# Stability and Dissociation of Adeno-Associated Viral Capsids by Variable Temperature-Charge Detection-Mass Spectrometry.

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**ABSTRACT:** Adeno-associated viral (AAV) vectors have emerged as gene therapy and vaccine delivery systems. Differential scanning fluorimetry or differential scanning calorimetry are commonly used to measure the thermal stability of AAVs, but these global methods are unable to distinguish the stability of different AAV subpopulations in the same sample. To address this challenge, we combined charge detection-mass spectrometry (CD-MS) with a variable temperature (VT) electrospray source that controls the temperature of the solution prior to electrospray. Using VT-CD-MS, we measured the thermal stability of empty and filled capsids. We found that filled AAVs ejected their cargo first and formed intermediate empty capsids before completely dissociating. Finally, we observed that pH stress caused a major decrease in thermal stability. This new approach better characterizes the thermal dissociation of AAVs, providing the simultaneous measurement of the stabilities and dissociation pathways of different subpopulations.

AAV vectors are currently being used as gene therapy and vaccine delivery systems for a range of diseases,[1-5] with multiple FDA-approved AAV therapies.[1,3,6] For example, Luxturna is an AAV2 therapeutic for hereditary blindness, and Zolgensma is an AAV9 therapeutic for spinal muscular atrophy.[7] One component in the quality control and development of AAV capsid therapeutics is the measurement of their thermal stability.[8-9] Typically, differential scanning fluorimetry (DSF) and differential scanning calorimetry (DSC) are used to characterize the stability of AAVs by measuring their melting temperature ($T_m$).[3,10-12] Most AAV serotypes have published $T_m$ values in different buffers and in different stressed conditions.[10,12] DSF and DSC experiments have shown that AAVs have $T_m$ values that typically range from 55–85 °C, and filled AAVs typically have similar or higher $T_m$ values compared to their empty counterparts.[10-12] These experiments provide simple measurements of the global thermal stability of AAV samples, but many preparations of AAVs have heterogeneous populations of empty, partially filled, and filled capsids that are not distinguishable by conventional methods.[13-16]

Native charge detection-mass spectrometry (CD-MS) has previously been used to measure the intact mass of AAV capsids and characterize the ratios of empty, partially filled, fully filled, and extra filled viral capsids.[13-14,17-18] Native MS retains non-covalent interactions in the gas-phase, which allows AAV capsids to remain intact in the mass spectrometer.[14,19] CD-MS simultaneously measures the *m/z* and charge of ions to directly determine the mass. CD-MS was pioneered using home built instruments,[14,20-21] but recent work has extended this to commercial Orbitrap instruments.[19,22]

Although CD-MS with AAVs has advanced significantly, our hypothesis was that CD-MS could be coupled with variable temperature electrospray ionization (VT-ESI) to characterize the thermal stability of AAV subpopulations during mass analysis. A previous CD-MS study[4] heated AAV capsids prior to mass analysis, and we wanted to build on this method by heating the capsids during continuous CD-MS analysis. By carefully controlling the temperature of the analyte solution in the needle during ionization, VT-ESI-MS with conventional native MS has previously been used to study protein unfolding[23] and thermodynamics of biomolecular interactions.[24-27] A recent review gives a comprehensive overview of variable temperature MS methods.[24]

Although it is possible to resolve empty AAVs and perform online thermal denaturing with conventional native MS (Figure S1–S3 and Supplemental Results), the raw data was noisy, and it was difficult to resolve the individual charge states for AAV capsids at higher temperatures. Furthermore, we were unable to resolve filled AAVs with native MS. To address these limitations, we used Orbitrap CD-MS, which uses the signal intensity of single ions to determine

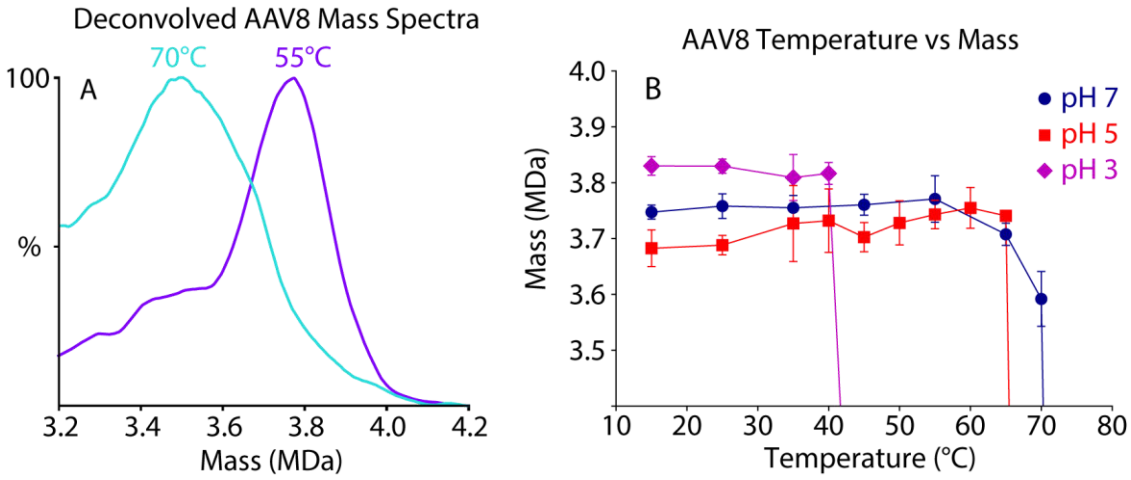


**Figure 1.** Thermal ramp of empty AAV8 capsids. A) Overlayed deconvolved CD-MS mass spectra of AAV8 capsids at 55 °C and 70 °C, which shows a loss of mass at higher temperatures. B) Temperature vs mass plot of AAV8 capsids at pH 7 as *blue circles*, pH 5 as *red squares*, and pH 3 in *magenta diamonds*. Stressed capsids dissociate at a lower $T_m$. Empty capsid masses were extracted using the data collector from UniDec by setting the extraction to the center of mass for high mass signals above 50% relative intensity, which extracts the middle of the AAV8 mass distribution for each temperature. Data is not shown beyond the temperature at which the high mass signal is lost.

the charge, to measure the mass distributions of AAV capsids.[17,19,28] We performed CD-MS at increasing solution temperatures to differentiate between the dissociation of empty and filled capsids based on their mass and relative signal intensity.

Detailed methods are provided in the Supporting Information. Briefly, AAVs were buffer exchanged by dilution in 0.2 M ammonium acetate and concentrated with a 100 kDa MWCO filter, which was done at least two consecutive times. Thermal ramps were performed from 15–75 °C, at 5 or 10 °C increments, allowing 20 seconds for the sample in the nano-ESI needle to equilibrate at each temperature, and then acquiring mass spectra at that temperature. Three replicate ramps with separate needles were completed for each temperature ramp, and error bars indicate standard deviations between replicates. VT-ESI was performed using the design published by McCabe et al.,[23] and PEG-coated needles were used to improve the sensitivity.[29]

**VT-CD-MS of Empty AAV8 Capsids Reveals Thermal Dissociation.** With increasing temperatures, VT-CD-MS showed a stable average mass for empty AAV8 capsids from room temperature up to around 55–65 °C (Figure 1, Figure S4, Figure S5). Around this point, the mass began to decrease, and the signal died for high mass species at 70–75 °C. Unfortunately, the loss of signal was too sudden to fit to a sigmoidal curve to measure a $T_m$ for AAV8, but we can say that AAV8 dissociated within the 70–75 °C range. This temperature range is similar to results with conventional native MS (Figure S2) and corresponds closely with the known $T_m$ of empty AAV8 capsids, which is 71 °C in phosphate-buffered saline (PBS).[10,12] Importantly, the loss of mass at 55–70 °C (Figure 1) indicates that the capsids become partially destabilized at a lower temperature than its known $T_m$. The mass loss between 55 °C and 70 °C is 179±83 kDa (p=0.009), which may correspond to different oligomers (dimers to tetramers) of viral proteins (VPs) dissociating from the capsid, but the standard deviation is too high to assign specific oligomer loss.

To explore how stress affects the thermal stability during VT-CD-MS, we buffer exchanged AAV8 capsids into ammonium acetate at pH 3 and pH 5 prior to the temperature ramp. Previous DSF and DSC studies have shown that lowering pH stresses AAV capsids and lowers the $T_m$.[10,15] At pH 5, the capsids dissociated at 65–70 °C, which is slightly lower than the pH 7 capsids and matches previous DSF studies.[10,12,15] In contrast, capsids at pH 3 had a loss of signal at 40–45 °C, significantly lower than the unstressed capsids. These results are lower than previous $T_m$ measurements of AAV8 at pH 3, which dissociated at around 60 °C in a mix of PBS and acetate buffer.[12] Because there is some dependence of AAV stability on the buffer conditions, some of the differences with previous studies may be due to using ammonium acetate or the ionic strength.[10,12] Nevertheless, these results show that the loss of signal in VT-CD-MS agrees with $T_m$ values measured by other techniques and provides useful information on the thermal stability of empty AAV capsids.

**Filled AAVs Eject their Cargo.** We then sought to characterize capsids with DNA cargo, and we chose commercially available AAV2 filled with the cytomegalovirus promoter labeled with green fluorescent protein (CMV-GFP) from Virovek. AAV2 and AAV8 capsids have similar stabilities, shown by DSF, and we chose this sample because it has very few empty capsids.[10] Under ambient temperatures, the capsids are almost fully filled but have an additional extra filled AAV mass distribution that was about 0.7 MDa higher in mass than the filled capsid, which has been previously seen by Jarrold and coworkers.[4] We then performed a temperature ramp on these AAV2 capsids from 15–75 °C. Interestingly, we found that filled AAVs with a mass around 4.6

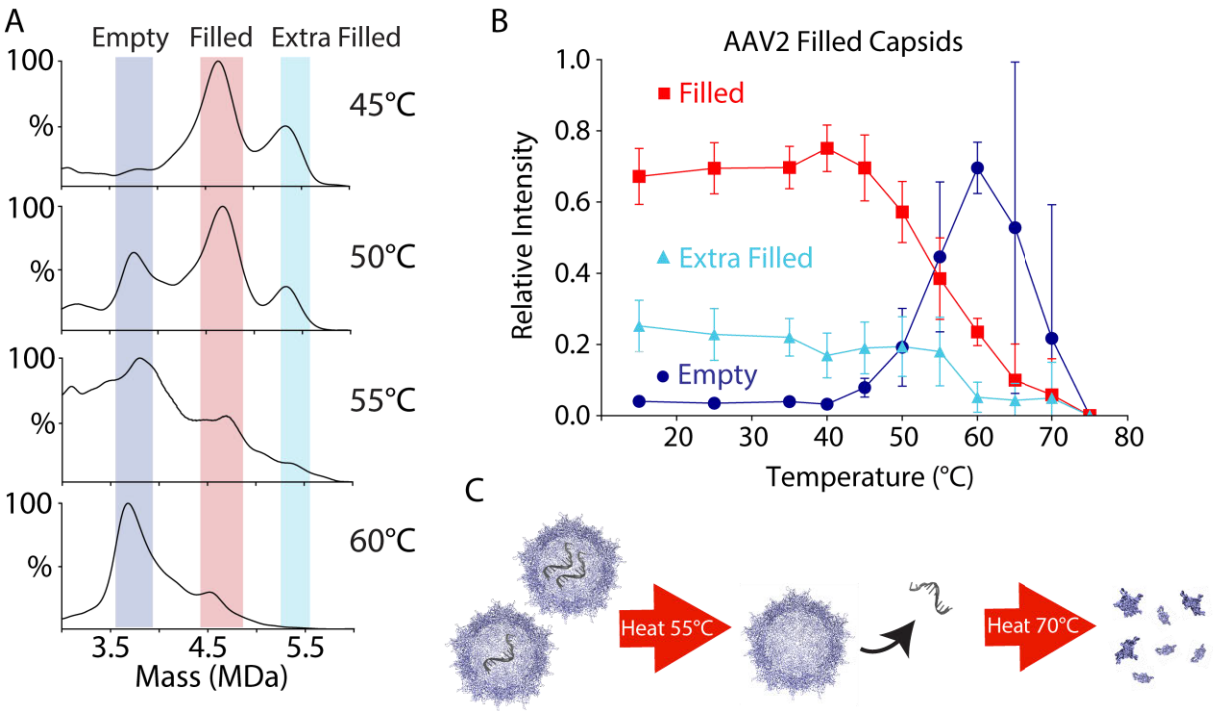


**Figure 2**. Thermal ramp of purified AAV2 capsids filled with CMV-GFP. A) Deconvolved CD-MS spectra of AAV2 filled capsids at various temperatures with the empty mass region shaded in *dark blue*, the filled AAV mass distribution in *red*, and the extra filled AAV mass distribution in *ice blue*. B) Thermogram of AAV2 CMV-GFP filled capsids with empty capsids as *blue circles*, filled capsids as *red squares*, and extra filled capsids as *ice blue triangles*. Relative intensities are calculated by sum normalizing each AAV subpopulation at a specific temperature. C) An artistic depiction of the data shown in part B, where the filled capsids eject their cargo prior to complete dissociation.

MDa lost their 0.9 MDa cargo at 55 °C, leaving an empty capsid that subsequently fully dissociates at 70–75 °C (Figure 2, Figure S6, & Figure S7). Unfortunately, the lower *m/z* regions of the CD-MS spectra during the thermal ramp were too messy to assign accurate masses (Figure S8), which may be due to CID fragmented protein and/or DNA released from the capsid in solution.

These data reveal that filled AAVs eject their cargo first before completely dissociating, which has previously been seen with atomic force microscopy and transmission electron microscopy.[30] Prior publications have shown that AAV capsids undergo conformational changes at higher temperatures but do not completely dissociate.[15,31-32] Our VT-CD-MS results confirm that AAV capsids lose their DNA cargo at lower temperatures than the $T_m$.

Examining the relative intensities of empty capsids versus filled capsids with increasing temperature, a single sigmoidal curve was fit to measure an ejection temperature ($T_E$)—the temperature where the DNA cargo was ejected—of 53.3±1.6 °C (Figure S9). This value provides different information than the melting temperature ($T_m$), which captures the temperature at which the capsid fully dissociates. VT-CD-MS offers a unique way to simultaneously measure both parameters.

Finally, similar results were observed for a complex pharmaceutical sample with a mixture of empty, partially filled, and fully filled AAVs. We found that signal for the partially filled and fully filled capsids was lost at a $T_E$ of 55–60 °C (Figure S10 & S11). The $T_m$ of the remaining emptied AAV capsids was close to the empty AAV8 at 65–70 °C (Figure 1). As the filled capsids eject their cargo, the relative intensity increased for empty capsids (Figure S10 & S11). Together, the VT-CD-MS data from filled AAV2 and AAV8 indicate that the partially, full, or extra filled capsids first eject their cargo and leave an intermediate empty capsid that overlaps in mass and stability with the original empty AAV population. Similar to the filled AAV2 capsids, we found that the lower *m/z* region was messy, and we were unable to assign confident masses for this region (Figure S12).

Overall, we demonstrated that VT-CD-MS is a useful tool for the characterization of AAV capsid thermal dissociation. The loss of signal for AAVs during VT-CD-MS closely corresponds with their known $T_m$. We also found that filled AAV2 capsids form an empty intermediate upon heating, which confirms that filled AAVs eject their cargo before completely dissociating.[30] VT-CD-MS thus provides simultaneous measurements of cargo ejection ($T_E$) and capsid dissociation ($T_m$) for different AAV subpopulations in the same sample, which offers a novel method for quality control and characterization of AAV capsids. Furthermore, the combination of VT-ESI with CD-MS offers the exciting possibility of probing the stabilities of other complex samples.

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## SUPPLEMENTAL INFORMATION

The supplemental information includes the materials and methods for sample preparation, mass spectrometry, and data analysis. It also contains supplemental figures of the VT-ESI-MS analysis of AAVs, VT-CD-MS analysis of mixed AAV8, and raw data figures. It also contains a supplemental results section describing the native MS analysis of empty AAV8 capsids.

## CONFLICT OF INTEREST

MMK and MTM have filed a provisional patent on VT-CD-MS analysis of AAVs and (with CAA and CCH) on coated nESI needles.

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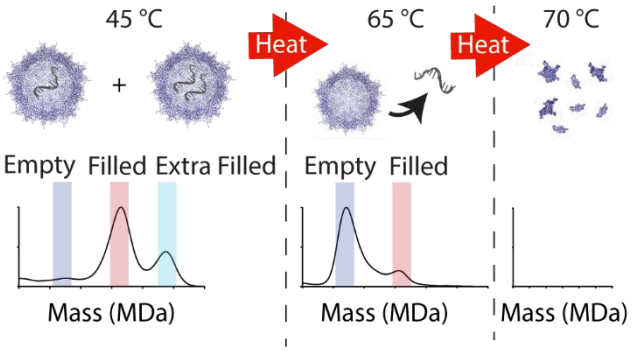
**TOC Figure:**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