12

Force Microscopy – A Tool to Elucidate the Relationship Between Nanomechanics and Function in Viruses

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1. Introduction

Within the last 20 years nanotechnology has broadened our perspective of different well established disciplines. One of these is virology, which through the development of techniques for the manipulation of single nanoobjects has evolved and acquired a new direction giving birth to the new emerging field of physical virology. An excellent review on this has been recently published (Roos, et al., 2010). Viruses, originally conceived only as harmful parasites, causatives of disease are now being investigated from a single particle point of view. The intrinsic mechanical properties of individual viruses have started to be unveiled by means of ultrasensitive surface probe techniques such as Atomic force microscopy (AFM) (Roos & Wuite, 2009). The profiler device is an ultrasharp tip which is also being used for probing viruses with piconewton (10^{-12} N) loading forces.

From the materials science point of view viruses can be considered as highly ordered nanostructured assemblies usually composed by no more than a handful of different components. Viruses can be either non-enveloped or enveloped viruses. In a non-enveloped virus the genome being either DNA or RNA is encaged inside a quasi-spherical protein shell, the capsid. In an enveloped virus the capsid is additionally covered with a lipid bilayer which contains proteins. This lipid envelope stems usually from the cellular membrane of the host. The viral capsid is on one hand a protective barrier which allows save transportation of the genome against chemical hazards, mechanical deformation, and drastic changes in environmental conditions. On the other hand the capsid is designed by nature for being capable of cell entry and cargo release. The capsid self-assembles without requiring external energy. One of the remarkable features of viral capsids is their symmetry which is a consequence of self-assembly from identical subunits (Baker, et al., 1999). The shape of a wide range of viruses can be described by the quasi-equivalence principle (Caspar & Klug, 1962). In this concept the shape of viral capsids is composed of a network of hexagons forming the facets and connected by pentagons at the vertices of the resulting icosahedra. The triangulation number T used to classify viral shapes can be calculated from a walk between two neighboring pentagons over the shortest possible distance performing h steps in one direction and k steps in the other direction as shown in Fig. 1. In this particular example h equals 1 and k equals 2. T is then given by

$$T = h^2 + k^2 + h \cdot k = 1 + 4 + 2 = 7 \quad (1)$$

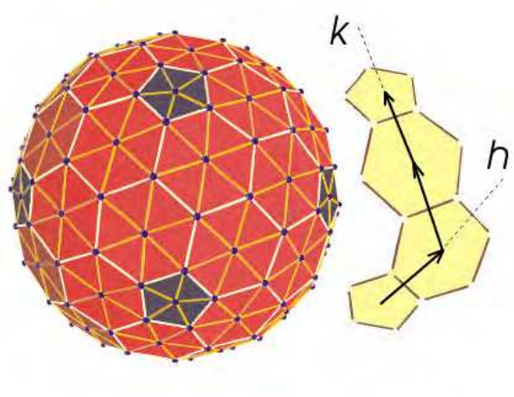

Fig. 1. Scheme of the Caspar & Klug construction for the calculation of triangulation numbers. With permission from (Siber, 2006).

However, there are some exceptions. For example, viruses within the family of Papovaviridae cannot be properly described by the Caspar & Klug principle. Recently developed concepts account for a wider gamma of features in viral capsid structure (Twarock, 2006).

Some viruses like the Tobacco mosaic virus require the presence of the genome for posterior capsid assembly (Fraenkel-Conrat & Williams, 1955). Other capsids, such as that of the Cowpea Chlorotic Mottle Virus (CCMV) may be reversibly assembled *in vitro* without the presence of the genome resulting in so-called virus-like particles (VLP) (Bancroft, et al., 1967). Empty capsids of several human viruses like papilloma virus, norovirus, rotavirus and Ebola virus have been used as vaccines (Nicollier-Jamot, et al., 2004, Schiller & Lowy, 2001, Warfield, et al., 2003) (Crawford, et al., 1999). Self assembly of viral capsids in the absence of the genome may lead to capsids with different triangulation numbers observing as a rule a reduced stability (White, et al., 1997). The surface of VLP´s can be engineered by bioconjugation approaches. As the position of every amino acid is known with subnanometer precision nanostructures with an unprecedented accurateness of the chemical properties of their surface can be fabricated. The use of viruses and VLPs extends well beyond medicine. Aided by modern genetic engineering techniques like the Baculo virus expression system the production of large amounts of recombinant proteins became possible (Ernst, et al., 1998, Kost & Condreay, 1999, Kost, et al., 2005). This has paved the way for using viral cages in nanotechnology, for example, as containers and templates for the assembly of hybrid materials (Graff, et al., 2002) (S. W. Lee, et al., 2003, Nam, et al., 2006). Viruses and VLP´s are currently being used as basic elements in electronic devices, as nanoreactors for catalysis, platforms for surface functionalization and display systems (Fischlechner & Donath, 2007, Fischlechner, et al., 2006, Singh, et al., 2006, Uchida, et al., 2007, Vriezema, et al., 2005).

The structural and material properties of viruses including their mechanical properties have developed through millions of years of interacting evolution with their hosts. Following the enormous progress in molecular virology, exploring their mechanical properties in relation to function is of great importance for understanding the biology of viruses. AFM could

reveal the mechanics during the first steps of viral entry. A nanoindentation experimental setup can uncover internal structural transformations. In most cases, it is not known how the capsid gets destabilized at the final stage of infection and how this is correlated with the release of the genome. This chapter focuses thus on the application of force microscopy as a novel powerful technique as applied to virology. Nanoindentation experiments may help to answer questions like how soft, hard or fragile is a single wild type virus or an empty viral capsid? How elastic or how stable are these assemblies under external applied forces? Do their material properties resemble similarities with those of macroscopic materials? Which external environmental conditions can change their mechanical properties? And finally, what can their mechanical properties tell us about their mechanism of infection. Also the newly developed field of single receptor-virus interactions is briefly introduced.

2. Mechanical properties of lipid layers and vesicles

With atomic force microscopy, the material properties of single nanoobjects can be probed directly after they have been localized with imaging. After positioning the cantilever above the desired object repeated approach-retraction cycles can be performed by controlling the movement of the piezo-crystal in the z direction. The tip is pressed against the sample until a predetermined cantilever deflection is attained. After an eventual dwell time the cantilever is retracted completing one approach-retraction cycle. With the cantilever spring constant the originally recorded deflection values can be converted into force. Finally, forces vs. separation curves are obtained. In commercial devices this procedure is usually provided by a software option. Force vs. separation curves provide a wealth of information about the details of interaction of the tip with the sample and the mechanical properties in the nanoscale (Capella & Dietler, 1999). In the force-mode the AFM tip can be used as an indenting tool to test deformability and compliance of soft homogeneous, layered or composite nanomaterials (Bhushan, 2007, Radmacher, et al., 1995). The Young modulus can be obtained from indentation experiments applying the Hertz model (Hertz, 1882). Refinements of this model take account for the finite thickness of layers on solid supports.

Of special interest for virus related studies are the mechanical behavior of supported lipid bilayers and the indentation of lipid vesicles, since supported lipid layers form a constitutive part of enveloped viruses. When the tip starts to compress the bilayer, repulsion is observed. If the load gets sufficiently large the tip may puncture the bilayer overcoming the intermolecular forces holding the lipid molecules together. The associated breakthrough forces have been measured (Loi, et al., 2002, Pera, et al., 2004, Richter & Brisson, 2003). After breakthrough, as a rule, regular desorption plateaus upon retraction can be observed. They are associated with pulling tethers of lipids connecting the tip with the substrate. The tips can be modified to control their interaction with lipid membranes. Dufrene and Schneider studied the mechanical behavior of supported bilayers in some detail (Dufrene, et al., 1998) At forces below 5 nN the lipid bilayer behaves as an elastic film. Repulsion was attributed to steric and hydration forces as well as to the elastic response after contact. The observed hardness was 108 Nm^{-2}. Distances traveled by the AFM tip within jumps into the substrate were of the order of ~ 6-7nm, which is close to the value of the bilayer thickness. Their studies established a connection between mechanical properties of lipid bilayers with the interaction forces between the lipid molecules within the bilayer and stressed their importance for adhesion of cells and membrane fusion. Recently, more sophisticated

theoretical and experimental approaches have been undertaken to extract mechanical parameters like the bending rigidity κ and the surface tension σ from indentation experiments (Norouzi, et al., 2006, Steltenkamp, et al., 2006). The mechanics of various vesicle structures has also been studied. When a small spherical vesicle of the size of the order of viruses is being indented at least at larger indentations the volume cannot be conserved as the area of the vesicle membrane can only slightly increase because of the high stretching resistance of bilayers. The elastic properties of cholinergic synaptic vesicles were first reported by the group of Hansma (Garcia, et al., 1998, Laney, et al., 1997). For Indentations of about 10 nm on vesicles with diameters of about 110 nm with AFM tips with a radius of 11-23 nm, Young moduli of 2-13 x 10^5 Pa were calculated. Studies on synaptic vesicles and their interaction with the Synapsin protein were also reported (Awizio, et al., 2007). Their stiffness was 0.2 pN/nm and the Young modulus was 75 kPa applying Reissner´s theory of deformation of thin elastic shells (Reissner, 1952). The deformation of closed shells depends significantly on whether the shells are empty or filled, because the response is given by an interplay of out of plane bending and in plane stretching that arises at the point of compression. The response of the material to the applied force is thus governed by its intrinsic elastic (Y) and bending (κ) material properties. When sufficiently high loading forces are applied buckling may occur (Helfer, et al., 2001, Tamura, et al., 2004). Buckling is an instability phenomenon which further complicates the analysis of indentation experiments. Liang et al. investigated the mechanical response of Egg yolk Phosphatidylcholine unilamellar vesicles by nanoindentations (Liang, et al., 2004). It was found that force distance curves showed two breakthrough forces which was interpreted as the puncturing the upper and lower bilayer of the vesicle. Values for the bending and Young modulus of 0.21×10^{-19} J and 1.97×10^6 Pa were reported using the Hertz model. The application of the Hertz to closed thin shells is, however, problematic. As an example of a biological vesicle the elastic properties of Clathrin-coated vesicles have been measured (Jin & Nossal, 2008, Jin, et al., 2006). A bending rigidity of about 285 k_bT was calculated for these protein-reinforced vesicles with diameters of about 100 nm. Delorme et al. fabricated hollow faceted polyhedrons from a mixture of cationic surfactants. The shape of them resembled that of viruses (Delorme, et al., 2006). Localized indentations in faces or vertexes exhibited pronounced differences in stiffness. Using the Reissner model a Young´s modulus of 180 MPa and a bending rigidity of 450 k_BT were obtained. Interestingly, concepts from continuum mechanics apply quite well to deformations in assembled nanostructured materials formed by subunits if they show a fluid behavior at the individual scale.

3. Imaging viruses and capsids with AFM

Atomic force microscopy has proved to be an efficient tool for imaging of soft nanomaterials in the dry state as well as under liquid conditions. Even though experimental techniques like X-ray diffraction, Scanning electron microscopy and Cryo-Transmission electron microscopy provide a wealth of information from external and internal features of virus morphology and structure with nearly atomic resolution, samples often require artificial staining or fixation methods which can be problematic with regard to virus characterization in the native state. With AFM viruses can be imaged under physiological conditions within minutes (Malkin, et al., 2002). AFM is thus as a complementary tool for the immediate identification of pathogens. Structural and morphological changes of the virus can be monitored in response to a change of the environmental conditions as the media can be

exchanged during the experiment. Another major advantage is that only minimal amounts of the sample are required for imaging. 50-100 µl of a concentrated sample are more than sufficient. The preparation of a virus sample for AFM imaging is straightforward. First, appropriate attachment of the viral particles to the substrate is required since shear forces present an unavoidable risk during scanning with the AFM tip even with soft cantilevers. This can be accomplished by proper selection of the substrate to ensure sufficient adhesion of the viruses or VLP´s. For example, the substrate can be coated with polymers or it can be made more hydrophobic by silanization (Zhao, et al., 2006). Viruses can be also covalently attached to previously functionalized surfaces. Anti-viral antibodies (McDonnell, et al., 2002) and patterned surfaces have been employed (Howorka, et al., 2009). However, it has to be taken into account that adhesion forces if strong enough could lead to flattening of VLP´s. This may lead to a subsequent underestimation of the diameter of the virus (Cuellar, et al., 2010). In the context of imaging it is thus advisable to study the affinity of the viruses to the surface, because if the attachment is too weak imaging might induce detachment. In the opposite case of a rather strong interaction the virus envelopes might be deformed or even destroyed. A related problem is, that the applied loading forces during imaging have to be kept as low as possible in order to reduce deformation of the virus shell. Imaging hollow VLP´s has to be performed with particular care. The absence of the genome renders them even more susceptible to deformation. In this case tapping mode should be preferred because shear forces and contact times with the sample are largely reduced (Moller, et al., 1999). Glass and mica are the most common substrates for particle immobilization as AFM is concerned. Both surfaces are easily available and are rather flat. While glass normally required mild cleaning procedures through well established protocols, mica has to be only cleaved (pulled apart) with the help of a commercial adhesive tape. This produces a molecularly flat and clean surface ready to use. In AFM imaging a superior subatomic resolution can be reached as far as height is concerned, but its lateral resolution is much lower being limited by the accuracy of the piezo scanner and the shape and dimensions of the tip. Even the sharpest AFM tip has a finite tip radius of the order of 10 nm. Therefore pits and slots with characteristic dimensions of the tip radius cannot be imaged. Furthermore, convolution caused by the tip size and shape leads to an overestimation of the width and makes measurements of shape of spherical objects below the equator impossible (Banin, et al., 2002). As a rule of thumb, for viruses, the measured width has been reported to be 2 to 5 times that of the real particle (Kuznetsov, et al., 2001). Enveloped viruses are more difficult to image than non-enveloped ones because the space between the soft lipid envelope and the capsid core is usually filled with a soft matrix protein layer which enhances the compliance of the virus and also increases the possibility of disruption of the surface by the tip while imaging. Also if the size of the capsid is much smaller than the entire particle, the capsid may be free to move inside the viscous matrix protein resulting in a further enhanced compliance of the virus. Viruses can be imaged both in the dry state (air) as well as under physiological conditions (liquid). Imaging in the dehydrated state is generally easier, and the resolution is higher but it has to be taken into account that during dehydration, the virus may loose mechanical stability and strength leading to shrinkage and collapse into oblong or flattened shapes because of strong capillary forces occurring during evaporation of the liquid. Crystallization of salt is another difficulty associated with drying from physiological solutions. Imaging in dry state can be advantageous in certain situations,

for example, when information of the general architecture of a virus is in the focus rather than the mechanical properties of the virus itself. Image acquisition can start right after placement of the sample under the AFM scanner since the sample must not be incubated to reach equilibrium due thermal motion within the fluid prior to imaging. However, important features of a virus can be only revealed under at least near physiological conditions, where the virus particle retains all its natural properties.

AFM has been applied to characterize the surface of tobacco mosaic virus, and the interaction with its genome for the full and partial coated case (Drygin, et al., 1998). The host-virus interaction was studied with Pox virus on living cells (Ohnesorge, et al., 1997). A detailed characterization of morphological and structural aspects of several plant, insect and animal viruses was undertaken by the group of A. McPherson. The successful attachment of a large amount of viral particles to flat substrates and their posterior growth into 2 and 3D crystals by a vapor diffusion approach permitted to investigate their nucleation mechanism into highly packed hexagonal lattices (Malkin, et al., 2002, McPherson, et al., 2003). It was found that sometimes aberrant sized virions where incorporated within the lattices, inducing defects on the surface and breaking the symmetry of the crystal. Tapping mode AFM yielded images of small viruses of about 28 nm with molecular resolution. Images of Brome mosaic virus and Turnip yellow mosaic virus showed the individual capsomers (see figure 2). However in other cases, like the satellite tobacco mosaic virus and the insect tipula Iridescent virus, the molecular details could not be resolved but the faceted shapes of the capsids were recognized.

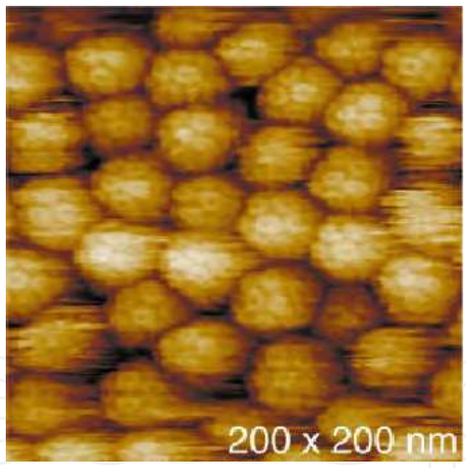

Fig. 2. High resolution AFM images of Brome mosaic virus. Capsomers at the surface can be identified. With permission from (Kuznetsov, et al., 2001).

They also studied animal viruses of relevance for health like Vaccinia, HIV, Herpes simplex virus and Murine Leukemia virus. Their external morphological features where comparable to those obtained by electron microscopy, and moreover, the internal structure of these viruses could be uncovered by using detergents to remove the lipid envelope, using proteolytic enzymes, low pH exposure or assisted particle disruption.

Decomposition of Herpes simplex virus allowed for identification of structural elements in every layer (Malkin, et al., 2002). Elongated glycoproteins protruding 10 to 25 nm out the

surface of the enveloped particle were detected. When the envelope was removed with detergents pentons and hexons forming the capsomers could be identified. Remarkable high resolution AFM images of wild type Herpes virus and a pentonless empty version of the capsids of the same virus were obtained after treatment with Urea (Sodeik, et al., 2009). Finally, the treatment of the virus with a strong ionic detergent like sodium dodecyl sulfate (SDS) induced extrusion of the viral DNA which was clearly seen in the AFM images. Images of the surface at high magnification of wild type Murine Leukemia virus and Human immunodeficiency virus revealed dense arrangements of protein tufts on their surface (McPherson, et al., 2004, McPherson, et al., 2004). For HIV, the protrusions on the surface of the envelope have been associated with aggregates of the gp120 protein. Blebs protruding out of the surface were observed and have been identified as being scars from the budding process of the virus. After detergent treatment a capsid size of 35 to 40 nm was determined from AFM images. The envelope formed a 35 nm thick layer around the capsid (McPherson, et al., 2005). The size distribution of MLV and HIV VLP´s was not consistent with a homogeneous population of VLP´s. The particle size ranged from 120 - 170 nm and 100 - 160 nm, respectively. These broad distributions in virus size may be a product of deviations in the self assembly process of the capsid proteins. This is more likely to occur in empty capsids where the genome is not involved during particle formation, because it is known that the genome forms the scaffold for assembly and induces stability.

The multilayered architecture of Vaccinia virus has been investigated in hydrated and dehydrated conditions. It was found that the outer corona with embedded proteins and its respective proteinaceous lateral bodies characteristic for this virus were clearly visible (McPherson, et al., 2008). Exposure of these virions to proteases yielded empty cores which when dried resembled flattened sacs with pancake shapes. From this study it was further concluded that the encased genome was moderately condensed within the capsid, rather than being in a dense and compact state. The group of P. Hinterdorfer investigated the topography and RNA release of Human Rhinovirus onto mica surfaces under physiological conditions (Hinterdorfer, et al., 2004). It was shown that polygonal subunits with sizes of about 3 nm could be discerned in individual particles and that exposure of the virions to acidic conditions lead to RNA release. The absence of loop formation of the RNA molecule in the case of partial release suggested that the observed features of release induced by acid conditions may be related to the mechanism of genome release from the viral capsid upon infection.

The capability of AFM to resolve fine topographical features at the surface of the viral particle strongly depends on the radius of the apex of the tip and on the particular molecular architecture on the external capsid surface. A sharper tip is obviously advantageous for imaging small pits and protrusions at the viral surface. Theoretically it should be possible to visualize the hexameric and pentameric arrangements of proteins within capsomers or the localization of spike proteins at the surface. Nevertheless, for viruses which have crowded proteins coronas with only narrow molecular canyons, like Norovirus and Cowpea Chlorotic Mottle virus, high resolution images at the surface are limited by the geometrical constraints of the tip (Cuellar, et al., 2010, Knobler, et al., 2006). In these cases, with typical commercial Silicon Nitride tips with a radius of about 15 nm the viruses appear as hemispherical caps as shown in figure 3. With the fast advancement of AFM technology, these limitations will be improved by the use of sharper probes fabricated

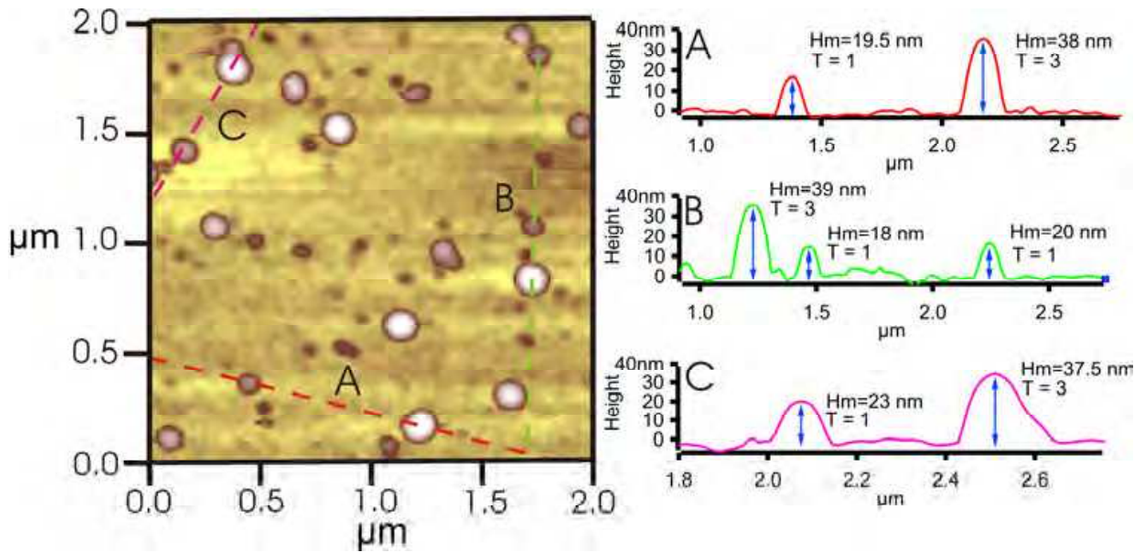

Fig. 3. Height image of coexisting Norovirus-like particles with T = 1 and T = 3 capsid number. The capsids appear as hemispherical caps without discernible substructure.

for example from carbon nanotubes. Nonetheless the positions of the symmetrical five, three and two-fold capsid axes with respect to the substrate was distinguishable with the current tips even for one of the smallest viruses like the Parvovirus minute virus of mice (MVM) with a average size of only about 25 nm (Pablo, et al., 2006). Refined studies on the protein organization of influenza A virus in neutral an low pH have been recently reported (Le Grimellec, et al., 2010). A clear honey comb organization of activated spike proteins at low pH was noticed possibly related to the initial steps of viral fusion with the endosomal membrane.

The two key steps of infection, membrane fusion during entry of the virus into the cell and release by budding may be explored by AFM. Fusion of enveloped viruses with membranes is usually a pH triggered mechanism that can be studied using supported lipid bilayers. AFM Images of fused Rubella-like particles onto lipid coated colloids have been reported in the context of engineering authentic viral building blocks on colloids (Fischlechner, et al., 2007). The particles appear as distinctive objects of smaller size than the original particles embedded into the lipid layer when imaged onto a solid substrate. The uptake of nanoparticles by cells via endocytosis has been followed by AFM (Labhasetwar & Vasir, 2008). These results encourage the use of AFM as a perspective technique to explore the entry mechanisms of a variety of non-enveloped viruses for which little is known. The process of viral budding has been recorded for several enveloped viruses in a real-time frame sequence (McPherson, et al., 2003). Massive numbers of virions have been seen appearing at the cell surface as shown in figure 4. Moreover, the interesting topographic changes a cell suffers at different stages of infection can be monitored, as has been done for West Nile virus and corona virus in Vero cells (J. W. M. Lee & Ng, 2004). An increase of filopodia formation was observed following infection by West Nile virus, accompanied by actin filament formation in the periphery of the infected cell membrane. Magnified cellular regions showed how newly formed viruses where transported into cell filopodia within sacs or envelopes. Cells infected with severe acute respiratory syndrome-associated corona virus

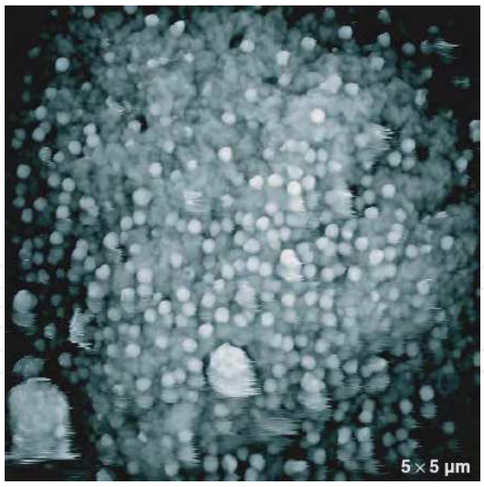

Fig. 4. AFM image of a massive number of HIV particles budding out the surface of a virus infected H9 human fibroblast in culture. With permission from (Kuznetsov, et al., 2004)

showed thickening at the edges of their pseudopodia, which are likely to be responsible for the extrusion of incipient particles (Ng, et al., 2004). The budding of retroviruses has been demonstrated (Rousso & Gladnikoff, 2008, Rousso, et al., 2009). The budding process of Moloney murine leukemia virus was continuously visualized as viral particles emerged out from the membrane within a time period of about 48 minutes. Budding dynamics of viral particles was observed to occur at two different rates. It was also found that budding take place at indifferent sites onto the cell membrane. Their subsequent study on budding of the HIV showed how the infection process alters the internal organization of the cell cytoskeleton in the immediate regions of virus release. This demonstrated that assembly and budding of retroviruses are a cytoskeleton mediated processes.

4. Nanointdentation studies in viruses and capsids

The mechanical properties of individual viruses and VLP´s can be obtained in a straightforward way from nanoindentation experiments. The force vs separation curves yield effective spring constants as a function of the applied force. The effective spring constant is a combination of the spring constants of the cantilever and that of the viral particle. This cantilever-capsid system can be treated as two springs in series. The spring constant of the employed cantilever is determined before by pressing it onto a hard surface and by analyzing its thermal noise spectrum in solution. The point stiffness or spring constant of the capsid itself can be thus obtained as follows $k_{Cap} = (k_{Cant} * k_{Eff})/(k_{Cant} - k_{Eff})$. The spring constant of the capsid depends both on the fundamental elastic material properties such as Young modulus and Poisson number as well as on the shape of the object and on the way how the force is applied. Extracting the elastic properties of the material itself from the experimentally obtained spring constants is a rather challenging task, especially in the case of more complicated geometries, where analytically tractable formulas are not available. Furthermore, there is always an uncertainty whether the tip was pressed onto the center of the nanoparticle or whether it was displaced from it by an unknown distance. It has also to be considered that in the case of facetted viral particles the mechanical response will be different depending on whether the tip was pressed onto a vertex or a facet. Although the

point stiffness already provides a mechanical feature of the capsid, it is nevertheless desirable to extract the fundamental elastic parameters of the capsid material itself. From the theory of elasticity it is known that the complete description of elastic deformations of bodies requires knowledge of two elastic constants related to the properties of the material itself. In most cases these two parameters are given by the Young modulus Y and the Poisson ratio μ. For example, the tensile strength of materials is usually characterized in terms of the Young modulus which provides a direct measure of the hardness of the material under consideration. The Young modulus is the ratio of stress applied to the face of a body with constant cross section to the resulting strain. Poisson´s number represents the negative ratio of the relative change in thickness to the strain being the relative change in length. In order to derive the Young modulus of virus particles or capsids some simplifying assumptions have to be made. Quite often the particle is approximated as a closed thin shell where the radius is much larger than shell thickness. This allows using the well elaborated theory of deformation of thin shells (Landau & Lifshitz, 1986). For small deformations ξ of the order of the shell thickness, d, applied on a spherical shell of radius R by a point loading force F a linear response is predicted. This linearity with the applied force is given by

$$F = \frac{\alpha \cdot Y \cdot d^2 \cdot \xi}{R} \tag{2}$$

here α is a proportionality factor close to unity which has been derived for viral capsids by means of a finite element analysis (Gibbons & Klug, 2007). Most studies related to mechanics of viral shells apply thin shell theory and/or numerical finite element analysis to interpret experimental Data (see figure 5) (Kasas & Dietler, 2008). The virus capsid is considered as a homogeneous elastic material with Hookean behavior.

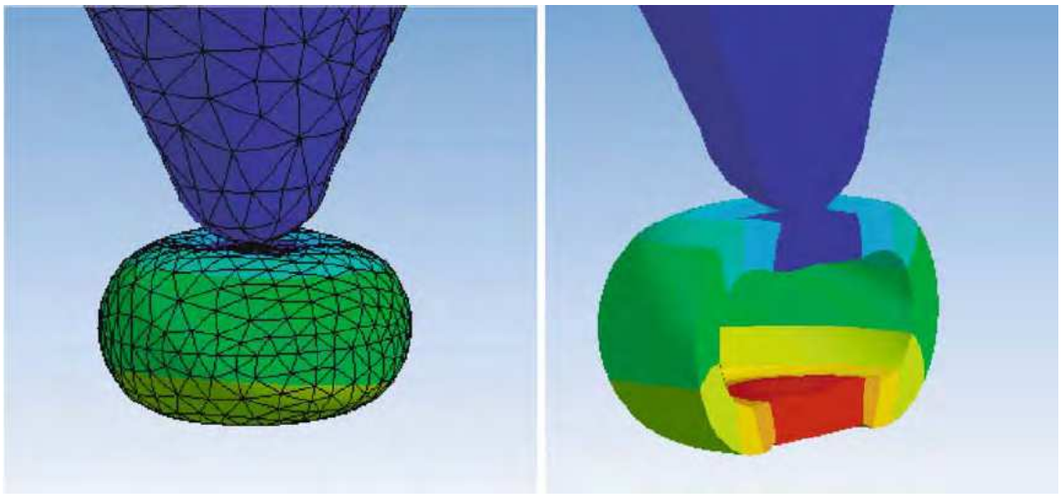

Fig. 5. Scheme of finite element analysis to model the compression of a shell by an AFM tip. With permission (Kasas & Dietler, 2008).

The first nanoindentation experiments on VLP´s were conducted by Ivanovska et al. on the φ29 bacteriophage capsid (Ivanovska, et al., 2004). The life cycle of this phage is rather interesting. The capsid is first assembled and then connected to a molecular motor which is used to pack the genome inside the assembled capsid. It has been shown that the capsid can

withstand internal pressures as high as 60 bar (Smith, et al., 2001). This stored pressure is subsequently used as an energy reservoir to provide the driving force for the infection process. After the virus attaches to a specific receptor at the surface of the bacterium a molecular gate opens and the genome is forced into the interior of the bacterium. Indentations on the φ29 capsids showed an elastic behavior at small loading forces. A bimodal distribution for the capsid stiffness was found, which was attributed to the inhomogeneous structural features in the capsid protein structure. Deformations of up to about 30% of the total height of the capsid observed a linear response. Repetitive indentations showed full recovery of the shell. Indentations with higher loading forces of about 2.8 nN led to a nonlinear behavior which usually terminated with a sudden collapse of the shell. Consecutive indentations finally resulted in breakage of the shell indicating induced fatigue of the material. A Young modulus of 1.8 GPa was found which is within the range of hard plastics. Two years later, the mechanical properties of the empty and wild type CCMV were investigated at different pH values.

The idea behind this was that the CCMV capsid shows a reversible expansion as a function of pH, which is thought to be caused by mutual electrostatic repulsion between internal residues of the capsid proteins (Michel, et al.,2006). It was found that the radial swelling was accompanied by a softening of the capsid material. In the swollen state at pH 6, the capsid responded linearly for deformations up to 70% of the capsid diameter followed by complete recovery. At pH 5, a three-fold increase in the capsid stiffness together with a drastic drop in the force was observed, which indicated a structural failure of the shell. The RNA containing capsid displayed a similar elastic behavior with a slight but yet measurable increase in stiffness. A single point mutation in the capsid increased its stiffness from 140 to 190 MPa. This is an important finding concerning the design of nanostructured materials with tunable properties. Stiffness values of the order of 10^2 MPa are comparable to soft plastics like Teflon.

For DNA - carrying viruses, an increase in stiffness of the native virus compared with the empty capsid can be expected, because the crowded inner state of its genome will resist externally induced compressions. This has been demonstrated for λ-phages (Evilevitch, et al.,2011, Ivanovska, et al.,2007). The virus was twice as strong as an empty capsid. A linear response of the capsid without hysteresis to the applied force was observed with fully reproducible elastic behavior for indentations of about 25% of the capsid radius. A critical maximal threshold force of 0.8 nN was required to break an empty capsid while 1.6 nN were needed to break DNA - filled capsids. This threshold force for rupturing empty λ-phage capsids is about half the value required to break the φ29 phage capsids. The capsid material had a Young modulus of 1.0 GPa. This study proved that viruses acquire structural support and stability against indentations due the internal osmotic pressure generated by the hydrated state of the highly packed genome within the capsid. Wild type minute virus of mice showed a similar reinforcement (Carrasco, et al.,2006). High resolution imaging and indentations performed precisely at the two, three and five-fold axes of the DNA-filled and empty viral capsids, permitted to discern an anisotropic behavior in the stiffness of capsid. Empty capsids showed an isotropic force response. It was therefore concluded that internal DNA interactions at specific sites of the capsid wall contributed differently to this mechanical reinforcement, decreasing the bending of the capsid wall during external induced deformations by the AFM tip. Replacement of a single amino acid was sufficient to remove specific non-covalent interactions between the DNA molecule and sixty equivalent binding sites within the capsid wall (Carrasco, et al., 2008).

The DNA-filled Herpes simplex virus-1(HSV-1) appeared to be the strongest virus tested to date by AFM nanoindentations. The envelope-free capsid could withstand maximal compression forces of about 6 nN (Liashkovich, et al.,2008). Mechanical failure of the DNA containing capsids was observed at loading forces of about 9 nN. This failure resulted in expulsion of the genome. For these large viral capsids of about 120-130 nm in diameter deformations slightly below the shell thickness (~16 nm) were linear, however, irreversible capsid damage was observe for forces above 7 nN. A noticeable decrease of stiffness observed for empty capsids was attributed to the absence of nucleic acids. Other data did not imply a difference in the stiffness and threshold breaking forces between empty and DNA-filled capsids (Roos, et al., 2009). One reason for this discrepancy concerning the influence of the content on capsid stiffness may be due to the presence of certain amount of tegument still attached to the particles. The presence of this tegument could probably reinforce the capsid resistance against deformation.

The first nanoindentation studies on mechanical properties of animal and enveloped viruses were performed by Kol et al. Murine Leukemia virus (MLV) and Human immunodeficiency virus (HIV) (Kol, et al.,2006, Kol, et al.,2007). The focus of this work was on detecting internal morphological alterations that these viruses undergo during the maturation process as a requirement for infectivity. A drastic change in the measured stiffness in both viruses between their mature and immature state was indeed observed. The stiffness of the MLV decreased three-fold and that of HIV more than 14-fold upon maturation, respectively. The mature shell of the MLV became brittle upon repetitive compression and by application of large loading forces. The higher stiffness of HIV particles in the immature state was attributed to the presence of a specific viral envelope protein cytoplasmic tail domain, since finite element calculations suggested that removal of this domain would result in an 8-fold decrease of the Young´s modulus of the particle. The decrease in stiffness goes along with a decrease in shell thickness during maturation. The authors conclude that the regulation of retroviral mechanical properties plays an important role in the life cycle of these viruses. They are stiff during viral budding and need to become soft during entry. In the above mentioned work, an eventual influence of the lipid envelope on the mechanical properties of viruses and VLP´s has been not taken into account. Recently, the mechanical properties of Rubella-like particles (RLPs) have been investigated (Cuellar, et al., 2012). Rubella viruses are enveloped viruses with a relatively thick matrix layer located between the envelope and the capsid (Risco, et al., 2003). Instead of a single linear regime, two consecutive linear force responses were obtained upon indentation. It was supposed that the first is related to the mechanical response of the envelope while in the second one the deformation is transmitted to the capsid (Figure 7C). It was found that RLP´s are very soft and can thus be easily deformed by external forces. Even when the particles were imaged with the lowest possible loading forces below 100 pN and with very soft cantilevers with a stiffness of 0.01 N/m the RLP´s were noticeably compressed during imaging. This followed from a comparison with indentation measurements which revealed the height at the first contact, which was comparable to electron microscopy data. The spring constant corresponding to the steeper linear regime was about 0.017 N/m. This is a quite small value compared with other viruses suggesting that the RLP´s represent rather soft particles. In a coming publication, the influence of membrane deformation and osmotic pressure due the entrapped matrix protein between capsid and envelope on the mechanical properties of RLP´s will be discussed.

AFM nanoindentation and imaging experiments with hepatitis B virus (HBV) capsids together with mass spectrometry of the HBV proteins showed that the proteins assemble into discernible T = 3 and T = 4 capsids (see figure 6) (Uetrecht, et al.,2008). The Young moduli of both structure variants were very close being in the range comparable to soft plastic materials. The capsids observed irreversible deformation for indentation forces close to 1 nN. Our own work on Norovirus (NV) virus-like particles showed a relationship between stiffness and size on one hand and the pH value on the other hand (Cuellar, et al., 2010). It was concluded that these changes are related to a modulation of non-covalent interactions among the capsomers by the pH. This pH dependence of the stiffness may play a role for infectivity. The Norovirus-like particles showed a remarkable elastic behavior upon repetitive indentations even for high loads as high as 8 nN, where the capsid was totally compressed and, nevertheless, the deformations were fully reversible upon retraction. Nonetheless, in certain occasions a progressive failure pattern developed in the force curves upon consecutive compressions. Even in this case the slope and the initial point of contact remained unchanged. The recovery of the particle stiffness after total compression suggests an inbuilt self-healing capacity of these capsids against externally induced deformations.

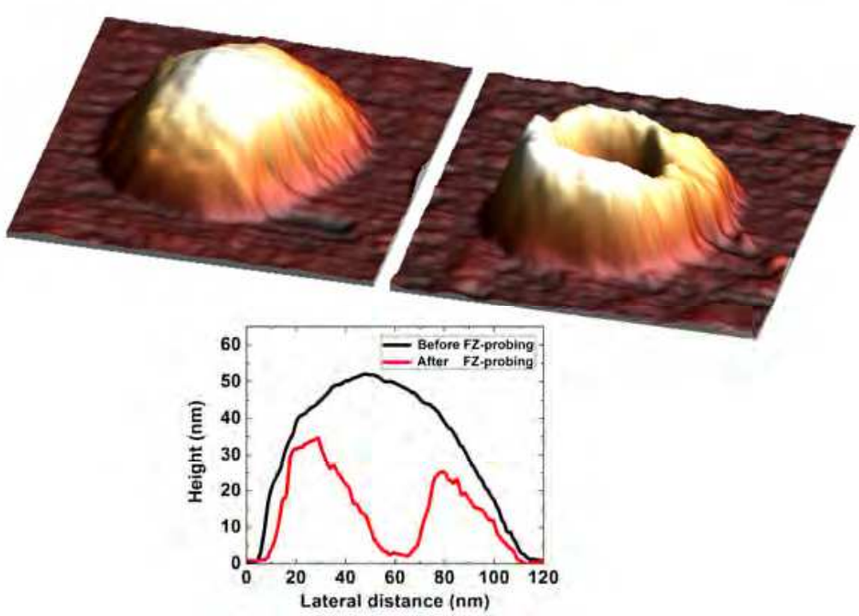

Fig. 6. A virus before and after indentation. Indentation with 4.5 nN resulted in capsid damage (right). The lower image provides height profiles across the capsid. With permission, W. H. Roos, G. J. L. Wuite, Nanoindentation studies reveal material properties of viruses. Adv. Mat., 2009, Vol. 21, 1187-1192

In parallel with experiments theoretical approaches have been developed to study the shape and deformation of viral capsids by taking into account both external loading forces and internal osmotic pressure exerted by the genome (Buenemann & Lenz, 2008, Zandi & Reguera, 2005, Zandi, et al., 2004). The indentation by an AFM tip has been simulated providing an understanding of the ongoing dislocation and the loss of stability on a molecular level (Ahadi, et al., 2009, Arkhipov, et al., 2009, Roos, et al., 2010, VIiegenthart & Gompper, 2007, Vliegenthart & Gompper, 2006, Zink & Grubmuller, 2009).

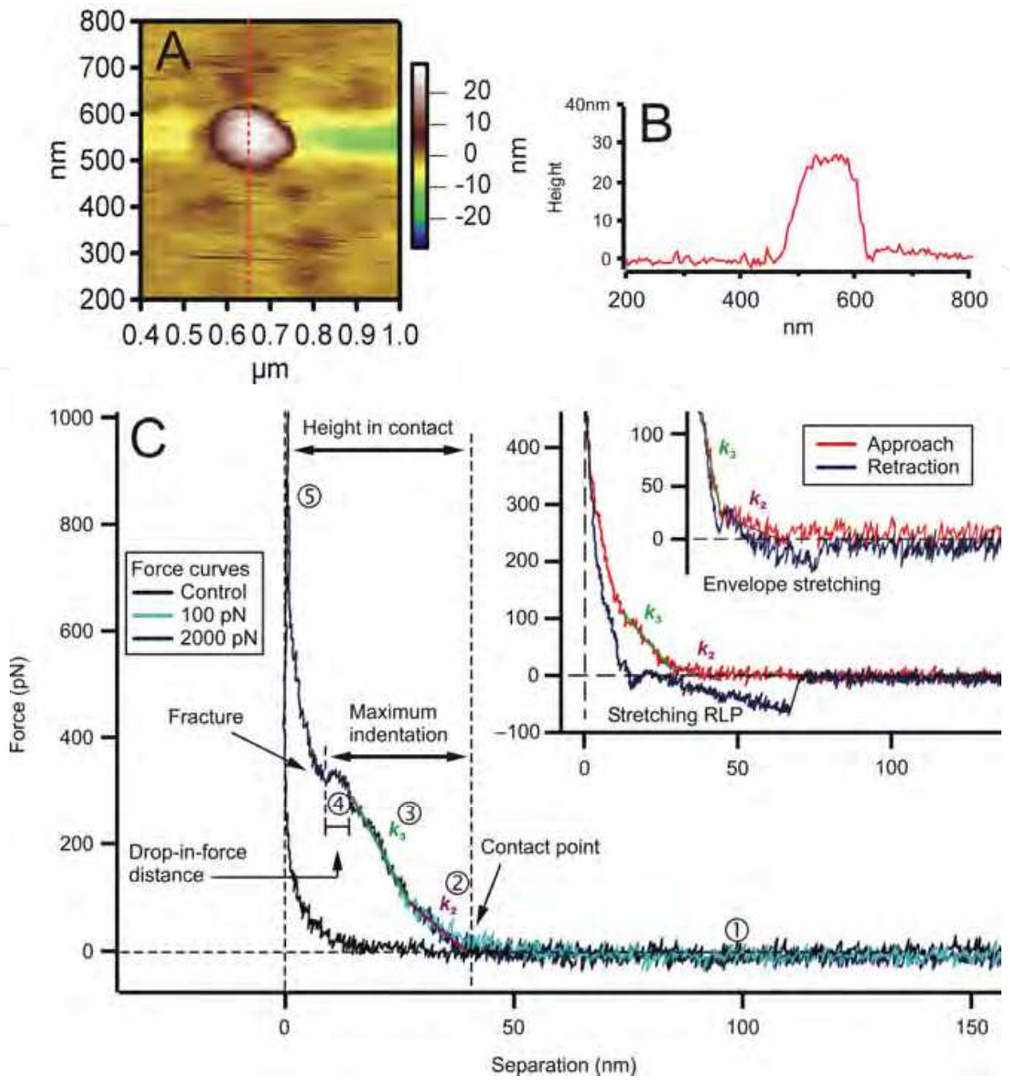

Fig. 7. Indentations on Rubella-Like particles. A and B show a height image of an RLP and its respective profile. In C, approach curves for small (light blue) and large (dark blue) indentations together with the control curve (black) are shown. The two insets show magnifications of approach and retraction curves for small loading forces.

An important parameter is the dimensionless Föppl-von Kármán number. It characterizes the balance between stretching and bending energies for large deflections of thin plates. If it becomes too large buckling occurs. It can thus be used to predict viral shapes and buckling transitions under high loads (Klug, et al., 2006, Lidmar, et al., 2003, Siber, 2006, Siber & Podgornik, 2009).

5. Force spectroscopy of receptor-virus interactions

In recent years, force spectroscopy has been applied for studying virus-host interactions at the level of single molecules. In this special AFM technique the properties of the single molecules themselves or their interaction with receptors are probed. A variety of chemical

functionalization approaches permit the firm attachment of molecules of interest to AFM tips to test their interaction with surfaces. Most of the original studies of this later known as single molecule force spectroscopy (SMFS) technique were devoted to investigate the minute forces required for single polymer stretching or adsorption (Hugel, et al., 2001, Hugel & Seitz, 2001). The group of Gaub was the first, who have successfully applied this approach to measure bond breakage for individual ligand-receptor interactions. For example, the work required for unbinding can be quantified (Florin, et al., 1994, Grandbois, et al., 1999, Moy, et al., 1994). Typically, forces of about 2 nN were required for bond dissociation in single covalent silicon-carbon pairs, while one of the strongest known and commonly used non-covalent biological complexes that of stretptavidin-biotin resist only about 257 pN before rupture occurs. In this way, the doors to the field of single molecular recognition spectroscopy were open as was shown by subsequent studies on the affinity between individual antibody-antigen pairs (Allen, et al., 1997, Browning-Kelley, et al., 1997, Hinterdorfer, et al., 1996) . No less interesting is the force mapping of cellular receptors directly at the cell surface (Gunning, et al., 2008). Such studies could identify regions with a high or low density of specific receptors (Horton, et al., 2002). Thereby, the evident potential of using the AFM tip together with the cantilever as a fishing device for testing individual binding forces between viral particles and cell surface receptors is evident. Likewise, the mechanical aspects behind the fusion process of single viral particles with cells can be studied (Skehel & Wiley, 2000). The experimental setup is straightforward. Effective immobilization of one of the test molecules to the AFM tip is required while the complementary molecule can be properly fixed to a flat substrate, onto a spherical colloid or can even be part of the surface of a cell. Stable decoration of the AFM tip with proteins and virions has been frequently carried out by means of heterobifunctional crosslinker molecules with reactive groups to amines like N-hydroxysuccinimide (NHS) esters which provide strong bridging via amide bond formation (Riener, et al., 2003). This covalent linkage has been further improved by adding poly(ethylene)glycol linkers providing flexibility and freedom of movement to the bound particle [(Hinterdorfer, et al., 1996). For sufficiently small interaction forces, this approach ensures long term measurements of repetitive approach-retraction cycles without loss of the binding complex. The kinetics of single bond formation and dissociation can be theoretically described as initially proposed by Kramers, extended later by Bell, and finally refined by Evans & Ritchie (Bell,1978, Evans&Ritchie,1997, Hanggi, et al.,1990). This concept describes the physics of bond rupture induced by an externally assisted pulling force. One of the major results of these theories is that it provides an expression for the mean rupture force required to break the bond as a function of the applied pulling force. The predicted logarithmic increase in the rupture force with the rate of pulling has indeed been frequently observed for different binding molecular systems (Evans, 2001, Hanley, et al., 2003, Noy, 2008, Sulchek, et al., 2005).

Applications in virology are still scarce. To date, measurements and quantitative analysis of single virus-receptor interactions have been carried out only for the HIV virus and the human Rhinovirus (HRV). Not only was the single binding interaction of the HIV-1 gp120 viral protein with its co-receptor CD4, CCR5 and CXCR4 at the surface of the cell studied in detail, but also the dynamics at the initial state of fusion as well as its inhibition (Chang, et al., 2005, Dobrowsky, et al., 2008). Their results showed that maximal bond strengths within the binding pocket gp120-CD4 were about 26 pN at pulling rates of 200 pN/s, and increased to 35 pN for pulling rates of 500 pN/second. The bond had a lifetime of 0.27 seconds with

an interaction length of 0.34 nm. After 0.3 seconds a decrease in tensile strength was observed which indicates a subsequent weakening of the bond. This destabilization of the bond was attributed to a conformational change that gp120 experiences when binding to the CCR5 receptor immediately before fusion. The free energy of the initial gp120-CD4 bond formation was 6.7 k_BT. It decreased to 3.9 k_BT during the destabilization phase to later increase to 7.6 k_BT after anchoring to CCR5. The use of molecules that could block fusion, but that do not interfere with the binding interaction between CD4 and CCR5, suggested that the apparent weakening of the initial bond between gp120 and CD4 was likely related to a conformational transition of the gp120-CD4 bond toward binding to CCR5.

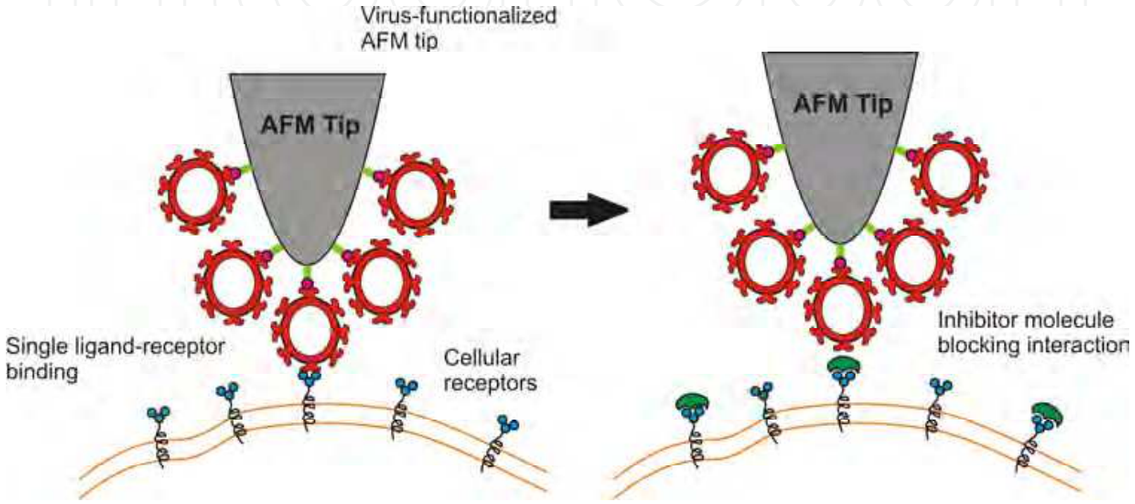

Fig. 8. Scheme of single virus-receptor binding measurements. Left: Specific binding between a virus epitope and a receptor. Right: An inhibitor molecule is used to block the interaction.

Hinterdorfer and coworkers have studied the binding of single human Rhino viruses to its lipoprotein (LDLR) receptor at the cell surface (Hinterdorfer, et al., 2008, Kienberger, et al., 2010). The viral particles were probed against receptor functionalized surfaces and living cells. Their applied protocol for virus attachment to the AFM tip resulted in a densely packed homogeneous monolayer of virions covering the tip and cantilever surface. They observed a time-dependent bond strength reinforcement as a result of consecutive binding of receptor molecules. Forces of about 82 pN were necessary for a single HRV-LDLR unbinding event; however analysis of multimodal force spectra showed peaks at 149, 203 and 273 pN for contact times of 31 milliseconds. It was thus determined that HRV can bind to up to 4 bonds at the initial state of attachment. In both studies the results are interpreted assuming that only one single viral particle being attached at the apex of the AFM tip interacted with the receptors. The pioneering work of these two groups undoubtedly has demonstrated that force spectroscopy is a rather useful tool for virology as it provides insight into the initial steps of virus binding and entry uncovering the mechanics of recognition, and entry in a time resolved fashion. This technique could also help to reveal details of the more intricate multistep binding processes that some other viruses undertake during cell entry. An interesting system worth of studying would be that of rotavirus or Herpex simplex virus A. It is known that these two viruses have to bind to more than two

receptors before entry, however the details of binding leading finally to virus entry are far from being understood (Lopez & Arias, 2004, Lycke, et al., 1991). Research into this perspective direction has just started. Understanding the first steps of virus entry may be of considerable importance for development of novel antiviral drugs. Nanomedicine, for example, promises to develop precise and flexible methods of pathogen detection against emerging and fast spreading diseases. Biosensors based on single molecule technologies could offer alternative solutions to tackle future challenges. Progress in the selective immobilization of molecular targets allows for the fabrication of micro- and nano-patterned surfaces. This may eventually lead to the fabrication of biosensor platforms, which are expected to magnify the capabilities of force spectroscopy toward the implementation of ultrasensitive virus assays (Donath, 2009).

6. Viral mechanics and the infection cycle

Understanding the material properties of viruses and capsids and their changes at various stages of their infection cycle could establish a link between structure and function. An outstanding review regarding mechanical properties of viral capsids and their biological implications has been given by W.H. Roos et al. (Roos, et al., 2007). Research into this exciting direction is still in its beginnings. It was shown that the retroviruses MLV and HIV switch from a stiff to a soft structure during maturation. This may have a direct impact on infectivity (Kol, et al., 2006, Kol, et al., 2007). A stiffer particle could probably facilitate engulfment of the capsid by the bilayer during budding. On the contrary, a softer particle would be able to tolerate deformation facilitating penetration during cell entry. The DNA-anisotropic mechanical reinforcement observed in the MVM (Carrasco, et al., 2006), lead to the proposition that the lower rigidity measured at the fivefold axes where the capsid pores are located, could possibly have an adaptive biological role useful for posterior infectivity. The exceptional mechanical stability found for the HSV-1 virus might be a key factor for the survival during transport over long distances of the axonal cytoplasm where is exposed to mechanical stresses by molecular motors before it reaches its final point for cargo release (Liashkovich, et al., 2008). The reported mechanical stability and self-healing mechanism in Norovirus capsids (Cuellar, et al., 2010) with regard to drastic changes in pH may be an essential requirement for infection. To succeed as a pathogen the capsid has to survive the highly acidic pH in the stomach and become infectious under the weakly basic conditions within the ileum. The measured softening and loss of stability of the capsid at higher pH values could have a direct relation to the virus entry and RNA release. In the case of phages nanoindentation experiments have shown that the mechanical support, provided by the internal highly packed genome to the whole particle, contributes to their survival to shear stresses experienced by the capsid during adhesion to the surface of bacteria. Phages as well as probably other viruses have been optimized by nature for maximum capacity of storage limited only by the mechanical strength of the shell (Roos, et al., 2007, Roos & Wuite, 2009).

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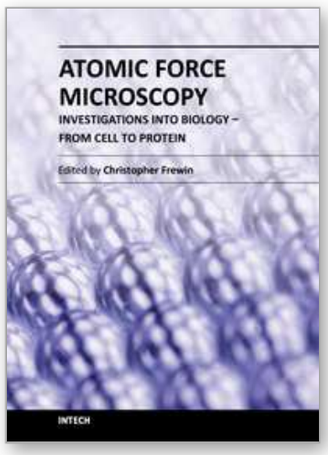

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The atomic force microscope (AFM) has become one of the leading nanoscale measurement techniques for materials science since its creation in the 1980's, but has been gaining popularity in a seemingly unrelated field of science: biology. The AFM naturally lends itself to investigating the topological surfaces of biological objects, from whole cells to protein particulates, and can also be used to determine physical properties such as Young's modulus, stiffness, molecular bond strength, surface friction, and many more. One of the most important reasons for the rise of biological AFM is that you can measure materials within a physiologically relevant environment (i.e. liquids). This book is a collection of works beginning with an introduction to the AFM along with techniques and methods of sample preparation. Then the book displays current research covering subjects ranging from nano-particulates, proteins, DNA, viruses, cellular structures, and the characterization of living cells.

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