Video Article

In Vitro Disassembly of Influenza A Virus Capsids by Gradient Centrifugation

Sarah Stauffer[1,2], Firat Nebioglu[3], Ari Helenius[1]

[1]Institute of Biochemistry, ETH Zurich

[2]Department of Biochemistry, University of Zurich

[3]Institute for Molecular Health Sciences, ETH Zurich

Correspondence to: Sarah Stauffer at sarah.stauffer@gmx.net, Ari Helenius at ari.helenius@bc.biol.ethz.ch

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Abstract

Acid-triggered molecular processes closely control cell entry of many viruses that enter through the endocytic system. In the case of influenza A virus (IAV), virus fusion with the endosomal membrane as well as the subsequent disassembly of the viral capsid, called uncoating, is governed by the ionic conditions inside endocytic vesicles. The early steps in the virus life cycle are hard to study because endosomes cannot be directly accessed experimentally, creating the need for an *in vitro* approach. Here, we describe a method based on velocity gradient centrifugation of purified virions through a two-layer glycerol gradient, which enables analysis of the IAV core and its stability. The gradient contains a non-ionic detergent (NP-40) in its lower layer to remove the viral membrane by solubilization as the virus sediments toward the bottom. At neutral pH, viral cores are pelleted as stable structures. The major core components, matrix protein (M1) and the viral ribonucleoproteins (vRNPs), can be clearly identified in the pellet fraction by SDS-PAGE. Decreasing the pH to 6.0 or lower in the bottom layer selectively removes M1 from the pellet followed by release of vRNPs at more acidic conditions. Viral protein bands on Coomassie-stained gels can be subjected to densitometric quantification to monitor intermediate states of IAV disassembly. Besides pH, other factors that influence viral core stability can be assessed, such as salt concentration and putative viral uncoating factors, simply by modifying the detergent-containing glycerol layer accordingly. Taken together, the presented technique allows highly reproducible and quantitative analysis of viral uncoating *in vitro*. It can be applied to other enveloped viruses that undergo complex uncoating processes.

Video Link

The video component of this article can be found at https://www.jove.com/video/53909/

Introduction

Influenza A virus (IAV) is an enveloped virus and belongs to the family of *Orthomyxoviridae*. Its genes are encoded on a segmented, negative-sense and single-stranded RNA genome. In humans, IAV causes respiratory infections, which occur in seasonal epidemic outbreaks and bears the potential for global pandemics[1]. Upon binding to sialic acid residues on the host cell surface[2], IAV is internalized by clathrin-dependent endocytosis and clathrin-independent pathways[3-8]. The acidic milieu (pH < 5.5) in the endocytic vacuoles triggers a major conformational change in the IAV spike glycoprotein hemagglutinin (HA), which results in fusion of the viral and the late endosomal membrane[9]. Once the IAV capsid (here also referred to as viral "core") has escaped from late endosomes (LEs), it is uncoated in the cytosol followed by transport of the viral ribonucleoproteins (vRNPs) into the nucleus — the site of virus replication and transcription[10-13]. Prior to acid activation of HA the virus experiences a gradual decrease in pH in the endocytic system, which primes the core for its subsequent disassembly[14-16]. In this "priming" step the M2 ion channels in the viral membrane mediate the influx of protons and K^+[10,14,16]. The change in ionic concentration in the virus interior disturbs the interactions build up by the viral matrix protein M1 and the eight vRNP bundle, and facilitates IAV uncoating in the cytoplasm following membrane fusion[10,14-17].

Direct and quantitative analysis of the priming step has been hampered by the fact that endosomes are difficult to access experimentally. The entry process is, moreover, highly non-synchronous. In addition, end-point assays, such as qRT-PCR of released viral RNA or infectivity measurements, do not provide a detailed picture about the biochemical state of the viral capsid at any given step of entry. While perturbation of endosomes by siRNA or drug treatment has significantly contributed to the understanding of IAV entry[18-20], fine-tuning is difficult and prone to unspecific side effects in the tightly controlled endosome maturation program.

To avoid these problems, we have adapted a previously developed *in vitro* protocol based on the use of velocity gradient centrifugation[17]. In contrast to other attempts [21,22,23,24], which were mostly based on combinations of proteolytic cleavage and detergent treatment followed by EM analysis, this approach enabled an easily quantifiable result. Centrifugation through different gradient layers allows the sedimenting particles to be exposed to and react with changing conditions in a controlled manner. In the presented protocol, IAV derived from clarified allantoic fluid or purified viral particles are sedimented into a two-layer glycerol gradient in which the bottom layer contains the non-ionic detergent NP-40 (**Figure 1**). As the virus enters the second, detergent-containing layer, the viral lipid envelope and envelope glycoproteins are gently solubilized and left behind. The core, composed of the eight vRNP bundle and surrounded by a matrix layer, sediments as a stable structure into the pellet fraction.

Viral core proteins, such as M1 and vRNP-associated nucleoprotein (NP), can be identified in the pellet by SDS-PAGE and Coomassie staining. In particular, taking advantage of commercially available gradient gels and staining with the highly sensitive colloidal Coomassie[25], enables high precision and detection of even small amounts of viral core-associated proteins.

This sets the basis for testing whether different conditions such as pH, salt concentration, and putative uncoating factors have effects on the sedimentation behavior of core components and core stability. To this aim, only the lower, detergent-containing layer in the glycerol gradient is modified by introducing the factor or condition of interest. The technique has been particularly valuable in investigating the effect of different pH values and salt concentrations on the integrity of the IAV core[16]. Intermediate steps of IAV uncoating could be monitored including dissociation of the matrix layer at mildly acidic pH (<6.5) followed by vRNP dissociation at pH 5.5 and lower[16,17] (**Figure 1**). The latter step was further enhanced by the presence of high K^+ concentration in the glycerol layer, reflecting a late endocytic environment[16]. Thus, as the virus and the core sedimented through the gradient they experienced a changing milieu that mimics conditions in endosomes. The outcome was a stepwise disassembly of the viral core *in vitro*, complementing results derived from cell biology assays.

The method presented here has enabled fast and highly reproducible analysis of IAV (X31 and A/WSN/33) and IBV (B/Lee/40) uncoating triggered by acidic pH and increasing K^+[16,17] as well as disassembly of paramyxovirus cores upon alkaline pH exposure[17]. It is conceivable that the approach can be adapted to other enveloped viruses to gain insights into the biochemical properties of the viral capsid structure and capsid disassembly during cell entry.

Protocol

1. Preparation of Buffers and Stock Solutions

1. Prepare MNT buffer (20 mM MES, 30 mM Tris and 100 mM NaCl) by dissolving 470 mg Tris Hydrochloride, 390 mg MES hydrate and 580 mg NaCl in 80 ml ddH_2O. Adjust the buffer to pH 7.4 and bring it to a final volume of 100 ml with ddH_2O.
2. Prepare three identical solutions of 500 mM MES buffer by dissolving 9.76 g MES hydrate in 80 ml dH_2O each. Adjust the buffers to pH 5.8, 5.4, and 5.0, respectively, by titrating with concentrated NaOH. Bring each buffer to a final volume of 100 ml with dH_2O.
3. Prepare two identical solutions of 500 mM Tris buffer by dissolving 7.88 g Tris Hydrochloride in 80 ml dH_2O each and adjust the pH to 7.4 and 6.4, respectively, by titrating with concentrated HCl. Bring the buffer to a final volume of 100 ml with dH_2O.
4. Prepare 50% glycerol solution in dH_2O and stir well until the glycerol is homogenously mixed. Filter the solution through a Steritop filter unit (0.22 µm pore size) into a glass bottle.
5. Prepare 10% NP-40 solution and 1 M NaCl in dH_2O.
6. Prepare 25x concentrated protease inhibitor by dissolving two tablets in 4 ml ddH_2O.

2. Preparation of Glycerol Gradients

1. Prepare 5 ml detergent buffer master mix (300 mM NaCl, 2% NP-40, 2x concentrated protease inhibitor) solution for each pH condition to test (pH 7.4, 6.4, 5.8, 5.4, and 5.0). For this purpose, mix 9 ml of 1 M NaCl, 6 ml of 10% NP-40, 2.4 ml of the 25x protease inhibitor stock, and 9 ml ddH_20.
2. For each pH condition pipet 4.4 ml of the master mix into a 50 ml conical tube.
3. For pH values above 5.8 add 0.6 ml of the respective pH-adjusted 500 mM Tris stock solution to the tube. For pH 5.8 and lower add 0.6 ml of the respective pH-adjusted 500 mM MES stock solution.
4. Make 5 ml of buffer solution containing 300 mM NaCl, 2x protease inhibitor, 60 mM Tris adjusted to pH 7.4 and ddH_2O. This will serve as the detergent-free control gradient buffer.
5. If necessary, fine-adjust the pH of the solutions to pH 7.4, 6.4, 5.8, 5.4, and 5.0 by adding concentrated HCl or NaOH solutions, respectively.
6. Add 5 ml of the 50% glycerol stock to 5 ml of the detergent-containing and detergent-free buffer mixtures resulting in six different 25% glycerol solutions. Verify the pH values by using pH indicator strips.
   NOTE: In case the measured pH differs significantly from the desired value, the respective solutions should be prepared again.
7. Prepare 15% glycerol solution by mixing 50% glycerol stock with dH_2O in a 3:7 ratio.

3. Ultracentrifugation of IAV

1. Add 3 ml 15% glycerol solution into ultra-clear centrifugation tubes (13.2 ml, 14 mm x 89 mm) by using a 5 ml syringe and a needle (21 G, 9 cm long). Do not leave drops at the inner wall of the tube as this might disturb the integrity of the gradient. Repeat this for a total of six centrifugation tubes, one for each of the five pH conditions to test and one for the control sample.
2. Carefully place 3.4 ml 25% glycerol solution under the 15% glycerol layer by using a 5 ml syringe and a long needle. Take care to not mix the two layers. Repeat this for all six conditions to test with the respective pH-adjusted glycerol solutions.
   NOTE: Work in class II biosafety cabinet for the following steps.
3. Gently overlay the glycerol gradients with 30 µl clarified allantoic fluid containing IAV (X31, H3N2) diluted in 1 ml MNT buffer (corresponds to around 20-30 µg of total viral protein) for each gradient.
4. Balance opposing tubes and place them into a SW41 ultracentrifugation rotor. Centrifuge for 150 min, at 55,000 x g, and 12 °C.
5. After the centrifugation, carefully remove the supernatant, *i.e.*, both glycerol layers by using a clean Pasteur pipette and an aspirator. Resuspend the pellet in 40 µl (1x) non-reducing LDS sample buffer. It is important to pipet up and down several times to dissolve the pellet completely. Transfer the sample into a 1.5 ml microcentrifuge tube.

4. SDS-PAGE of Pellet Fractions and Coomassie Staining

1. Heat all samples at 95 °C for 10 min. At this point the samples could be stored at -20 °C until they are analyzed by SDS-PAGE.

2. Load 20 µl of the dissolved pellets onto a pre-cast gradient (4-12%) Bis-Tris mini gel and run for 1 hr at 200 V in 1x MOPS SDS running buffer.
3. Make fixation solution with 40% methanol and 10% glacial acetic acid in ddH_2O.
4. Incubate the gel in fixation solution for 1 hr and stain overnight in a 15 cm cell culture dish with a sufficient volume of colloidal Coomassie solution while gently shaking at room temperature.
   NOTE: It is important to close the dish in order to avoid evaporation of the staining solution.
5. Destain the gel in ddH_2O. Replace the ddH_2O every 15-20 min until the gel background becomes clear. Store the gel in ddH_2O at 4 °C until it is scanned for band quantification.
6. Scan the gel at high resolution and use commercially available or custom-made software for quantification of protein band intensities. Subtract the background signal from a region close to the respective bands and normalize these values to the detergent-free control samples (at pH 7.4).

Representative Results

As already discussed, priming in endosomes is required to render the IAV core uncoating competent. Protonation of the core weakens interaction of M1 and vRNPs (composed of the viral RNA, NP, and the polymerase complex PB1/PB2/PA). This process is initiated when incoming virus is exposed to a pH of 6.5 (or lower) in early endosomes (EEs) and continues until the virus fuses at around pH 5.0 in LEs.

In order to mimic the decrease of pH, the bottom layer of the glycerol gradients were adjusted to values between pH 7.4 and 5.0 at a constant salt concentration. X31 derived from clarified allantoic fluid was subjected to velocity gradient centrifugation and analyzed by SDS-PAGE (**Figure 2A**, **B**). Without detergent present in the gradient (**Figure 2A**, lane1) intact virions are pelleted, as reflected by the characteristic pattern of bands representing HA (HA1 and HA2; 63 kDa), NP (56 kDa), and M1 (27 kDa) in the Coomassie stained gel. Since the gel was run under non-reducing conditions, proteolytically cleaved HA1 and HA2 are still connected by disulfide bonds and run at approximately 64 kDa. Under reducing conditions the bands corresponding to HA1 and HA2 would appear very close to the NP and M1 bands, respectively, making it difficult to faithfully interpret and determine band intensities that are solely derived from the viral core (**Figure 2B**).

Upon addition of NP-40 to the bottom (25%) glycerol layer the viral envelope including HA, NA, and M2 were solubilized and the viral core alone sedimented into the pellet during ultracentrifugation (**Figure 1**; **Figure 2**, lane 2). Starting with a pH below 6.5, M1 is gradually lost from the pellet fraction, reaching a minimum between pH 5.8 and 5.4 (**Figure 2A**, **B**). Below pH 5.8 vRNPs were dissociated and thus lost from the pellet. Previously, it has been shown that, in fact, whole vRNPs are released into the upper layer, as their release correlated with the loss of PB2 from the pellet fraction[16]. Protein band intensities were determined, revealing two distinct pH thresholds for disassembly of the M1 layer and dissociation of vRNP bundles, respectively (**Figure 2**). Additional bands could be observed on the gel, which might correspond to the polymerase proteins PB1 (87 kDa), PB2 (86 kDa), PA (83 kDa), and M2 (11 kDa). Due to their lower copy numbers inside IAV virions we could not reliably determine their identity and they were consequently not taken into consideration for the quantification.

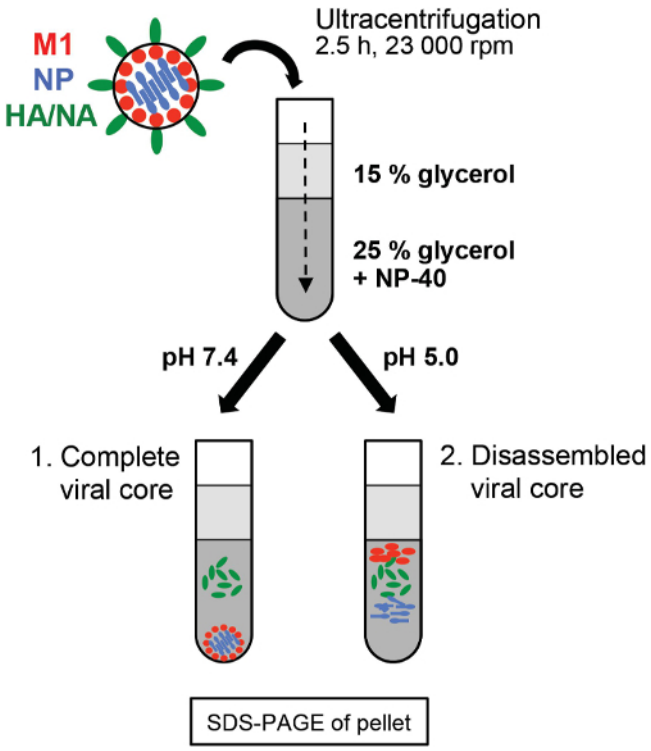

Figure 1: Schematic representation of the IAV *in vitro* core disassembly assay. Briefly, purified viral particles are loaded on a two-step glycerol gradient. The bottom layer contains the detergent NP-40 and is adjusted to the desired pH and salt concentration while the upper, detergent-free layer is kept at a constant pH and salt concentration. Depending on the condition in the lower glycerol layer, complete viral cores sediment to the bottom (1) or are dissociated and remain in the glycerol layer (2). Neutral pH leads to sedimentation of intact viral cores composed of M1 and vRNPs. Conditions favorable for uncoating, such as acidic pH (5.0), results in dissociation of the viral core components and therefore loss from the pellet fraction. Following ultracentrifugation, glycerol supernatants are removed and pellets are analyzed by SDS-PAGE. Please click here to view a larger version of this figure.

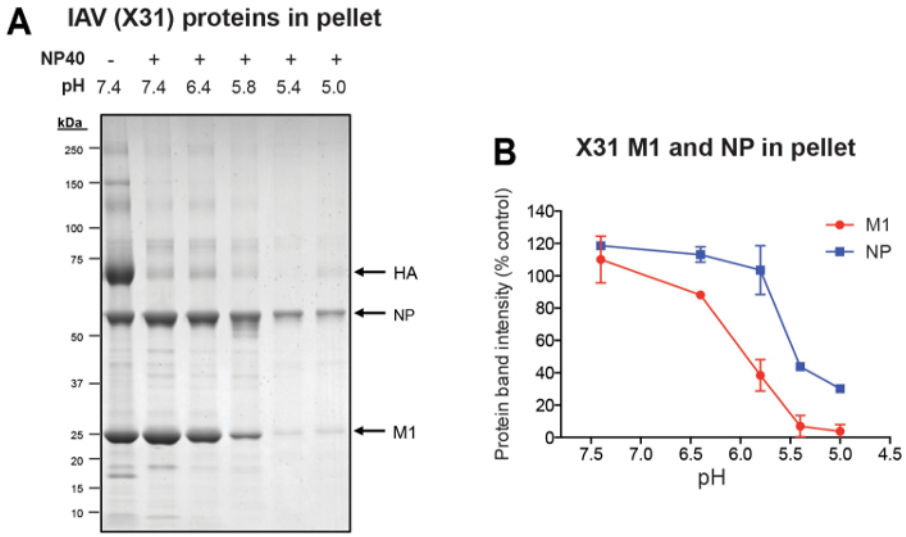

Figure 2: IAV core disassembly *in vitro* upon acidification. (**A**) *In vitro* disassembly of X31 cores at different pH conditions in the lower glycerol layer. As a control, NP-40 was omitted from the bottom glycerol layer (first lane). Samples were separated by SDS-PAGE under non-reducing conditions followed by Coomassie staining. Viral protein bands are indicated on the right. (**B**) Densitometric quantification of the intensities of viral protein bands shown in (A). Protein band intensities for M1 and NP were normalized to those at pH 7.4 without NP-40. Data are represented as means of duplicate experiments ± standard deviation (SD). Adapted from Stauffer *et al.*, 2014[16]. Please click here to view a larger version of this figure.

Discussion

Viral capsids are metastable macromolecular complexes. While assembly of virions requires the encapsidation and condensation of the virus genome, initiation of the next round of infection depends on disassembly of this compact capsid structure. Viruses have evolved to exploit

various cellular mechanisms to control the coating-uncoating cycle, including cellular receptors, chaperones, proteolytic enzymes, physical forces provided by motor proteins or helicases as well as pH and ionic switches[26,27]. Here, we describe an *in vitro* approach based on a glycerol gradient centrifugation to specifically test the effect of factors promoting disassembly of the IAV core. The technique can be potentially adapted for dissecting uncoating processes of other enveloped viruses.

By using colloidal Coomassie, the major structural IAV proteins M1, NP, and HA can be clearly detected in SDS-PAGE even with a relatively small virus particle number. Yet, a more in-depth characterization of the sedimented capsid structure and its composition would require follow-up analyses, such as electron microscopy (as previously presented[28]), Western blotting, dynamic light scattering or mass spectrometry. Further extension of the protocol could include fractionation of the lower (25%) glycerol phase and analysis of the specific core components.

Adjustment of the lower glycerol layer to the optimal fusion pH of 5.0 (for X31) led to an almost complete dissociation of the matrix layer (**Figure 2**)[16]. However, around 30% of the input NP signal can still be detected at this pH. It is possible that due to the absence of cellular uncoating factors in this setup, such as recently identified histone deacetylase 6 (HDAC6)[18], incomplete disassembly occurs. Inside the cell, slow acidification of the viral core and exposure to a switch from Na^+ to K^+ primes the virus for complete vRNP release controlled by uncoating factors[16]. We cannot exclude that in our setup, the non-ionic detergent partially disrupts the acid-exposed and destabilized core. However, no effect on the core sedimenting at neutral pH is observed indicated by similar protein band intensities as compared to non-solubilized samples (**Figure 2A**, compare lanes 1 and 2). Although, the assay presented here proved to be highly reproducible and (as such is) robust enough for band quantification, a certain range of variation could be observed.

The use of glycerol in this protocol is critical for the outcome of the experiment. As previously reported[17], ultracentrifugation through a sucrose gradient destabilizes the IAV core, which is likely due to a high osmotic force created by sucrose. Purification of IAV via sucrose gradients might exert similar effects. Therefore we compared egg-grown X31 derived from clarified allantoic fluid and sucrose-purified stocks. No major difference could be observed with respect to the influence of acidic pH on core stability (data not shown). Nevertheless, in order to exclude potential side effects of the osmotically active sucrose, we recommend performing the *in vitro* uncoating assay with clarified allantoic fluid or concentrated cell culture supernatants.

Besides the choice of gradient material, it is important to maintain the integrity of the gradient to not influence the sedimentation behavior of the lysed viral cores and ensure reproducible results. In addition, incomplete resuspension of the pellet fraction after ultracentrifugation likely leads to different outcomes. Finally, we strongly recommend adjusting the pH of the detergent-containing buffer solutions ideally on the same day of the experiment as small changes in ionic concentration might have a drastic effect on the viral core stability.

It is important to note that depending on the particular IAV strain, different disassembly efficiencies might occur based on the properties of the respective M1 and NP variants. This might also apply for mutant viruses. Thus, pH thresholds for matrix layer and vRNP dissociation have to be determined for the respective IAV strain before testing additional influences. For the analysis of specific conditions on viral core stability we suggest adjusting the bottom glycerol layer to a pH value so that half-maximal disassembly of M1 is achieved. It is possible to investigate the influence of specific ions or reducing agents by modifying the detergent-containing layer accordingly. Putative cellular uncoating factors could be added directly to the glycerol gradient. In case of cytoplasmic factors, a third glycerol layer at the bottom of the gradient could be introduced, which is adjusted to neutral pH, contains the cellular factor of interest, and is free of detergent. In this way, the three-layer gradient would even more closely mimic the passage of the virus through the endocytic system and release of gene segments into the neutral cytoplasm.

Taken together, we present a robust yet simple *in vitro* assay to study IAV priming and uncoating, which has the potential for versatile modifications in order to address diverse questions in the context of IAV entry. In addition, it can be applied to investigate entry processes of other enveloped viruses with related uncoating mechanisms.

Disclosures

The authors have nothing to disclose.

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