challenge to interpret the results of mechanical measurements on enveloped particles as they are composites of nucleic acids, proteins and lipids. This might be an explanation why the body of nanoindentation studies on capsids alone is so much larger than on enveloped viruses. However, given the examples above and the increased insights that have been created over the last years, the scene is set to explore the material properties of other viral particles with lipid bilayers.

4. Nanoindentation on non-viral protein shells

While the initial indentation studies on protein nanoshells were all performed on viral particles, recently more and more non-viral nanoshells have been mechanically probed by AFM. Such shells are identified in both prokaryotic as well as eukaryotic cells [74–76]. Some of these nano-sized compartments encapsulate enzymes that catalyse metabolic steps. For others, the exact function is up-to-date unknown. An example of such a particle is the Vault particle. Vault particles are ribonucleoprotein particles present in eukaryotic cells. The cellular function of these particles remains unclear and they are, among others, studied as potential drug delivery vehicles. The structure of the vault particles can be approximately described by two double cylinders of which one is capped and that are linked to each other. The dimensions of these elongated assemblies are ~ 40 nm in diameter at the central barrel portion, narrowing to ~ 20 nm towards the capped region and ~ 67 nm in length [76]. The mechanical properties of the vault particles are probed by AFM and in particular the elasticity of complete as well as half vaults was probed [77]. Furthermore, the particles were indented until fracture and the particles were imaged

Fig. 4. Vault particles. a) AFM image of a vault particle before indentation. b) AFM image after indentation. c) Corresponding Force-deformation curves. The black curve is the indentation curve, which shows a clear drop in the force at 150 pN indicating a fracture event. The grey curve is the retraction curve and the jump-up to ~75 pN reveals the healing of the fracture. The particles were adsorbed on HOPG surfaces and imaged in liquid. Reprinted from Ref. [77], Copyright 2014, with permission from Elsevier.

after indentation. Sometimes a fracture was observed. Remarkably, these fractures disappeared over-time, i.e. they were self-healing. In other cases the fracture already disappeared before the next image was taken, but the Force-deformation curve revealed the presence of a transient fracture, as can be seen in Fig. 4. Overall recovery typically occurred on time scales of less than a few seconds, but the temporal resolution of the set-up did not allow for detailed studies on this process. Experiments by High Speed AFM could probably provide more accurate information onto the recovery behaviour of the vault particles. Follow-up studies scrutinized the pH dependent stability and revealed how low pH is destabilizing the central barrel region of the vault particles [78]. The authors speculate that the destabilisation is linked to a weakening of the lateral protein-protein interactions at low pH. In another approach, the mechanics of Lumazine Synthase from *Aquifex aeolicus* (AaLS) was probed [79]. Lumazine synthase is a bacterial enzyme involved

in riboflavin synthesis. It is present in various bacteria and in different morphologies. AaLS was found to possess a $T = 1$ icosahedral structure, i.e. it consists of 60 identical subunits that aggregate in 12 pentamers which together form the ~16 nm nanoshell (pdb: 1HQK) [80]. The AaLS wild type particles (wt) showed a high mechanical resilience, revealing that the extreme thermostability (melting temperature of 120 °C [80]) is reflected in the strength of the AaLS particles. Next to the wt particles also AaLS-13 mutant particles with negative residues on the inside were tested. Likely as a result of the negative charges on the inside, these particles form in a different morphology and the measured height (~37 nm) would fit with a $T = 3$ structure. The mutant particles were tested in an empty state as well as in a loaded state. The load was supercharged GFP (+36). No difference in material properties could be observed for the empty and cargo-containing particles indicating that loading of this cargo does not influence the material properties of the particles. However, a clear difference was found between the wt and mutant particles. Both the spring constant and the break force were at least an order of magnitude lower for the AaLS-13 particle with respect to the AaLS particle. The approximate Young's modulus dropped from ~520 MPa for the wt particle to ~23 MPa for the mutant particle. The recently resolved structure of the AaLS-13 particles revealed that there are large openings in the cage [81], providing an explanation for the observed very low spring constant of the particles. The paper on AaLS also introduced a new way of analysing nanoindentation curves [79]. In this approach, inspired by indentation analyses of HBV particles [44], first all the indentation curves are averaged and next the mechanical properties were retrieved. This in contrast to curve-by-curve analysis as traditionally is performed for nanoindentation experiments. It was confirmed that the new approach results in a similar outcome with respect to the normally adopted way of analysis, but one of the additional advantages is that another way of representing the data, in density plots, gives a direct overview of the distribution of all indentation curves. Fig. 5 shows an AFM image of an AaLS-13 particle and the density plot of indentation curves on a wt AaLS particle.

In a combined AFM nanoindentation, native Mass Spectrometry and modelling approach the ~22 nm bacterial nanocompartment Encapsulin (pdb: 3DKT) was probed [82]. The MS experiments revealed that encapsulin only carries a single copy of the hexameric cargo enzyme. While for the cargo-free and the cargo-loaded AaLS mutant particles no clear difference in mechanical properties was found, this was not the case for encapsulin. The cargo-loaded particles broke at a much lower force than the empty encapsulins. This difference was independent on whether native or non-native cargo was encapsulated. It seems that the local binding of the cargo is frustrating the shell integrity and that such a defect would nucleate mechanical failure upon deformation. Detailed molecular dynamics simulations would be able to shed light onto possible differences between the empty and loaded encapsulin particles. Bacterial microcompartments such as the carboxysome are bacterial metabolic nanocages that are involved in carbon fixation. Despite their name, they are rather nanocompartments than microcompartments as the typical diameter is roughly 100 – 150 nm [75]. Imaging by High Speed AFM revealed the dynamics of facet assembly, indicating that assembly is governed by weak inter-protein interactions [83]. Nanoindentation experiments showed that the ~150 nm carboxysomes are relatively soft (~10x softer than phage P22) [84]. However, as the particles are much larger than phage P22 the reported Young's modulus, determined using the thin-shell model, of both particles is similar. Next to naturally occurring protein shells, recently also artificial protein shells such as the octahedral ~13 nm in diameter O3-33 cages (pdb: 3VCD) have been designed and assembled [85]. These particles were the subject of nanoindentation studies [79]. Here the particles were adhered onto a mica surface and subsequently imaged and deformed. Fig. 5 shows

Fig. 5. AaLS and O3-3 particles. a) AFM image of an AaLS-13 particle. The recorded height is ~ 30 nm, but this is not yet corrected for the indentation during imaging and the actual height is therefore higher (see for instance ref. [19] for a discussion on this topic). The inset shows a negative stain electron micrograph of an AaLS-13 particle. Particles are absorbed onto HOPG and imaged in 25 mM sodium phosphate buffer, 0.5 M NaCl, 1 mM EDTA at pH 7.8. b) Image of an O3-33 artificial nanocage. The image height is ~ 13 nm. The inset shows a reconstruction of the octahedral particle. Particles are absorbed onto mica and imaged in 25 mM Tris-HCl buffer, 150 mM NaCl, 1 mM DTT at pH 8. c) Density plot of all indentation curves on wt AaLS particles with in black the average indentation curve. Modified and reprinted with permission from Ref. [79]. Copyright 2016, American Chemical Society.

an image of the adhered artificial nanocage. The particles could deform up to $\sim 16\%$ before breakage and the estimated Young's modulus was in the range as found for CCMV.

5. Conclusion

While initially the protein nanoshell AFM indentation field was mainly curiosity driven research on the astonishing rich and complex material properties of viruses, we now see the field maturing and expanding. First of all we see that a variety of biological questions on viruses are now addressed by AFM nanoindentation, second we see that a variety of non-viral nanoshells are recently being probed as well. This development reveals the broad applicability of the AFM nanoindentation approach and it is expected that this powerful technique will be at the basis of a multitude of discoveries yet to come.

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