# Combining Surface-Induced Dissociation and Charge Detection Mass Spectrometry to Reveal the Native Topology of Heterogeneous Protein Complexes 

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#### Abstract

Charge detection mass spectrometry (CDMS) enables the direct mass measurement of heterogeneous samples on the megadalton scale, as the charge state for a single ion is determined simultaneously with the mass-to-charge ratio $(m / z)$. Surface-induced dissociation (SID) is an effective activation method to dissociate non-intertwined, non-covalent protein complexes without extensive gas-phase restructuring, producing various subcomplexes reflective of the native protein topology. Here, we demonstrate that using CDMS after SID on an Orbitrap platform offers subunit connectivity, topology, proteoform information, and relative interfacial strengths of the intact macromolecular assemblies. SID dissects the capsids ( $\sim 3.7 \mathrm{MDa}$ ) of adenoassociated viruses (AAVs) into trimer-containing fragments (3mer, 6 mer, 9 mer, 15 mer , etc.) that can be detected by the individual ion mass spectrometry (I2MS) approach on Orbitrap instruments. SID coupled to CDMS provides unique structural insights into heterogeneous assemblies that are not readily obtained by traditional MS measurements.




## INTRODUCTION

Native mass spectrometry ( nMS ) has emerged as a powerful tool for structural biology that has advantages in sensitivity, speed, and tolerance of sample heterogeneity. It is complementary to classical techniques, ${ }^{1-4}$ such as nuclear magnetic resonance, X-ray crystallography, and cryo-electron microscopy. The ability of nMS to preserve non-covalent interactions in the gas phase allows the determination of the native structure and stoichiometry of large protein complexes. ${ }^{5,6}$ To determine this stoichiometric information of a given analyte, the charge state distribution in the mass-to-charge ( $\mathrm{m} / \mathrm{z}$ ) spectrum must be resolved to determine the mass. ${ }^{7}$ However, resolving the charge state distribution can be challenging for heterogeneous macromolecular assemblies. The heterogeneity arising from sequence variants, post-translational modifications, proteoforms, counterions, and residual salts/solvents causes peak broadening and sometimes makes it impossible to resolve charge states. ${ }^{8}$ Single-particle charge-detection mass spectrometry (CDMS) can circumvent this limitation by determining the charge and $m / z$ of individual ions simultaneously. ${ }^{9,10}$ Previously, CDMS has been conducted on mainly house-built ion traps, ${ }^{11-15}$ but recent work has emerged on commercial Orbitrap platforms. ${ }^{16-19}$ The ability to directly detect charges extends the application of nMS to highly heterogeneous complexes in the megadalton range, including ribosomes, ${ }^{20}$ vaccines, ${ }^{21}$ and intact virus particles. ${ }^{22,23}$ Due to the growing applications in gene therapy, CDMS is used in the characterization of adeno-associated
viruses (AAVs), including capsid assembly, ${ }^{24}$ thermal stability, ${ }^{25,26}$ genome truncations, ${ }^{27}$ and product heterogeneity. ${ }^{28,29}$
Gas phase dissociation is commonly employed in nMS to gain structural information, including subunit connectivity, ligand binding sites, and topology of macromolecular complexes. ${ }^{30-33}$ Collision-induced dissociation (CID), one of the most common activation methods, produces unfolded, highly charged monomers and the complementary ( $N-1$ )mers through multiple-step, low-energy collisions with background gas molecules. ${ }^{34,35}$ CID allows multi-step accumulation of internal energy and results in the restructuring of isolated analyte ions. ${ }^{36}$ Although it provides useful stoichiometry information, limited direct information on subunit connectivity is revealed. To probe the quaternary structure of protein complexes, surface-induced dissociation (SID) has emerged as an alternative dissociation method. Unlike CID, SID involves a faster, high-energy deposition process and assists analytes to overcome energy barriers. ${ }^{37,38}$ In SID, analyte ions are typically accelerated and directed to collide with a gold surface coated with a monolayer of fluorocarbon chains. ${ }^{39,40}$ Stainless steel, coated with background contaminants, is also used as the

[^0]
collision surface in the recent development of the SID device. ${ }^{41}$ SID enables the dissociation of a complex prior to extensive restructuring, and the resulting subcomplex identities are more reflective of the solution phase structure. ${ }^{42-45}$ At low collision energies, SID tends to break the weakest non-covalent interfaces, making it a powerful tool to determine relative interfacial strengths and reflect the subunit connectivity of the protein complexes. ${ }^{46}$
As part of nMS, SID is also hampered by sample heterogeneity. The SID-produced fragments of a heterogeneous sample are structurally informative but can be difficult to distinguish from each other or precursor ions if they overlap in $m / z$. To overcome these issues, CDMS offers an alternative to separate overlapping species, even for instruments where ion mobility is not commercially available or isotopes are not resolved. Here, we take advantage of both technologies (SID and CDMS) and integrate them into one workflow to determine detailed structural information for intact macromolecular assemblies. The CDMS approach used here has been developed and described by Kelleher and colleagues as individual ion mass spectrometry (I2MS) and selective temporal overview of resonant ions (STORI). ${ }^{18,47}$ This approach has been recently commercialized as Direct Mass Technology (DMT) Mode. We first performed SID-CDMS on both homomeric and heteromeric complexes, GroEL, and human 20S proteasome, respectively. We then applied this approach to the megadalton range and characterized the quaternary structure of empty adeno-associated viral (AAV) capsids, which are currently being used as gene therapy delivery systems. ${ }^{48-50}$ A house-built algorithm was employed to plot the resulting $m / z$ values in 2D against the resulting charge and mass of all the data collected during the SIDCDMS experiment. The SID-CDMS spectra were further processed and deconvolved using UniDec Charge Detection (UniDecCD) to remove some of the charge measurement uncertainty and increase the mass accuracy. ${ }^{51}$

## - EXPERIMENTAL SECTION

Samples. GroEL was purchased from Millipore Sigma (St. Louis, MO, USA) and refolded with the presence of ATP using a published protocol. ${ }^{52}$ Human 20S proteasome was purchased from Enzo Biochem (New York, NY, USA). Empty AAV8 was purchased from Virovek (Hayward, CA, USA). GroEL and AAV samples were buffer-exchanged by resuspending the particles in 200 mM ammonium acetate and concentrating/ diluting with 30 or 100 kDa molecular weight Amicon Ultra centrifugal filters (Merck Millipore, Burlington, MA, USA), at least three consecutive times. The proteosome was bufferexchanged into 150 mM ammonium acetate using Micro BioSpin P6 spin columns with a 6 kDa cutoff (Bio-Rad, Hercules, CA, USA).

Streptavidin (SA) was purchased from Thermo Scientific Pierce (Rockford, IL, USA). C reactive protein human recombinant (CRP), pyruvate kinase (PK) from rabbit muscle, and glutamate dehydrogenase (GDH) from bovine liver were purchased from Millipore Sigma (St. Louis, MO, USA). SA, CRP, PK, and GDH were buffer-exchanged into 200 mM ammonium acetate using Micro Bio-Spin P6 spin columns with a 6 kDa cutoff (Bio-Rad, Hercules, CA, USA). SA, CRP, PK, GDH, and GroEL were used to construct the charge calibration function.

Mass Spectrometry. All experiments were performed on a Q Exactive Ultra High Mass Range (UHMR) Orbitrap MS
(Thermo Fisher Scientific, Bremen, Germany), which has been modified to include SID. ${ }^{39}$ All single-particle charge-detection data were acquired using the Selective Temporal Overview of Resonant Ions (STORI) analysis by Kafader et al. ${ }^{18,47}$ An aliquot of $1-2 \mu \mathrm{~L}$ sample was loaded into borosilicate capillaries (prepared in-house) for nano-ESI. Instrument parameters were optimized for the transmission of high-mass ions. Therefore, ion transfer target $m / z$ and detector optimization were set to high $m / z$. In-source trapping was enabled with a desolvation voltage ranging between -5 and -100 V . The ion transfer optics (injection flatapole, inter flatapole, and bent flatapole lens) were set to 5,4 , and 2 V for GroEL and proteosome and 10,10 , and 9 V for AAV8. $\mathrm{N}_{2}$ was used as collision gas for all experiments, resulting in a UHV pressure of $2 \times 10^{-10}$ to $5 \times 10^{-10}$ mbar. Typical settings of transmission mode and SID experiments can be found in the Supporting Information (Table S1). Data were acquired at resolution settings corresponding to 2 s transients with transient averaging disabled. To lower the number of ions entering the Orbitrap analyzer, samples were diluted to concentrations between 50 and 500 nM , while sample concentrations for SID were in the $1-3 \mu \mathrm{M}$ regime. In addition, automatic gain control (AGC) was disabled, and injection times were manually set between 0.03 and 1 s to maximize individual ion acquisition by optimally populating available charge state and isotopic channels while minimizing multi-ion events, which correspond to the collection of multiple ions at a singular frequency value. To achieve single ion measurement, the number of individual ion signals collected was decreased to $\sim 100$ per scan event. The charge calibration was generated by nanoelectrospray of various proteins with known charge states: streptavidin, C reactive protein, pyruvate kinase, glutamate dehydrogenase, and GroEL.

Data Processing. All raw signal ion data were first filtered by STORIboard software (Thermo Fisher Scientific) using an R squared threshold of 0.99 , duration threshold of 0.42 s , maximum time of birth of 0.1 s , and signal-to-noise threshold of 1 . R squared ensures a linear STORI slope, and the duration threshold ensures that the ion persists long enough to enable accurate charge assignment. The maximum time of birth ensures that the birth of that new frequency value is excluded from processing if any fragmentation occurs within the Orbitrap. The STORI slopes for unknown species were converted to charges using a calibration generated from protein standards with known charge states, as described in Figure S1. The charge vs $m / z$ and mass vs $m / z$ were plotted using a house-built Python-based data processing algorithm. The CDMS data were processed and deconvolved using UniDecCD. ${ }^{51}$ Detailed data processing parameters are described in the Supporting Information.

## ■ RESULTS AND DISCUSSION

CDMS Resolves the Extensive SID Fragments of GroEL. The SID-CDMS approach was first applied on a homomeric protein standard GroEL, a tetradecameric protein complex ( 801 kDa ) consisting of two stacked heptamer rings. ${ }^{53}$ Upon activation by SID, GroEL dissociates into a wide variety of products, ranging from monomers to tridecamers and including heptamers that reflect the stacked-heptamer structure. ${ }^{54}$ Those extensive fragments are informative for characterizing the structure, but they also add complexity to the spectra due to overlapping charge states of different


Figure 1. Charge $-m / z$ plot (A) and mass- $m / z$ plot (B) of the SID-CDMS spectra for GroEL tetradecamer at "normal" charge (as sprayed from ammonium acetate), acquired with an IST voltage of 50 V and an SID voltage of 135 V . The separation in charge states in the vertical axis discriminates species that are overlapping in $m / z$. The charge distributions of ions are labeled at their approximate location with transparent white lines. The oligomeric states of GroEL from monomer to tetradecamer are indicated by a number from 1 to 15 . (C) Zoomed-in view of charge $-\mathrm{m} / \mathrm{z}$ plot at 11220-11700 Da. The $m / z$ overlapping hexamer, pentamer, tetramer, trimer, and dimer are well resolved by charge states. (D) The SIDCDMS spectrum was processed and deconvolved by UniDecCD to better view the relative intensity of SID products. The red inset structure is a cartoon representation of the GroEL tetradecamer.
oligomers, making the product assignment ambiguous, especially for the hexamers, which can overlap with dimers and trimers. Previously, SID of GroEL was done on a quadrupole time-of-flight (Q-TOF) instrument, and ion mobility was used to separate $m / z$ overlapping fragments. ${ }^{54}$ Although this identified different fragments of GroEL, it is still difficult to resolve all possible oligomers in the overlapping region because of the low $m / z$ resolution and arrival time distribution broadening due to the partial restructuring of the ions. To overcome those challenges, SID on the Orbitrap platform was coupled with CDMS, which nicely resolved the overlapping $m / z$ fragments.

GroEL tetradecamer in 200 mM ammonium acetate solution results in a charge state distribution with the 66+ charge state as the dominant peak. The whole charge state distribution was selected and subjected to an SID voltage of 135 V , yielding a variety of fragments ranging from monomers to tetradecamers, which is consistent with the observation of SID for GroEL in AmAc on the Q-TOF platform. ${ }^{54}$ These extensive fragments, along with the charge-stripped tetradecamers, are well resolved and separated by charge and $m / z$, as demonstrated in Figure 1A. The overlapping product peaks at $m / z 11,440 \mathrm{Da}$ have been resolved as hexamer 30+, pentamer $25+$, tetramer 20+, trimer 15+, and dimer $10+$ in CDMS (Figure 1C), which were ambiguous in past SID experiments with TOF platforms. ${ }^{54}$ The monomer $5+$ at that $m / z$ value is
not detected due to the low charge detection limit of proteins ( $\sim 5+$ to $6+$ ). At $m / z 11,440$ and other regions with fortuitous overlap of $m / z$, the direct measurement of the charge state allows for the direct observation of oligomeric states that overlap in $m / z$. The monomer and dimer product ions are dominant fragments. We have not seen dominant dimers in past experiments on GroEL; while it is tempting to suggest that the dissociation pathway follows the ejection of small subunits (monomers and dimers) from the taut allosteric state of GroEL, ${ }^{55,56}$ we do not currently have additional evidence to support this speculation. Restructuring can be suppressed with charge reduction in the solution phase (Figure S2), resulting in heptamers present in higher abundance compared to the adjacent fragments (trimers, tetramers, pentamers, and hexamers) in the spectrum (Figure 1A,B) as shown previously. ${ }^{54}$ The heptamer from normal charging peaks within the $m / z$ range of $11,000-16,000$ correspond to charge states from $36+$ to $26+$, which are around half the charge states of the tetradecamer precursor $(69+$ to $62+)$. This indicates that there is a portion of tetradecamer undergoing the dissociation pathway that produces heptamers in a symmetric manner, representative of its native quaternary structure (two stacked heptamer rings). The dominant charge state of heptamer is $31+$, a value smaller than half of the dominant charge state of tetradecamer 66+. This pathway is possible from the dissociation of precursors with lower charge states, consistent


Figure 2. (A) Mass-m/z plot of the SID-CDMS spectra for human 20 S proteasome, acquired with an SID voltage of 105 V. The charge distribution of ions is designated at their approximate location with white lines. The structure of the precursor and major dissociation products, $\alpha_{7}$ and $\alpha_{7} \beta_{7}$, is graphically depicted in cartoon representation. (B) Zoomed-in view of the low mass region of the SID-CDMS spectrum processed and deconvolved by UniDecCD, highlighting $\alpha$ monomers/dimers/trimers. The inset cartoon representation shows the connectivity of the $\alpha_{7}$ ring determined by the SID-CDMS spectra and consistent with the crystal structure.
with the previous observation that the precursors undergo charge stripping prior to dissociation. ${ }^{57}$ The lower charge states help suppress the restructuring of the precursor and allow the native-like fragmentation pathway. The expected fragmentation pattern for GroEL shown in SID-CDMS demonstrates that this method enables the separation of the overlapping fragments from more complicated biological assemblies, providing useful structural information even in the absence of ion mobility.

SID-CDMS Separates in Charge and $m / z$, Reflecting the Structural Topology of the 20S Proteasome. The human 20 S proteasome is a hetero-oligomer ( $724 \mathrm{kDa}, 28$ subunits) composed of two outer heptameric rings containing $\alpha$-type subunits and two inner rings consisting of $\beta$-type subunits, stacked into a cylindrical structure $\left(\alpha_{7} \beta_{7} \beta_{7} \alpha_{7}\right) .{ }^{58}$ For human proteasomes, the $\alpha$ - and $\beta$-subunits of heptameric rings are closely related but non-identical, resulting in complexity in the tandem mass spectra. Multiple proteoforms have been observed for both $\alpha$ - and $\beta$-subunits, bringing an additional level of heterogeneity to characterization. ${ }^{59,60}$
The whole charge state distribution of the proteasome was isolated, dissociated using SID at 105 V , and then detected by CDMS (intensity $-m / z$ and charge $-m / z$ plots shown in Figure S3). The unresolved intensity $-m / z$ spectrum was separated by adding charge detection and converted to mass outputs using the house-built Python algorithm. Multiple SID fragments were detected and plotted in mass $-m / z$ as shown in Figure 2A: free $\alpha$-containing subunits, $\alpha_{7}$ rings, half proteasomes $\left(\alpha_{7} \beta_{7}\right), \beta_{7} \beta_{7} \alpha_{7}$ particles ( $20 S-\alpha_{7}$ ), and subcomplexes of the proteasome that are missing one or two $\alpha$-subunits. The most abundant fragments are the free $\alpha_{7}$ rings, revealing that the $\alpha-\beta$ interface is relatively weaker than the $\beta-\beta$ interface. This is consistent with a previous PISA interfacial analysis that showed that the $\alpha-\beta$ interface has a smaller interface area, fewer hydrogen bonds, and fewer salt bridges compared to the $\beta-\beta$ interface. ${ }^{58,60,61}$ Half proteasomes $\left(\alpha_{7} \beta_{7}\right)$ are also observed, suggesting that the dissociation energy is sufficient to cleave the $\beta-\beta$ interface, either from the intact precursor or after loss of the $\alpha_{7}$ ring. 20S- $\alpha_{7}$ are the complementary fragments of the $\alpha_{7}$ ring, and without CDMS or IM, those peaks can be difficult to assign with confidence because their $m / z$ range overlaps
with the region of the charge-stripped precursor, half proteasomes, and 20S- $\alpha$ and 20S-2 $\alpha$. The charge measurement capability of CDMS allows for the direct observation of those SID products that overlap in $m / z$ space. 20S- $\alpha$ and 20S-2 $\alpha$ fragments have been respectively reported in SID results using ion mobility or Orbitrap without CDMS. Due to the heterogeneity of $\alpha$-subunits and salt adducts, broader peaks are usually observed for 20S- $\alpha$ and 20S- $2 \alpha$ fragments. The two distributions of 20S- $\alpha$ and 20S-2 $\alpha$ can be discriminated in CDMS, but the accurate mass assignment is still tough due to their low intensity. In addition to the cylindrical structure of the full proteasome, further structural information can be revealed by $\alpha$-containing subunits. The $\alpha$ monomer, dimer, trimer, and tetramer peaks clustered in the $m / z$ region 3000 to 7000 are directly differentiated in CDMS (Figure 2B). All seven $\alpha$ monomers are observed, and the proteoforms of $\alpha$ monomers are identified by the exact masses (Table S3). The observed masses are slightly higher than the theoretical masses due to adducting of solvents/salts. The complete connectivity of the $\alpha_{7}$ ring, as demonstrated in the inset of Figure 2B, can be mapped by the $\alpha$ dimers and trimers. All surface collision products of proteosomes separated and characterized by CDMS together provide multiple layers of structural information for the hetero protein complexes, including overall structure, connectivity, and proteoforms.

SID-CDMS Dissects AAV Capsids into Multimers of Trimeric Fragments. Adeno-associated viral (AAV) vectors are currently used as gene therapy delivery systems for various diseases. ${ }^{62-65}$ The AAV capsid packages a 4.7 kb singlestranded DNA (ssDNA) genome. AAV capsids ( $\sim 3.7 \mathrm{MDa}$ ) contain 60 subunits of 3 distinct viral proteins (VPs) with an approximate ratio of 1:1:10. ${ }^{66} \mathrm{VP} 2$ and VP 3 are truncations of VP1; VP3 is the shortest among the VPs and is a truncation of VP2. ${ }^{67}$ Only the overlapping region in VP3 is resolved in the atomic resolution structures of AAVs determined by cryoelectron microscopy and X-ray crystallography due to the low abundance of VP1/2 and the disordered nature of the extra sequence stretches in VP1/2. ${ }^{68}$ In the resolved structures of capsids, VP monomers assemble into a $T=1$ icosahedral capsid shell with 2 -, 3 -, and 5 -fold symmetry axes. ${ }^{66}$ There is a depression at the 2 -fold symmetry axes contributing to


Figure 3. Low- and high-energy SID-CDMS of empty AAV8 capsids. (A) Mass-m/z plot of the SID spectra for AAV, acquired with an SID voltage of 125 V . The inset is a cartoon representation of the empty AAV8 capsid composed of 60 copies of viral proteins: VP1 (yellow), VP2 (blue), and VP3 (white). The approximate icosahedral 2-, 3-, and 5-fold axes are labeled. (B) Mass-m/z plot of the SID spectra for AAV, acquired with an SID voltage of 225 V . (C) The inset is the zoom-in of the low mass region, showing the smaller oligomeric fragments from monomers to hexamers. (D) Deconvolved SID-CDMS at an SID voltage of 225 V by UniDecCD. Numbers indicate the oligomeric states. The inset is a cartoon representation showing the representative fragmentation pathway at low SID energy. The 60 mer capsid dissociates along $2 / 5$-fold axes into 15 mer and 45 mer ; 45 mer continues to dissociate into 15 mer and 30 mer. (E) Deconvolved SID-CDMS at an SID voltage of 225 V by UniDecCD. Numbers indicate the oligomeric states. The inset is a cartoon representation showing the representative fragmentation pathway at high SID energy. Small VP subunits ( 1 mer , 2 mer , 3 mer , 6 mer , 9 mer , and 15 mer ) are observed. The interface areas of two trimers are determined by PISA analysis (PDB ID: 2QA0).
conformational changes in response to $\mathrm{pH} .{ }^{69}$ The trimers with a 3 -fold axis of symmetry protrude from the capsid surface and are responsible for receptor binding and antibody recognition. Five VP monomers form a pentamer with a 5 -fold axis of symmetry, leaving a channel pore in the center to link the inside virus to the outside environment. ${ }^{70}$

Characterization of AAVs, including the stoichiometry of VPs and the purity of genome-loaded AAV, is essential in AAV preparations, but unfortunately, many AAV companies currently make their measurements by AUC (analytical ultracentrifugation) with a relatively large sample requirement. With low sample consumption and high accuracy, CDMS can resolve intermediate mass species, making it a unique technique for the quantitation of capsid populations. ${ }^{71}$ Previous studies have focused on using CDMS to measure the intact mass of AAVs to determine the levels of empty, partially filled, and fully filled capsids. There are few structural studies of AAVs using mass spectrometry because AAVs are found to be stable enough to survive traditional gas-phase activation, with little to no fragmentation seen. ${ }^{23,24,28}$ SID, which provides a larger collision target and higher-energy deposition, is found to be able to dissociate the intact capsids efficiently. The SID fragments of AAVs can be well resolved when SID is coupled with CDMS, providing unique insights
into the AAV capsid structure. The intact mass of empty AAV8 was measured first by CDMS (intensity $-m / z$ and mass- $m / z$ plots shown in Figure S4), and the major population was 3.72 MDa corresponding to an empty capsid. There is also a minor population (4.14 MDa) with a mass higher than empty AAV8, possibly due to the incomplete removal of the genome. Ions in the $m / z$ range of $20,000-25,000$ for empty AAV8 were isolated and subjected to SID, resulting in a series of different fragments, each aligning with a different oligomeric state of the viral protein. At low SID voltage ( 125 V ), fragments associated with trimer, hexamer, $9 \mathrm{mer}, 15 \mathrm{mer}, 30 \mathrm{mer}$, and 45 mer were observed (Figure 3). All those fragments are composed of VP trimers, which is consistent with the native topology of AAV, in that 20 VP trimers assemble into a 60 mer capsid. Based on these results, trimers are predicted to be the relatively stable building block (the inner interface of trimers is slightly stronger than the outer). The fragmentation pattern matched the calculated interface areas of the capsid remarkably well. When considering the top 3 interfaces of the crystal structure using the PDBePISA, ${ }^{61,72}$ the total interface area required to cleave the VP trimer to monomers is $14,880 \AA^{2}\left(3 \times 4960 \AA^{2}\right)$, which is much higher than the total interface between two trimers ( $6271 \AA^{2} ; 2 \times 2364 \AA^{2}+1 \times 1543 \AA^{2}$ ). This shows the tendency to cleave along the $2 / 5$-fold wall primarily when
colliding with the surface, releasing 15 mer and 45 mer . Both 15 mer and 45 mer can further dissociate at the weaker interfaces, producing additional fragments. Increasing the SID voltage allows higher-intensity monomers, dimers, trimers, tetramers, pentamers, and hexamers to be observed in the low $m / z$ region. Some percentage of those extensive fragments may be due to the secondary dissociation from SID fragments, e.g., monomers and dimers are possible from trimers. Similarly, 9 mers are likely to be the complementary fragments of hexamers released from the 15 mer based on the fact that the relative intensity of 15 mer decreases compared to that in lowenergy SID. Validation of the secondary dissociation requires the collection of SID energy-resolved mass spectra or tandem SID in future work. In addition to verifying AAV topology, SID-CDMS also shows the possibility of the direct examination of VP composition from intact AAVs. The fragments smaller than 15 mer contain predominantly VP3 with different combinations of VPs found in 24 mer , 30 mer , and 45 mer (Table S4). This is consistent with the fact that the VP3s are the most abundant viral proteins with a theoretical percentage of over $83 \%$. The additional sequences in VP2 and VP3 might also provide extra interactions locally, making them harder to cleave.

## - CONCLUSIONS

SID has been shown previously to produce structural details for protein complexes with informative fragments that are not observed in traditional activation methods. CDMS provides a straightforward solution to resolve extensive SID fragments on the Orbitrap platform by directly measuring the charge states of fragments that would otherwise overlap in nominal $\mathrm{m} / \mathrm{z}$ space. In this work, we applied a house-built Python algorithm for plotting the CDMS data ( $w /$ SID) in charge $-m / z$ or mass $-m / z$ to effectively extract meaningful mass information directly from the otherwise unresolved and uninterpretable mass spectra. More importantly, we demonstrate that SID coupled with CDMS is a powerful tool for studying substructure and connectivity, especially for large and heterogeneous complexes. For GroEL, SID-CDMS reflects the stacked-ring structure, which is consistent with previous results on time-of-flight platforms coupled with ion mobility. In addition to the native structure of the 20 S proteasome, multiple layers of structural information are provided in SIDCDMS, including subunit connectivity and proteoform information. For AAV capsids, SID-CDMS suggests that the 60 mer capsid is assembled from 20 VP trimers as the monomer-monomer interface of the trimers has a higher interfacial strength than other interfaces in the capsid. The SID-CDMS integrated approach provides a means to better define the assembly of capsids, perhaps leading to the engineering of more stable vectors. SID-CDMS shows the potential to investigate the viral protein composition of intact capsids and will benefit from future improvements to the accuracy of charge assignment.

## - ASSOCIATED CONTENT

## (5) Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.3c02185.

Additional data analysis parameters; supporting figures of SID-CDMS plots of GroEL with charge-reducing reagents, raw SID-CDMS plots of human 20 S
proteasome, raw CDMS plots of empty AAV8 capsids, and zoomed-in SID-CDMS of human 20S proteasome; supporting tables summarizing the theoretical and measured masses of SID fragments of human 20S proteasome and empty AAV8 capsids (PDF)

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## Author Contributions

C.D. collected and analyzed the CDMS-SID experiments. S.P.C. and B.J.J. designed and wrote the Python-based data processing algorithm. M.M.K. initiated the SID analysis of AAV and helped with the UniDec data processing. J.O.K. assisted in the optimization of the CDMS tuning and raw data processing. V.H.W. conceived and directed the project. The manuscript was written through the contributions of all authors. All authors have given approval of the final version of the manuscript.

## Notes

The authors declare no competing financial interest.

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