Full length article

Acidification induces condensation of the adenovirus core

Marta Pérez-Illana^{a,1}, Natalia Martín-González^{b,1}, Mercedes Hernando-Pérez^{a,2},
Gabriela N. Condezo^a, José Gallardo^a, Margarita Menéndez^{c,d}, Carmen San Martín^{a,*},
Pedro J. de Pablo^{b,e,*}

^a Department of Macromolecular Structures, Centro Nacional de Biotecnología (CNB-CSIC), Madrid 28049, Spain

^b Department of Condensed Matter Physics, Universidad Autónoma de Madrid, Madrid 28049, Spain

^c Instituto de Química Física Rocasolano (IQFR-CSIC), Madrid 28006, Spain

^d Centro de Investigación Biomédica en Red de Enfermedades Respiratorias (CIBERES-ISCIII), Madrid 28029, Spain

^e Instituto de Física de la Materia Condensada (IFIMAC), Universidad Autónoma de Madrid, Madrid 28049, Spain

ARTICLE INFO

Article history:

Received 24 January 2021

Revised 11 August 2021

Accepted 12 August 2021

Available online 15 August 2021

Keywords:

Adenovirus stability

Acidification

Mechanical properties

Fluorescence spectroscopy

Adenovirus core

ABSTRACT

The adenovirus (AdV) icosahedral capsid encloses a nucleoprotein core formed by the dsDNA genome bound to numerous copies of virus-encoded, positively charged proteins. For an efficient delivery of its genome, AdV must undergo a cascade of dismantling events from the plasma membrane to the nuclear pore. Throughout this uncoating process, the virion moves across potentially disruptive environments whose influence in particle stability is poorly understood. In this work we analyze the effect of acidic conditions on AdV particles by exploring their mechanical properties, genome accessibility and capsid disruption. Our results show that under short term acidification the AdV virion becomes softer and its genome less accessible to an intercalating dye, even in the presence of capsid openings. The AFM tip penetrates deeper in virions at neutral pH, and mechanical properties of genome-less particles are not altered upon acidification. Altogether, these results indicate that the main effect of acidification is the compaction of the nucleoprotein core, revealing a previously unknown role for chemical cues in AdV uncoating.

Statement of significance

Studying the behavior of virus particles under changing environmental conditions is key to understand cell entry and propagation. One such change is the acidification undergone in certain cell compartments, which is thought to play a role in the programmed uncoating of virus genomes. Mild acidification in the early endosome has been proposed as a trigger signal for human AdV uncoating. However, the actual effect of low pH in AdV stability and entry is not well defined. Understanding the consequences of acidification in AdV structure and stability is also relevant to define storage conditions for therapeutic vectors, or design AdV variants resistant to intestinal conditions for oral administration of vaccines.

© 2021 The Authors. Published by Elsevier Ltd on behalf of Acta Materialia Inc.

This is an open access article under the CC BY-NC-ND license

(<http://creativecommons.org/licenses/by-nc-nd/4.0/>)

1. Introduction

Virus particles have to find the balance between the stability needed to survive harsh environments during transmission

(extreme temperatures, osmotic pressure, ionic strength or pH) and the ability to release their genome in the appropriate time and place [1]. To achieve this goal, virus particles adopt metastable conformations, primed to disassemble under the appropriate cues provided by a host cell. These cues —receptor binding, proteases, acidification, among others— induce the conformational changes required for genome uncoating, and depend on the virus and cell nature [2]. In animal viruses, after attachment to the cell receptor, virus particles cross the cell membrane barrier by fusion (in enveloped viruses) or by hijacking the host endocytic mechanisms [3,4]. Disassembly triggering signals include virus-receptor

* Corresponding authors.

E-mail addresses: carmen@cnb.csic.es (C. San Martín), p.j.depablo@uam.es (P.J. de Pablo).

¹ Both authors contributed equally.

² Present address: Department of Physics of Materials, Universidad Autónoma de Madrid, Madrid 28049, Spain.

interactions or exposure to low pH in the endocytic compartment, among others. For example, influenza virus uses the low pH in late endosomes as a cue for penetration by membrane fusion [5], and poxviruses undergo structural changes in the capsid under acidification [6]. Flaviviruses, such as Dengue and Zika virus, undergo low pH-driven endosomal membrane fusion to release their genome into the cytoplasm during entry. Later, the immature virions undergo maturation during transport through the secretory pathway triggered by exposure to the more acidic pH environment of the trans-Golgi network [7].

Human AdVs are large non-enveloped viruses (950 Å diameter from vertex to vertex) with a *pseudoT* = 25 icosahedral capsid composed by three major (hexon, penton and fiber) and five minor coat proteins (IIIa, IVa2, VI, VIII and IX) [8]. The human AdV capsid encapsulates a linear, 35 kbp dsDNA genome bound to numerous basic, virus encoded proteins (150 copies of protein V, 500–800 copies of protein VII and 100–300 copies of protein X/μ), in a nucleoprotein core whose exact organization remains unknown [9–12]. AdV virions must undergo a controlled disassembly to simultaneously ensure genome protection while traveling from the plasma membrane to the nucleus, and efficient release of the viral DNA upon arrival to the nuclear pore [2]. Human AdV type 5 (HAdV-C5, or Ad5 for short) naturally infects respiratory epithelial cells, entering via receptor mediated endocytosis after binding to its two co-receptors: CAR (Coxsackievirus and AdV Receptor) and $\alpha_v\beta_5$ integrin [13]. Following internalization, the internal minor coat protein VI has to be exposed to mediate membrane disruption of the early endosome (pH 6), facilitating AdV escape to the cytosol (pH 7.8) to continue its travel to the nuclear pore [14]. It seems logical, therefore, to expect that the acidic nature of the early endosome could play a role in the initial stages of AdV uncoating, as shown for other viruses [5,15].

There have been two major approaches for investigating the role of acidification in AdV uncoating: looking at capsid disruption in purified virus particles, and analyzing virus entry in the cell. It has long been known that overnight dialysis against Tris-maleate buffers in the pH range 6.0–6.6 induces release of vertex proteins from the virions [16]. Additionally, extrinsic fluorescence spectroscopy assays, in which AdV particles are subjected to acidic conditions overnight, have shown that low pH destabilizes AdV particles [14,17]. On the contrary, other thermostability studies have demonstrated that overnight exposure to acidic conditions enhances Ad5 thermostability [18–20]. Conversely, when a buffer with high salt concentration was used in the same study, it was observed that virions were less thermostable upon acidification [18]. Finally, in-cell assays have shown that weak amines do not affect AdV genome delivery to the nucleus, and membrane penetration during endosome escape does not require acidic endosomal pH, but other yet-unknown cellular mechanism [21]. Therefore, there is uncertainty regarding the role of acidification on AdV stability and function.

Measurement of mechanical properties in liquid using Atomic Force Microscopy (AFM) provides a new avenue to examine the response of single virus particles to variation in the environment [55]. AFM studies have shown changes in the mechanical response of influenza virus across a pH range from early to late endosome [5]. In the dicistrovirus *Triatoma* virus, basic pH triggers uncoating by relaxing the stabilizing capsid-genome interactions, which results in a softening effect in the capsid [22]. Computational simulation studies complemented these observations by showing that genome deprotonation at basic pH generated electrostatic repulsion that triggered particle disassembly [23]. Similarly, cowpea chlorotic mottle virus particles undergo swelling and softening transitions when the pH is raised from 5 to 7 [24].

Here we analyze how Ad5 mechanical properties and thermostability change upon acidification, simulating the conditions

the particle may find as it traffics in the cell. We investigate the effect of short (~1 h) exposure to moderately low pH on Ad5 particles combining AFM (to explore changes in mechanical properties) with extrinsic fluorescence spectroscopy and electron microscopy (to interrogate virion thermostability and genome exposure). We use mild acidic conditions mimicking the early endosome (pH 6), from where Ad5 has been demonstrated to escape [25]. We also use pH 5.5 that resembles the late endosome, motivated by the fact that the native trafficking pathway for HAdV-B7 involves residence in late endosomes and lysosomes [26]. Finally, our more extreme condition (pH 4), could resemble the harsh conditions of the gastro-intestinal tract, as not only the enteric HAdV-F41/40 but also Ad5 and other serotypes have been found in feces and sewage waters [27]. Our results show a correlation between acidic pH, particle stiffness, capsid disruption, and genome accessibility compatible with changes in the condensation state of the core.

2. Materials and methods

2.1. Virus production and purification

For Ad5 virions, we have used the constructs Ad5/attP [28] and Ad5GL [29], which are E1 and E3-deleted but wild type for all structural proteins. Ad5/attP, which contains an attP sequence insertion and a Green Fluorescent Protein (GFP) cassette flanking the packaging signal, was used for fluorescence spectroscopy and electron microscopy studies. Ad5GL, which carries a GFP (G) cassette and the firefly luciferase gene (L), was employed for AFM assays. Genome-less capsids were obtained with the delayed-packing mutant Ad5/FC31 [28], which is an E1, E3-deleted construct carrying GFP and the φ C31 attB and attP sequences flanking the AdV packaging signal Ψ . These insertions hinder genome packaging, leading to the generation of an excess of empty particles. In particular, here we use the Ad5/FC31-L3 particles, which are completely sealed [30].

Ad5/attP, Ad5GL and Ad5/FC31 were propagated at 37 °C in HEK293 cells. Infected cells were harvested at 36 hours post infection (hpi) for Ad5/attP and Ad5GL, and 56 hpi for Ad5/FC31. Viral particles were purified by equilibrium centrifugation in double CsCl gradients, desalted on a Bio-Rad 10 DC column, and stored in 20 mM HEPES, pH 7.8, 150 mM NaCl (HBS) plus 10% glycerol at –80 °C, as previously described [30].

2.2. Atomic force microscopy

For AFM assays, stock virus samples were dialyzed against HBS for one hour at 4 °C to remove glycerol and stored in the same buffer in single use aliquots (5 μl) at –20 °C. Immediately previous to the experiment, samples were further diluted in HBS with 5 mM NiCl₂ to firmly attach the viral particles on the mica surface, obtaining a final concentration of approximately 1×10^{11} viral particles (vp)/ml. A volume of 20 μl of this virus solution was deposited on freshly cleaved mica and incubated for 20 min at 4 °C before washing with 5 mM NiCl₂ in HBS with the pH adjusted to the desired value for each experiment (7.8, 6, 5.5 or 4). Finally, a 500 μl drop of HBS-NiCl₂ at the corresponding pH was deposited on the sample. During the experiment the liquid chamber is rehydrated with the pipette once an hour, to avoid dehydration of the sample due to evaporation, and the experiments are performed in a box to keep the temperature constant. The Debye length, which is the distance from the surface at which the AFM cantilever bends due to the electrostatic force, is $\sim 1/[\text{ions}]^d$, where d is a parameter that depends on the valence of ions. If the force curves acquired at the beginning and at the end of the experimental session are similar, then the electrostatic force is the same, and

we can be sure that the salt concentration did not change during the experiment. The AFM (wsxm.es) was operated in jumping plus mode in liquid [31], using RC800PSA (Olympus) cantilevers with nominal spring constant of 0.05 N/m. Cantilevers were routinely calibrated using Sader's method [32]. AFM exploration using imaging forces up to 70 pN indicated a homogeneously distributed population of virus particles on the mica surface. For the nanoindentation experiments, virus particles were deformed with the AFM tip by performing single indentations (force versus z-piezo displacement (FZ)) experiments. A high-resolution image (128×128 pixels) of each particle was recorded before and after particle deformation (Fig. 1A). The maximum force applied during each nanoindentation was high enough to induce capsid disruption (Fig. 1A, after). Mechanical properties such as particle stiffness and breaking force were derived from the FZ curves (Fig. 1B). Changes in particle height were calculated from a profile line across the imaged area.

2.3. Fluorescence spectroscopy

To determine the accessibility of the virus genome to the medium, extrinsic fluorescence spectra were obtained from samples subjected to different pH conditions, in the presence of an intercalating dye. These assays were carried out as described [33] with minor modifications as follows. Stock virus samples ($\sim 10^{12}$ vp/ml) were diluted to 5×10^9 vp/ml in a final volume of 800 μ l of buffer (HBS adjusted to pH 7.8, 6, 5.5 or 4 with 1 M HCl) and further incubated during 1 hour at 4 °C at 300 rpm shaking. A volume of 8.8 μ l of YOYO-1 diluted 1:100 in milliQ water from the initial stock (Thermo Fisher Scientific, stock composition: 1 mM YOYO-1 in DMSO) was added to the virus sample and equilibrated for 5 min at 30 °C before data acquisition. Fluorescence emission spectra were obtained employing a Horiba FluoroLog spectrophotometer equipped with a Peltier temperature control device and continuous shaking. The dye was excited at 490 nm wavelength, and fluorescence emission was monitored from 500 to 580 nm using an excitation slit of 2 nm and an emission slit of 5 nm, integration time 2 s and 950 V voltage. Maximal emission intensity occurred at 509 nm. The temperature in the sample cell was increased from 30 to 70 °C, with readings every 2 °C after holding for 5 min for equilibration. Each run took approximately one hour and a half to be completed. The fractional change in fluorescence (normalized fluorescence, $F_n(T)$), was calculated according to Eq. (1):

$$F_n(T) = \frac{F_{exp} - F_i(T)}{F_f(T) - F_i(T)} \quad (1)$$

where T indicates the independent variable temperature (°C) and F_{exp} refers to the raw fluorescence signal in counts per second (c.p.s) at each temperature. F_i and F_f represent the linear extrapolates, at this temperature, of pre-transition and post-transition base lines, respectively.

A total of three independent experiments were considered to obtain the average curve of each pH condition, except for pH 7.8 and time point 62 °C of pH 4, were two experiments were considered. The half transition temperatures ($T_{0.5}$) were calculated from the fitting of the averaged fluorescence change fraction (F_n) as a function of temperature (T) to a Boltzmann sigmoid (Origin software package) according to Eq. (2):

$$F_n(T) = F_{max} + \frac{F_0 - F_{max}}{1 + \exp\left(\frac{T - T_{0.5}}{dT}\right)} \quad (2)$$

where $F_0 \approx 0$, $F_{max} \approx 1$ and dT = steepness of the curve.

Fig. 1. Effect of acidic pH on the mechanical properties of Ad5 particles.

A. Topography images of two Ad5 particles before and after performing an indentation.

B. Indentation curve computed from a force distance experiment (FZ). The images in (A) and the indentation curve in (B) correspond to Ad5 particles at pH 7.8.

C. Mechanical properties of Ad5 virions (left, Ad5-V) and empty particles (right, Ad5-E). Red symbols represent the spring constant, and blue symbols represent the breaking force, corresponding to the left and right axes, respectively.

D. Summary table of the mechanical properties plotted in panel C. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.4. Transmission electron microscopy

To visually assess particle disruption in thermostability assays, stock virus samples ($\sim 10^{12}$ vp/ml) were diluted to a concentration of 5×10^{10} vp/ml in HBS at different pH values (7.8, 6, 5.5 and 4) and incubated for 1 h at 4 °C and 300 rpm shaking. Twelve microliter aliquots were loaded on a Mastercycle Neux (Eppendorf) thermocycler to simulate the temperature ramp performed in the extrinsic fluorescence assays. At the desired temperature point, samples were cooled down on ice and adsorbed onto glow-discharged, collodion/carbon-coated electron microscopy (EM) grids, washed with Milli-Q H₂O drops and negatively stained with 2% uranyl acetate. Grids were imaged in a JEOL JEM 1011 transmission electron microscope with a Gatan ES1000Ww camera.

2.5. Theoretical estimation of protein and DNA charge

The theoretical net charge of core proteins at different pH values (7.8, 6, 5.5 and 4) was calculated using the online tool “Protein Calculator v3.4” (<http://protcalc.sourceforge.net/>), employing the sequence of the core proteins (NCBI Reference Sequences: V [AP_000208.1], VII [AP_000207.1] and μ [AP_000209.1]) and the copy numbers according to [11]: 148 copies for protein V, 527 copies for protein VII, and 290 copies for protein μ . The theoretical charge of the dsDNA Ad5/attP genome (32,422 kbp), at different pH values was calculated according to the formula and pKa values of 5'-nucleotides reported in [34]. Values reported for uridine monophosphate were assumed to be equal to those of thymidine monophosphate in our estimations. The charges of core proteins and DNA were summed for each pH condition to obtain the net core charge. The estimated percentage (%) of non-screened negative DNA charges was calculated as follows in Eq. (3):

$$\% = \frac{\text{Core proteins charge} + \text{DNA charge}}{\text{DNA charge}} \times 100 \quad (3)$$

2.6. Statistical analysis

Particle properties estimated from AFM data (spring constant, breaking force and height) are expressed as mean $\pm$ standard error of the mean (SEM). A one-way ANOVA test was performed on the height after breakage values at different pH conditions. Values of $P < 0.05$ are indicative of significant differences in the mean values. Normalized fluorescence ($F_n(T)$) curves are plotted as average values $\pm$ standard deviation. Half transition temperatures ($T_{0.5}$) are expressed as fit values of the averages $\pm$ standard error.

3. Results

3.1. Acidification softens the Ad5 virion

To examine the Ad5 mechanical properties in acidic conditions we performed nanoindentation experiments at four different pH values. A first AFM exploration showed predominantly intact particles adsorbed to the mica in all conditions tested, indicating that particle morphology is not affected by acidification (Fig. 1A, before), in contrast to other viruses [35]. We then measured the spring constant (k) of Ad5 virions (Ad5-V) by single nanoindentation assays. During the first stage of indentation, the virus particles undergo a linear deformation from which their stiffness can be derived (Fig. S1) [36,37]. The elastic constant of virus particles can be obtained by fitting the linear slope of the force-indentation curve (Fig. 1B, red line), while the breaking force is obtained from the drop point (Fig. 1B, blue circle). The elastic constant for Ad5 virions at pH 7.8 was $k_{\text{Ad5-V}} = 0.58 \pm 0.02$ N/m

(mean $\pm$ SEM; $N = 42$), as previously described [10,38]. In acidic conditions, the values obtained were: $k_{\text{Ad5-V}}$ (pH 6) = 0.49 ± 0.02 N/m ($N = 56$), $k_{\text{Ad5-V}}$ (pH 5.5) = 0.50 ± 0.01 N/m ($N = 60$), and $k_{\text{Ad5-V}}$ (pH 4) = 0.44 ± 0.02 N/m ($N = 47$) (Fig. 1C left, red symbols). These data show that acidification makes Ad5 virions softer. To discern whether this softening is related to the capsid or to its contents (genome and core proteins), we analyzed empty capsids (Ad5-E). The stiffness value for empty particles at pH 7.8 was $k_{\text{Ad5-E}} = 0.44 \pm 0.03$ N/m ($N = 54$) as previously described [10,38], and remained unchanged for all pH conditions tested (Fig. 1C right, red symbols and Fig. 1D). These results demonstrate that virion softening induced by acidification is caused by changes in the core, or in the interactions between core and capsid. Breaking force values were largely unaffected by acidification, with a value of 4.7 ± 0.2 nN for virions except for pH 4 where it decreased to 4.1 ± 0.1 nN (Fig. 1C left, blue symbols). The breaking force value for empty particles was constant at $\sim 2.6 \pm 0.2$ nN (Fig. 1C right, blue symbols). The fact that absence of the core results in a $\sim 50\%$ decrease in the breaking force seems to indicate that the presence of the core significantly strengthens the capsid, as found previously for other viruses [39,40], and in agreement with previous observations indicating that genome length plays a role in stabilization of the AdV particle [41]. However, AdV immature particles contain all core components and present a breaking force similar to that of empty capsids, while the stiffness is intermediate between those of empty and mature capsids [42]. In immature particles, the genome is highly condensed due to the absence of proteolytic processing of core proteins VII and μ [17,42–44]. These observations suggest that it is not the presence of core components, but changes derived from maturation, which actually reinforce the Ad5 capsid. Interestingly, our results show that at pH 4 the spring constant is similar in full and empty capsids, meaning that strong acidification causes an effect similar to the absence of genome and core proteins in capsid stiffness. This effect can be related to the moderate decrease in breaking force observed for virions at pH 4.

3.2. Acidification increases core condensation in Ad5 virions

Since acidification softens Ad5 virions but not empty capsids, we wondered if low pH has a role in the physical state of the core, similarly to observations previously described for immature particles, where high core condensation results in lower internal pressure and a softer particle than in the mature virion [17,42]. We assessed core condensation by analyzing the particle height (h) at the cracks created by single nanoindentations. The plot in Fig. 2A, right shows an example of height profiles before (purple) and after (black) an indentation experiment. The profiles were obtained at the lines defined by arrows in Fig. 2A, left. The height of intact particles, measured at the apex, was similar for all the pH conditions (~ 85 nm), and close to the nominal Ad5 height [8] (Fig. 2B, before). After nanoindentation, the height of the lowest point in the crater created by the AFM tip at the different pH conditions was: $h_{\text{pH 7.8}} = 63.7 \pm 1.8$ nm (mean $\pm$ SEM; $N = 48$), $h_{\text{pH 6}} = 72.0 \pm 0.9$ nm (mean $\pm$ SEM; $N = 34$), $h_{\text{pH 5.5}} = 69.7 \pm 0.7$ nm (mean $\pm$ SEM; $N = 50$), and $h_{\text{pH 4}} = 71.3 \pm 0.8$ nm (mean $\pm$ SEM; $N = 41$) (Fig. 2B, after). ANOVA test indicates that all the populations are significantly different at a 0.05 level. That is, the AFM tip penetrates deeper (~ 20 nm) in the particles at neutral pH than at any other pH condition tested (≤ 15 nm) (Fig. 2C). These results suggest that, upon shell breakage at pH 7.8, part of the core contents leave the capsid, allowing the tip to penetrate deeper than at the rest of conditions. Or, alternatively, in acidic conditions the core is more condensed, and hinders tip penetration inside the particle once the capsid shell breaks open.

Fig. 2. Height distribution before and after breakage of Ad5 particles.

A. Topography images of a single Ad5 particle before (top left) and after (bottom left) an indentation assay at pH 7.8. Arrows indicate the line at which the height profile before (purple) and after (black) an indentation curve was measured.

B. Heights measured before (average $\pm$ SEM) and after (boxplots) an indentation for the different pH conditions tested.

C. Cartoon showing the AFM tip reaching different depths in nanoindentation experiments. BEFORE the indentation, the measured height (cyan arrows) corresponds to the nominal diameter of the intact particle. AFTER the indentation, the measured height is lower for pH 7.8 than for the rest of conditions, indicating a larger penetration (red arrows). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3. Acidification decreases Ad5 genome accessibility

To obtain additional information on the effect of acidification on Ad5 virions, we carried out thermostability assays under different pH conditions, using extrinsic fluorescence and electron microscopy as indicators of genome accessibility and capsid integrity. Incubation at the desired pH values was kept short (~ 1 h) to mimic the AFM assays. At the initial temperatures (~ 30 °C), we did not find significant differences in fluorescence emission from particles treated at different pH values (Fig. 3A), indicating that the amount of dsDNA accessible to the dye was not increased at the tested acidification conditions. In the same line, negative staining electron microscopy showed that particle morphology remained intact after 1 h treatment with acidic pH (Fig. 4, 4 °C and 36 °C). These results were in agreement with the AFM

Fig. 3. Effect of acidification and heating on Ad5 virions analyzed by extrinsic fluorescence.

A. Raw fluorescence signal of YOYO-1 (F_{exp}) as a function of temperature for Ad5 virions after incubation for 1 h at the pH values indicated. The plot shows examples of single experimental curves recorded on the same experimental set. Raw data points are indicated with hollow circles. Of note, the fall in the fluorescence emission observed in the post-transition region reflects the behavior of the YOYO-1 signal in the presence of naked DNA [47].

B. Extrinsic fluorescence measurements normalized as described in Materials and Methods. $N = 3$ for pH 6, 5.5 and 4; $N = 2$ for pH 7.8 and pH 4 at 62 °C). Lines correspond to the Boltzmann sigmoid fitting to estimate the $T_{0.5}$ for each condition tested. Normalized data points are indicated with filled circles. For pH 7.8, fluorescence normalization was performed only until 60 °C since the post-transition base line is not considered linear anymore above 60 °C.

Fig. 4. Structural effects of acidification and heating on Ad5 virions analyzed by negative staining electron microscopy. Lines indicate approximately the experimental temperature point nearest to the calculated $T_{0.5}$ for each pH condition. Scale bar = 50 nm. For 48 °C pH 6 and pH 5.5, four different fields of view were selected to represent the various particle populations observed.

observations showing mostly intact particles for all pH values before nanoindentation (Figs. 1A and 2A, panels labeled “before”). Analysis of the average fluorescence curves shown in Fig. 3B yielded the following transition temperatures: $T_{0.5}$ of 46.9 ± 0.8 °C; 54.2 ± 0.5 °C; 56.0 ± 0.3 °C; 56.5 ± 0.1 °C for pH of 7.8, 6, 5.5 and 4, respectively; suggesting that acidification increases the thermostability of Ad5 virions. Additionally, lower increases in fluorescence emission were observed for decreasing pH values (Fig. 3A). Taking into account that the AdV genome is heavily bound to core proteins, such decrease in fluorescence emission could be due to a lack of capsid dismantling events, or to a tighter interaction of core proteins with the AdV genome hampering the intercalation of the fluorophore.

TEM images showed that at pH 7.8, Ad5 particles appear significantly disrupted starting from 48 °C (above the $T_{0.5}$), in agreement with the rapid increase in YOYO-1 fluorescence (Fig. 4). In the case of acidic pH (6, 5.5, 4), we started finding disrupted particles also at 48 °C. Although the disruption patterns observed varied, and not all particles appeared damaged, this observation indicates that in acidic conditions at 48 °C there are openings in the capsid shell through which YOYO-1 could penetrate. However, fluorescence does not appreciably increase (Fig. 3). This observation supports the hypothesis that changes induced by acidification are largely due to a different, more condensed state of the Ad5 nucleoprotein core. At pH 4, both the maximum change in the raw fluorescence signal and the raw fluorescence at endpoint temperature (70 °C, well beyond $T_{0.5}$) are notably lower than at the other pH conditions (Fig. 3A). Additionally, capsid damage at pH 4 seems lower than for all other conditions tested throughout all the temperature range studied (Fig. 4). That is, both capsid stability and shielding of dsDNA by core proteins seem to be maximally enhanced at this extreme pH.

4. Discussion

The results presented here indicate that short term acidic treatment of Ad5 particles by itself does not compromise capsid integrity, but has an impact on the core conformation, that reflects in capsid stiffness and genome accessibility to intercalating agents upon heating. We find a correlation between the elasticity of the Ad5 virion and genome exposure under acidic pH (Fig. 5): the rigid particles at pH 7.8 present higher genome accessibility upon capsid disruption, while in the soft particles at pH 4 the genome is less accessible to YOYO-1 intercalation even in the presence of cap-

sid openings. The fluorescence spectroscopy and transmission electron microscopy experiments were carried out in the same buffer (HBS). For AFM, we used NiCl_2 to attach adenovirus particles to the mica surface. These conditions ensure the stable attachment of the virus particle to the substrate required for AFM measurements, while guaranteeing the minimum deformation of the particle [10,17,38,44,45]. Our observations indicate that even if the Ni^{2+} cations were inducing some genome condensation, we can still detect the influence of acidification when all specimens are prepared at the same Ni^{2+} salts concentration.

Our observation that acidification softens the Ad5 particle while increasing core condensation is reminiscent of the behavior found for immature AdV particles, which are softer than the mature ones and also more thermostable [17,42]. Furthermore, when the capsid of immature particles is peeled away under mechanical fatigue, height measurements are compatible with an intact core (~ 70 nm), while for mature particles the remaining debris was only ~ 30 nm high, showing that most of the core components had diffused away [44]. The mechanical properties of the AdV immature particles produced by the *ts1* mutant have not been studied in acidic conditions. However, previously reported extrinsic fluorescence assays indicate that *ts1* particles can withstand harsher acidification conditions than the mature Ad5. Wiethoff et al [14] used extrinsic fluorescence to compare the thermostability of Ad5 and *ts1* capsids at various pH values, and found that below pH 5.5 the transition temperature of Ad5 particles decreased from 42.5 °C to 37.5 °C, while for *ts1* the low pH appeared to have no destabilizing effect. Pérez-Berná et al. [17] showed that DNA accessibility in Ad5 occurred as a highly cooperative transition centered at pH 6.1, while for *ts1* a broad transition was observed, centered at pH 4.1. Upon acidification, Ad5 particles were prone to more drastic capsid dissociation events (penton release, capsid curvature loss) than *ts1* [17]. These differences have been attributed to the higher condensation of the genome by the immature core proteins, which decreases internal capsid pressure [42]. The changes we observe in the mechanical properties of Ad5 virions subject to acidic pH also resemble those occurring in mature virions in the presence of spermidine, a 3^+ charged polyamine known as a dsDNA condensing agent [42]. Studies on an Ad5 mutant lacking polypeptide VII, the major core protein, also showed that particle softening and higher thermostability are directly related to higher core condensation [10,33,46].

Why would acidification have a tightening effect on the Ad5 core similar to that of condensing counterions or proteins? One hypothesis is that the increment of proton concentration itself can promote DNA condensation. However, it has been shown that monovalent cations do not cause condensation of dsDNA molecules in solution [47]. It is also conceivable that proton uptake by histidine residues in acidic milieu would increase the positive net charge of core proteins, strengthening their condensation action by screening the DNA negative charges [42,48]. Additionally, acidic residues (GLU, ASP) will reduce their negatively charged forms upon acidic conditions [48,49], which would further increase the positive charge of core proteins, and decrease their repulsion with the negatively charged DNA. However, it is also important to bear in mind that these changes in protein charges might also have counteracting effects. For example, neutralization of negatively charged forms by acidification could lead to a loss of salt bridges or favorable electrostatic interactions, thus having a negative impact on capsid and core protein stability. To evaluate the possible effect of pH changes in the capacity of core proteins to counteract the electrostatic repulsion of the confined DNA molecule, we estimated the charge of core proteins and genome at different pH values. As shown in Fig. 6A, at neutral pH the positive charges of core proteins can neutralize 45% of the DNA negative charges, with 55% remaining unscreened. As the pH decreases, both proteins and genome become more positive, but at a different

Fig. 5. Summary of the effects of acidic pH on Ad5 virions observed by nanoindentation and extrinsic fluorescence experiments. Comparison of $T_{0.5}$ as an indicator of genome accessibility (extrinsic fluorescence, black symbols) and spring constant k (nanoindentation, red symbols) values obtained for all conditions tested. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 6. Effect on net charge of core proteins and DNA upon acidification. **A.** Percentage of non-screened negative DNA charges in the Ad5 core as a function of pH. The table shows the estimated net charge values for core proteins and genome that were used to calculate the values in the plot. **B.** Cartoon representing the condensation of the AdV core upon acidification. See main text for details. Core proteins are represented as blue spheres, indicating their positive charge, while DNA is represented as a red scribble, indicating its negative net charge. The different shades of red and blue follow the charge scale at the right hand side: red (negatively charged) and blue (positively charged). Charge is expressed in elementary units (i.e. multiples of the proton charge). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

rate, so that the fraction of non-screened negative charges becomes 48% at pH 6, and 45% at pH 5.5. Finally, at pH 4, only 13% of the DNA negative charges remain unscreened by proteins.

It must be emphasized that these estimations can only be very approximate, given the current lack of knowledge on core architecture, protein structures, and stoichiometry. The actual copy number of the core proteins is not well defined, ranging approximately between 150 and 180 copies for protein V, 500–1000 copies for protein VII, and 50 to 300 copies for protein μ [11,12,50–52]. Furthermore, we have not taken into account the charges of the minor coat proteins that line the interior capsid surface, and it is not clear up to which point the core components are accessible to the solvent in the crowded interior of the virion. Finally, our theoretical estimates assume that amino acids and nucleotides in the core have pKa values equivalent to those of the isolated residues, which is only a moderately valid assumption for folded proteins or DNA. Nevertheless, our experimental results, together with the net charge estimations, are consistent with a model where acidification increases the capacity of core proteins to screen the negative charges of the DNA molecule, thus enhancing their genome condensation function (Fig. 6B). Recent studies on enteric HAdV-F41, which must resist the acidic conditions in the digestive tract, show no significant differences in capsid structure when moving from neutral to acidic pH, but extrinsic fluorescence assays indicate that at neutral pH HAdV-F41 is more thermostable than Ad5 [53,54]. Our present observations suggest that in HAdV-F41, the core may also play a role in the endurance of this enteric virus to the extreme conditions in the gut. Exploration of the mechanical

properties of HAdV-F41 would help understand the stability characteristics of the enteric virus, and therefore its specific intestinal tropism.

5. Conclusions

Previous studies showed that both particle morphology and genome accessibility were affected by overnight acidification of Ad5 virions at 4 °C [14,16,17]. Here we do not detect capsid disruption and genome accessibility by either TEM or extrinsic fluorescence after short term acidification (1 h), although AFM analysis indicates that changes in the core have already started, causing particle softening and a moderate reduction of capsid strength (Fig. 1). Only after additional input of stress by heating do Ad5 capsids start to open (Fig. 4), facilitating access of the intercalating dye to the genome (Fig. 3). Stability modulation in viral particles is a complex problem where many factors play a role: ionic strength, divalent ions, pH, time, buffering capacity of the solution, age, and previous history of the specimen. In this regard, more studies with different conditions and techniques are needed to solve the discrepancies on the role of acidic pH on AdV capsid stability. Nevertheless, from the present work we can conclude that acidification for time periods similar to those encountered while trafficking in the cell is not the sole responsible for Ad5 disassembly: other biological or chemical cues seem to be required to trigger uncoating. Our results also suggest that, in the endosome, acidification may play a role in keeping the Ad5 genome condensed, so that while the capsid starts to open and release internal components such as protein VI, the genome would stay protected inside the remaining shell to avoid premature release. This role, however, seems to be dispensable, as indicated by previous studies where lack of acidification did not stop virus entry. Nevertheless, understanding the effects of acidification in AdV stability modulation may help design better vectors for programmed delivery in environments of therapeutic interest.

Author contributions

N.M.G. performed AFM experiments. M.H.P. and M.P.I. carried out extrinsic fluorescence experiments. M.H.P. and M.P.I. performed transmission electron microscopy studies. G.N.C., J.G. and M.P.I. produced virus samples. M.H.P., N.M.G., and M.P.I. analyzed data. N.M.G. and M.P.I. initially wrote the manuscript, with significant inputs from M.H.P., M.M. P.J. P. and C.S.M. C.S.M. and P.J.P. coordinated the study.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank M. G. Mateu (CBMSO-CSIC-UAM) for careful reading and insightful comments on early drafts, M. Castellanos and L. A. Campos (CNB-CSIC) for advice with fluorescence measurements and analyses, and M.I. Laguna (CNB-CSIC) for expert technical help.

This work was supported by grants from the Spanish Ministry of Economy, Industry and Competitiveness (FIS2017- 89549-R; “Maria de Maeztu” Program for Units of Excellence in R&D MDM-2014-0377; and FIS2017-90701- REDT) and from the Human Frontiers Science Program (HFSPO RGP0012/2018) to P.J.P.; as well as grants PID2019-104098GB-I00/AEI/10.13039/501100011033 and BFU2016-74868-P, co-funded by the Spanish State Research Agency and the European Regional Development Fund, and 2019AEP045

from the Agencia Estatal CSIC to C.S.M. The CNB-CSIC is further supported by a Severo Ochoa Excellence grant (SEV 2017-0712). MM was funded by grant RTI2018-099985-B-I00, (MICINN/FEDER, UE) and the Ciber of Respiratory Diseases (CIBERES), an initiative from the Spanish Institute of Health Carlos III (ISCIII).

M.H.-P. was a recipient of a Juan de la Cierva Incorporation postdoctoral contract funded by the Spanish State Research Agency. M.P.-I. held a predoctoral contract from La Caixa Foundation (ID 100010434, under agreement LCF/BQ/SO16/52270032). J. G. is a recipient of a FPI predoctoral contract (BES-2017-079868) funded by the Spanish State Research Agency.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:[10.1016/j.actbio.2021.08.019](https://doi.org/10.1016/j.actbio.2021.08.019).

References

- [1] M.G. Mateu, Assembly, stability and dynamics of virus capsids, *Arch. Biochem. Biophys.* 531 (1–2) (2013) 65–79.
- [2] Y. Yamauchi, U.F. Greber, Principles of virus uncoating: cues and the snooker ball, *Traffic* 17 (6) (2016) 569–592.
- [3] S. Lopez, C. Arias, How viruses hijack endocytic machinery, *Nat. Educ.* 3 (9) (2010) 16.
- [4] D.S. Dimitrov, Virus entry: molecular mechanisms and biomedical applications, *Nat. Rev. Microbiol.* 2 (2) (2004) 109–122.
- [5] S. Li, C. Sieben, K. Ludwig, C.T. Hofer, S. Chiantia, A. Herrmann, F. Eghiaian, I.A. Schaap, pH-Controlled two-step uncoating of influenza virus, *Biophys. J.* 106 (7) (2014) 1447–1456.
- [6] F.I. Schmidt, C.K.E. Bleck, J. Mercer, Poxvirus host cell entry, *Curr. Opin. Virol.* 2 (1) (2012) 20–27.
- [7] T.Y. Tan, G. Fibriansah, S.M. Lok, Capsid protein is central to the birth of flavivirus particles, *PLoS Pathog.* 16 (5) (2020) e1008542.
- [8] C. San Martín, Latest insights on adenovirus structure and assembly, *Viruses* 4 (5) (2012) 847–877.
- [9] A.J. Pérez-Berná, S. Marion, F.J. Chichón, J.J. Fernández, D.C. Winkler, J.L. Carrascosa, A.C. Steven, A. Šiber, C. San Martín, Distribution of DNA-condensing protein complexes in the adenovirus core, *Nucl. Acids Res.* 43 (8) (2015) 4274–4283.
- [10] N. Martín-González, M. Hernando-Pérez, G.N. Condezo, M. Pérez-Illana, A. Šiber, D. Reguera, P. Ostapchuk, P. Hearing, C. San Martín, P.J. de Pablo, Adenovirus major core protein condenses DNA in clusters and bundles, modulating genome release and capsid internal pressure, *Nucl. Acids Res.* 47 (17) (2019) 9231–9242.
- [11] M. Benevento, S. Di Palma, J. Snijder, C.L. Moyer, V.S. Reddy, G.R. Nemerow, A.J. Heck, Adenovirus composition, proteolysis, and disassembly studied by in-depth qualitative and quantitative proteomics, *J. Biol. Chem.* 289 (16) (2014) 11421–11430.
- [12] J. van Oostrum, R.M. Burnett, Molecular composition of the adenovirus type 2 virion, *J. Virol.* 56 (2) (1985) 439–448.
- [13] U.F. Greber, J.W. Flatt, Adenovirus Entry: From Infection to Immunity, *Annu. Rev. Virol.* 6 (1) (2019) 177–197.
- [14] C.M. Wiethoff, H. Wodrich, L. Gerace, G.R. Nemerow, Adenovirus protein VI mediates membrane disruption following capsid disassembly, *J. Virol.* 79 (4) (2005) 1992–2000.
- [15] B. Mani, C. Baltzer, N. Valle, J.M. Almendral, C. Kempf, C. Ros, Low pH-dependent endosomal processing of the incoming parvovirus minute virus of mice virion leads to externalization of the VP1 N-terminal sequence (N-VP1), N-VP2 cleavage, and uncoating of the full-length genome, *J. Virol.* 80 (2) (2006) 1015–1024.
- [16] L. Prage, U. Pettersson, S. Höglund, K. Lonberg-Holm, L. Philipson, Structural proteins of adenoviruses. IV. Sequential degradation of the adenovirus type 2 virion, *Virology* 42 (2) (1970) 341–358.
- [17] A.J. Pérez-Berná, A. Ortega-Esteban, R. Menéndez-Conejero, D.C. Winkler, M. Menéndez, A.C. Steven, S.J. Flint, P.J. de Pablo, C. San Martín, The role of capsid maturation on adenovirus priming for sequential uncoating, *J. Biol. Chem.* 287 (37) (2012) 31582–31595.
- [18] J. Rexroad, R.K. Evans, C.R. Middaugh, Effect of pH and ionic strength on the physical stability of adenovirus type 5, *J. Pharm. Sci.* 95 (2) (2006) 237–247.
- [19] J. Rexroad, T.T. Martin, D. McNeilly, S. Godwin, C.R. Middaugh, Thermal stability of adenovirus type 2 as a function of pH, *J. Pharm. Sci.* 95 (7) (2006) 1469–1479.
- [20] J. Rexroad, C.M. Wiethoff, A.P. Green, T.D. Kierstead, M.O. Scott, C.R. Middaugh, Structural stability of adenovirus type 5, *J. Pharm. Sci.* 92 (3) (2003) 665–678.
- [21] M. Suomalainen, S. Luisoni, K. Boucke, S. Bianchi, D.A. Engel, U.F. Greber, A direct and versatile assay measuring membrane penetration of adenovirus in single cells, *J. Virol.* 87 (22) (2013) 12367–12379.
- [22] J. Snijder, C. Uetrecht, R.J. Rose, R. Sanchez-Eugenía, G.A. Marti, J. Agirre, D.M. Guerin, G.J. Wuite, A.J. Heck, W.H. Roos, Probing the biophysical interplay between a viral genome and its capsid, *Nat. Chem.* 5 (6) (2013) 502–509.
- [23] M. Martínez, C.D. Cooper, A.B. Poma, H.V. Guzman, Free energies of the disassembly of viral capsids from a multiscale molecular simulation approach, *J. Chem. Inf. Model.* 60 (2) (2019) 974–981.
- [24] B.D. Wilts, I.A.T. Schaap, C.F. Schmidt, Swelling and softening of the cowpea chlorotic mottle virus in response to pH shifts, *Biophys. J.* 108 (10) (2015) 2541–2549.
- [25] M. Gastaldelli, N. Imelli, K. Boucke, B. Amstutz, O. Meier, U.F. Greber, Infectious adenovirus type 2 transport through early but not late endosomes, *Traffic* 9 (12) (2008) 2265–2278.
- [26] N. Miyazawa, R.G. Crystal, P.L. Leopold, Adenovirus serotype 7 retention in a late endosomal compartment prior to cytosol escape is modulated by fiber protein, *J. Virol.* 75 (3) (2001) 1387–1400.
- [27] N.M. Hartmann, M. Dartscht, R. Szewzyk, H.-C. Selinka, Monitoring of adenovirus serotypes in environmental samples by combined PCR and melting point analyses, *Virol. J.* 10 (2013) 190.
- [28] R. Alba, P. Hearing, A. Bosch, M. Chillon, Differential amplification of adenovirus vectors by flanking the packaging signal with attB/attP-PhiC31 sequences: implications for helper-dependent adenovirus production, *Virology* 367 (1) (2007) 51–58.
- [29] T. Seki, I. Dmitriev, E. Kashentseva, K. Takayama, M. Rots, K. Suzuki, D.T. Curiel, Artificial extension of the adenovirus fiber shaft inhibits infectivity in coxsackievirus and adenovirus receptor-positive cell lines, *J. Virol.* 76 (3) (2002) 1100–1108.
- [30] G.N. Condezo, R. Marabini, S. Ayora, J.M. Carazo, R. Alba, M. Chillon, C. San Martín, Structures of adenovirus incomplete particles clarify capsid architecture and show maturation changes of packaging protein L1 52/55k, *J. Virol.* 89 (18) (2015) 9653–9664.
- [31] A. Ortega-Esteban, I. Horcas, M. Hernando-Pérez, P. Ares, A.J. Pérez-Berná, C. San Martín, J.L. Carrascosa, P.J. de Pablo, J. Gómez-Herrero, Minimizing tip-sample forces in jumping mode atomic force microscopy in liquid, *Ultra-microscopy* 114 (0) (2012) 56–61.
- [32] J.E. Sader, J.W.M. Chon, P. Mulvaney, Calibration of rectangular atomic force microscope cantilevers, *Rev. Sci. Instrum.* 70 (10) (1999) 3967–3969.
- [33] M. Hernando-Pérez, N. Martín-González, M. Pérez-Illana, M. Suomalainen, G.N. Condezo, P. Ostapchuk, J. Gallardo, M. Menéndez, U.F. Greber, P. Hearing, P.J. de Pablo, C. San Martín, Dynamic competition for hexon binding between core protein VII and lytic protein VI promotes adenovirus maturation and entry, *Proc. Natl. Acad. Sci.* (2020) 201920896.
- [34] J.C. Cameselle, J.M. Ribeiro, A.J.B.E. Sillero, Derivation and use of a formula to calculate the net charge of acid-base compounds. Its application to amino acids, *Proteins Nucleotides* 14 (3) (1986) 131–136.
- [35] D. Roshal, O. Konevtsova, A.L. Božić, R. Podgornik, S. Rochal, pH-induced morphological changes of proteinaceous viral shells, *Sci. Rep.* 9 (1) (2019) 1–9.
- [36] W.H. Roos, R. Bruinsma, G.J.L. Wuite, Physical virology, *Nat. Phys.* 6 (10) (2010) 733–743.
- [37] O. Kononova, Y. Kholodov, K.E. Theisen, K.A. Marx, R.I. Dima, F.I. Ataullakhanov, E.L. Grishchuk, V. Barsegov, Tubulin bond energies and microtubule biomechanics determined from nanoindentation in silico, *J. Am. Chem. Soc.* 136 (49) (2014) 17036–17045.
- [38] A. Ortega-Esteban, G.N. Condezo, A.J. Perez-Berna, M. Chillon, S.J. Flint, D. Reguera, C. San Martín, P.J. de Pablo, Mechanics of viral chromatin reveals the pressurization of human adenovirus, *ACS Nano* 9 (11) (2015) 10826–10833.
- [39] M. Hernando-Pérez, R. Miranda, M. Aznar, J.L. Carrascosa, I.A.T. Schaap, D. Reguera, P.J. de Pablo, Direct measurement of phage phi29 stiffness provides evidence of internal pressure, *Small* 8 (15) (2012) 2366–2370.
- [40] M. Jiménez-Zaragoza, M.P. Yubero, E. Martín-Forero, J.R. Castón, D. Reguera, D. Luque, P.J.J.M. de Pablo Rodríguez, Biophysical properties of single rotavirus particles account for the functions of protein shells in a multilayered virus, *Elife* 7 (2018) 37295.
- [41] B. Saha, C.M. Wong, R.J. Parks, The adenovirus genome contributes to the structural stability of the virion, *Viruses* 6 (9) (2014) 3563–3583.
- [42] A. Ortega-Esteban, K. Bodensiek, C. San Martín, M. Suomalainen, U.F. Greber, P.J. de Pablo, I.A. Schaap, Fluorescence Tracking of genome release during mechanical unpacking of single viruses, *ACS Nano* 9 (11) (2015) 10571–10579.
- [43] A.J. Pérez-Berná, R. Marabini, S.H.W. Scheres, R. Menéndez-Conejero, I.P. Dmitriev, D.T. Curiel, W.F. Mangel, S.J. Flint, C. San Martín, Structure and uncoating of immature adenovirus, *J. Mol. Biol.* 392 (2) (2009) 547–557.
- [44] A. Ortega-Esteban, A.J. Pérez-Berná, R. Menéndez-Conejero, S. Flint, C. San Martín, P.J. de Pablo, Monitoring dynamics of human adenovirus disassembly induced by mechanical fatigue, *Sci. Rep.* 3 (1) (2013) 1–7.
- [45] N. Martín-González, P. Ibáñez-Freire, Á. Ortega-Esteban, M. Laguna-Castro, C. San Martín, A. Valbuena, R. Delgado-Buscalioni, P.J. de Pablo, Long-range co-operative disassembly and aging during adenovirus uncoating, *Phys. Rev. X* 11 (2) (2021) 021025.
- [46] P. Ostapchuk, M. Suomalainen, Y. Zheng, K. Boucke, U.F. Greber, P. Hearing, The adenovirus major core protein VII is dispensable for virion assembly but is essential for lytic infection, *PLoS Pathog.* 13 (6) (2017) e1006455.
- [47] V.A. Bloomfield, DNA condensation by multivalent cations, *Biopolymers* 44 (3) (1997) 269–282.
- [48] A.C. Dumetz, A.M. Chockla, E.W. Kaler, A.M. Lenhoff, Effects of pH on protein-protein interactions and implications for protein phase behavior, *Biochim. Biophys. Acta* 1784 (4) (2008) 600–610.
- [49] D. Voet, J.G. Voet, C.W. Pratt, Fundamentals of biochemistry: life at the molecular level, John Wiley & Sons, 2016.
- [50] K. Hosokawa, M.T. Sung, Isolation and characterization of an extremely basic protein from adenovirus type 5, *J. Virol.* 17 (3) (1976) 924–934.

- [51] E. Everitt, B. Sundquist, U. Pettersson, L. Philipson, Structural proteins of adenoviruses. X. Isolation and topography of low molecular weight antigens from the virion of adenovirus type 2, *Virology* 52 (1) (1973) 130–147.
- [52] P.L. Stewart, R.M. Burnett, Adenovirus structure by X-ray crystallography and electron microscopy, *Mol. Repertoire Adenoviruses I* (1995) 25–38.
- [53] K. Rafie, A. Lenman, J. Fuchs, A. Rajan, N. Arnberg, L.A. Carlson, The structure of enteric human adenovirus 41—A leading cause of diarrhea in children, *Sci. Adv.* 7 (2) (2021) eabe0974.
- [54] M. Pérez-Illana, M. Martínez, G.N. Condezo, M. Hernando-Pérez, C. Mangroo, M. Brown, R. Marabini, C. San Martín, Cryo-EM structure of enteric adenovirus HAdV-F41 highlights structural variations among human adenoviruses, *Sci. Adv.* 7 (9) (2021) eabd9421.
- [55] A. Llauró, P. Guerra, R. Kant, B. Bothner, N. Verdaguer, P.J. de Pablo, Decrease in pH destabilizes individual vault nanocages by weakening the inter-protein lateral interaction, *Sci. Rep.* (2016) 6–34143.