ENHANCED DECLUSTERING AND CHARGE-STRIPPING ENABLES MASS DETERMINATION OF AAVS IN TOF MS

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INTRODUCTION

Adeno Associated Virus (AAV) capsids are the most attractive candidate for gene therapy vectors. These ball-like particles consist of 60 subunits of three viral proteins (VP1, VP2 and VP3) assembled in a variety of stoichiometries (average VP ratio of [5 : 5 : 50]),[1] giving an average mass ~3.7 MDa. "Full" capsids encapsulate ~4.7 kb single-stranded DNA. Precise determination of capsid mass remains challenging due to the high level of adduction and heterogeneity in the VP ratio which yields broad distributions of overlapping charge states. Herein, we demonstrate a method of enhanced declustering using a modified StepWave™ ion guide as well as extensive charge stripping using an electron capture dissociation (ECD) device, allowing resolution of charge states and deconvolution of mass.

AAV TRANSMISSION

AAV samples (Virovek, LakePharma and UNC Vector Core) were dialyzed (Amicon™ 10K, Merck) and buffer exchanged (Bio-Spin™, Bio-Rad) into 150 mM AmAc before infusion via nESI emitters (PicoTip™, New Objective). The transmission and detection of both full and empty AAV capsids can be achieved on the SELECT SERIES™ Cyclic™ IMS instrument without any modifications. These spectra contain featureless distributions (m/z FWHM ~ 1500) centred at m/z ~23,000 and ~30,000 for empty and full AAV8 capsids, respectively (Figure 1). Ratios of empty : full capsids can be established rapidly (Figure 1A - 5 min, Figure 1B - 2 min), but lack of charge state resolution precludes the determination of the molecular weights.

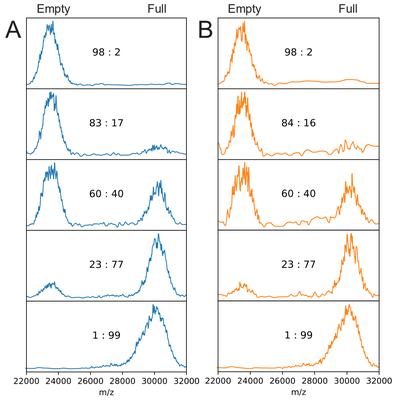

Figure 1. *Spectra of empty and full AAV8 capsids (different empty : full ratios, UNC Vector Core and Lake Pharma) obtained on an unmodified Cyclic IMS instrument after 5 min (**A**) and 2 minutes (**B**). Data were smoothed (Savitzky-Golay, window of 200). Concentration <1.4x10^{12} particles/mL.*

INSTRUMENTATION

Enhanced Declustering

The Q-cIMS-ToF instrument was fitted with a modified StepWave ion guide (Figure 2). It features two parallel plates oriented along the normal ion path (Figure 2B, C). An alternating voltage is applied to these plates inducing activation of ions via off-axis oscillations, enhancing declustering. Ions are propelled by the gas flow and focussed in a third direction by a series of electrodes stacked along the normal ion path. RF and traveling waves applied to those electrodes confine high m/z ions (Figure 2D). Previously demonstrated as highly efficient in removing detergent adducts from membrane proteins,[2] here we explore its applicability in resolving features in crowded m/z distributions of AAV ions.

Electron Capture Dissociation Device

An electromagnetostatic ECD cell (ExD WK-150, eMSion) was installed between the Cyclic IMS device and ToF analyser (Figure 2A).[3] The ions pass through a cloud of electrons confined by the magnetic field (Figure 2E), before m/z measurement in the ToF analyser. Despite the 64,000 m/z range limit of the instrument, higher m/z ions may be detected in the subsequent ToF cycle. The inclusion of the subsequent cycle duration into the time-to-m/z conversion allows an extended range spectrum to be plotted.

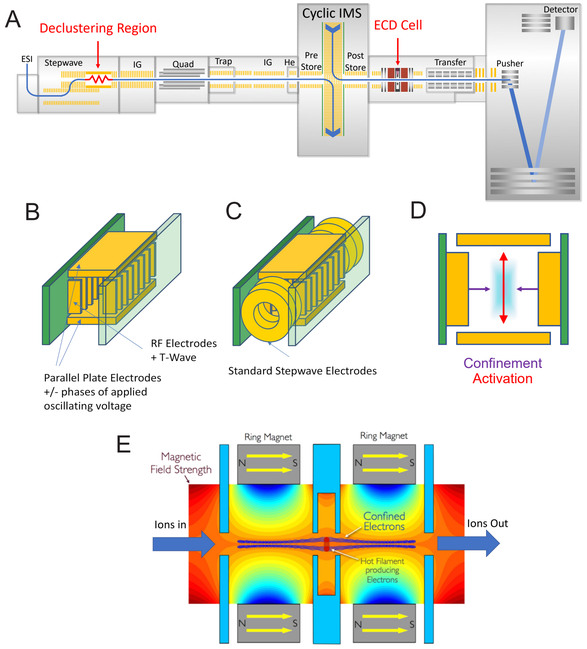

Figure 2. ***A****: Instrument schematic showing the Q-cIMS-ToF instrument geometry and location of the declustering region and ECD cell.* ***B,C****: Electrode arrangement in the declustering region.* ***D****: Schematic of directions in which ions are confined (purple) and activated (red).* ***E****: Electrode structure and magnetic field map in the ECD cell.*[3]

RESULTS

Enhanced Declustering

Previous work of Pierson *et al.* using CDMS indicate that the AAV assembly process results in a mixture of particles with up to 1891 different VP ratios (i.e. masses).[4] Each species is expected to present in a range of charge states, resulting in a crowded m/z distribution. Even a moderate amount of adduction can "blur" such m/z profiles (Figure 3A). Application of the alternating voltage (~50 kHz, 300 Vp-p) in the modified StepWave revealed features spaced by m/z ~50 on the broad m/z envelope of AAV5 ions (Figure 3B). We concluded that these peaks may constitute a pattern of interfering resonances akin to those reported by Todd *et al.*[5] Despite the resolution of such a pattern, simultaneous deconvolution of all underlying masses challenges current deconvolution algorithms.

Electron Capture no Dissociation

One way to simplify crowded m/z profiles of AAVs is to reduce the average charge such that m/z spacings between the charge states are larger than the widths of mass distributions with a common charge. To achieve this, we passed the AAV5 ions through the ECD device and observed a significant m/z shift (from m/z ~20,000 to ~90,000), and distinct peaks with increasing m/z spacing, characteristic of a charge state distribution (Figure 4A, B, blue trace). Using a modified version of the MaxEnt 1 algorithm, data were deconvolved to the corresponding mass and charge components (Figure 4C, red trace). The most abundant mass is ~3.580 MDa. This is somewhat smaller than the expected value of ~3.7 MDa perhaps due to the altered ratio of VP proteins, as discussed below. Importantly, the deconvolved mass profile is asymmetric, which again may be explained by some of the lighter assemblies being the most abundant ones.

Mass Distribution Modelling

The assembly process of capsids was proposed to be stochastic, with the probability of formation of "different-ratio" capsids approximated by a multinomial distribution, where multinomial probabilities reflect the VP expression levels, in solution.[4,6] The deconvolved data was fitted with multinomial distribution, constrained to the total of 60 samples (i.e. 60 VPs in a capsid). The "best fit" multinomial probabilities were found at [0.01 : 0.03 : 0.96], accounting for 2000 Da mass shift due to adduction (Figure 4C, grey bars vs red trace). The two most probable masses predicted by the above method (3.576 and 3.581 MDa) correspond to capsids with VP ratios of [0 : 1 : 59] and [0 : 2 : 58], respectively. The spacing between the most abundant peaks is ~5000 Da which is significantly higher than the estimated amount of adduction. Thus, it may be possible that some of the features in the deconvolved mass distribution are "real" (Figure 4C, red trace), and we will evaluate the reproducibility of this result in the future work. The resulting mass profile may be convolved with charge state distribution to model the resonance pattern in the "native" m/z distribution (Figure 3B, red line). Finally, we note that the asymmetric mass distribution may be caused by the biological/process artifact (e.g. "correlated" assembly, mixed batches), in which case a mix of multinomial (or other) distributions may be more appropriate to describe the data.

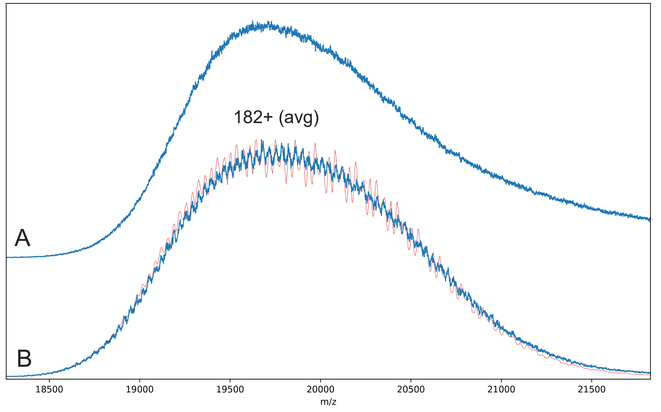

Figure 3. *Spectra of empty AAV5 capsids (Virovek) obtained with the enhanced declustering.* ***A:*** *Spectrum obtained with declustering device in "OFF" mode.* ***B:*** *Blue spectrum obtained following the application of 50 kHz square wave with 300 Vp-p to the parallel plate electrodes. Red trace corresponds to m/z distribution computed by convolving charge state distributions to mass distribution from deconvolved ECD data (Figure 4C, grey bars). Concentration <2x10^{13} particles/mL. Data were acquired for 15 minutes and smoothed (Savitzky-Golay, smooth window of 20).*

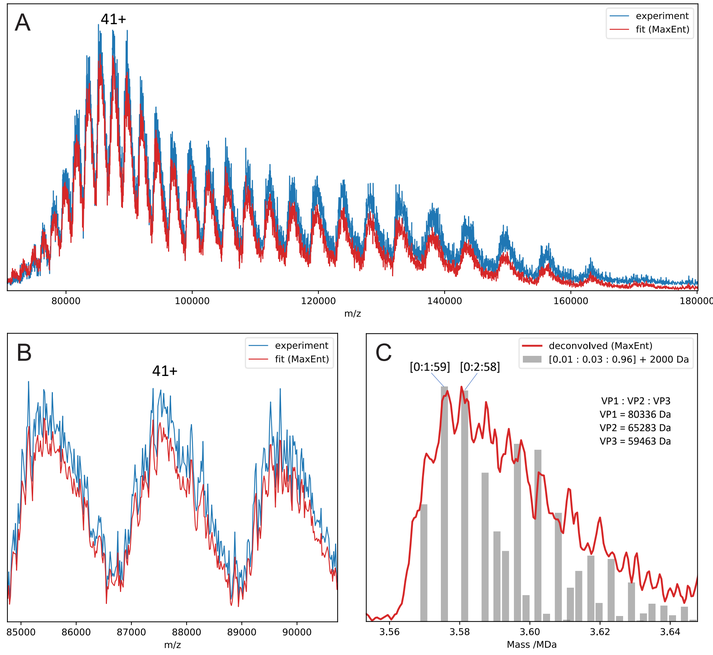

Figure 4. *Spectra of empty AAV5 capsids (Virovek) recorded following interaction with electrons inside the ECD cell.* ***A:*** *Blue trace corresponds to experimental spectrum. Red is the fit from the deconvolved data.* ***B:*** *Zoom in, comparing experimental and fit data.* ***C:*** *Deconvolved mass distribution (red). The grey bars correspond to the mass distribution computed from sets of 60 samples from a multinomial distribution having probabilities of [0.01 : 0.03 : 0.96]. The width of the bars approximates the estimated amount of adduction (2000 Da). The two most abundant masses correspond to capsids with VP ratios of [0 : 1 : 59] and [0 : 2 : 58]. Inset: masses of VP proteins*[6] *used to model mass distributions. Concentration <2x10^{13} particles/mL. Data were acquired for 2h (down-sampled by 100, no smoothing).*

SUMMARY AND OUTLOOK

- **A modified StepWave with enhanced declustering capability enables resolution of fine structure in native AAV spectrum.**
- **Charge reduction via ECnoD allows resolution of charge state peaks and deconvolution of AAV mass.**
- **Future work will investigate the presence of ECD fragments, further explore the accuracy of the measured mass and compare the results with protein-level data.**

References:

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