Mass Spectrometry of Proteins

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Studying 18 MDa Virus Assemblies with Native Mass Spectrometry**

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Following John Fenn's Nobel Laureate lecture ("Electrospray Wings for Molecular Elephants"),[1] native mass spectrometry (MS) has been developed as a powerful analytical technique to study large noncovalent protein complexes.[2] Using soft nano-electrospray ionization (nESI), these protein complexes can be transferred intact into the gas phase without significant loss of quaternary or tertiary structure. Minor modifications of commercially available MS instruments[3] can greatly improve the transmission of large ions, therefore yielding accurate and precise mass analysis of intact protein complexes. As a result, the stoichiometry of protein complexes can be readily determined in a native MS approach. The study of solution-phase and gas-phase dissociation along with the combination of MS with ion mobility can also yield valuable information on the stability and topology of a protein complex.[4]

Native MS has previously been applied to the study of intact viruses and virus capsids.[5] Apart from their role in viral pathogenesis, capsids are interesting because of their potential applications in nanotechnology and medicine as nanocontainers and nanoreactors and as assembly scaffolds.[6] Native MS enabled the study of intact virus capsids in which (nonnative) cargo encapsulation was quantified and capsid assembly was monitored.[7] These previous studies focused on relatively small capsids with Caspar–Klug triangulation numbers of $T=1$, 3, and 4, ranging up to 10 MDa in mass. Although the 10 MDa norovirus capsids could be detected with MS, their precise mass could not be determined due to a lack of resolution of individual charge states.[7b] Charge-resolved spectra were limited to particles up to roughly 5 MDa, which was hypothesized to be the upper size limit achievable.

Most current native MS studies are carried out on quadrupole time-of-flight (QToF) instruments, operating at higher pressures to facilitate transmission of large ions. The QToF is typically the setup of choice because of the superior sensitivity of the ToF analyzer at high mass-to-charge ratios. Although there is no theoretical upper mass limit for a ToF analyzer, achieving good-quality spectra of assemblies several MDa in size has proven not to be trivial.

We used a modified QToF instrument for the analysis of intact 18 MDa capsids from the bacteriophage HK97. HK97 is an important model system for studying bacteriophage assembly and maturation.[8] In vitro, the $T=7$ capsid assembles from a mixture of pentameric and hexameric capsomers (made of the capsid protein gp5) and the viral protease gp4 to form a first icosahedral intermediate termed Prohead-1. Upon assembly, the encapsidated protease molecules are activated and prime the maturation process that is characterized by a swelling transition yielding the mature capsid (Figure 1a).[9] Overexpression of the capsid protein in the absence of the viral protease results in the formation of a virtually identical icosahedral particle stalled at the Prohead-1 stage (Prohead-1^{gp5}).[10] We report here the analysis by native MS of Prohead-1^{gp5} as well as of the free capsomers (resulting from disassembly induced at high pH), providing for the first time mass spectra with charge-state resolution for a particle of such a size.

Figure 1 shows mass spectra of free capsomers and the Prohead-1^{gp5} particle. The fraction of pentons in the capsomer mixture, judged by the MS signal is 0.25 ± 0.04 (SD, $n=3$) (Figure 1b), which is slightly higher than expected because the capsid consists of 12 pentons and 60 hexons. Partially disassembled complexes and free capsid protein (gp5) monomers also appear in the spectrum, when the capsomer mixture is analyzed under alkaline conditions; this allows us to determine an accurate monomer mass of 42151 ± 1 Da (Figure S1 in the Supporting Information). From the expressed capsid protein sequence, a theoretical mass of 42243 Da was calculated, indicating that the N-terminal methionine is cleaved and that the resulting N-terminus is acetylated (yielding a theoretical mass of 42154 Da). The masses determined for the pentameric and hexameric capsomers are 210807 ± 9 Da and 252973 ± 9 Da, respectively, corresponding to 5.001 and 6.002 times the experimentally determined monomer mass. Prohead-1^{gp5} consists of 420 gp5 copies and its expected mass is thus close to 18 MDa. As illustrated in Figure 1c, the particle could be detected in our mass spectrum despite its tremendous size. Transmission of such large particles could be improved twofold by tuning the "steering" parameter of the mass spectrometer, which controls the angle of the ion beam into the pusher region of the ToF analyzer (Figure S2 in the Supporting Information).

Poor desolvation of large protein complexes often results in poor spectral quality. To improve desolvation and resolve individual charge states, we applied the maximum collision voltage achievable on our instrument (400 V).[11] In ammonium acetate buffer of a typically used ionic strength (50–200 mM) this was still not sufficient to achieve resolution of

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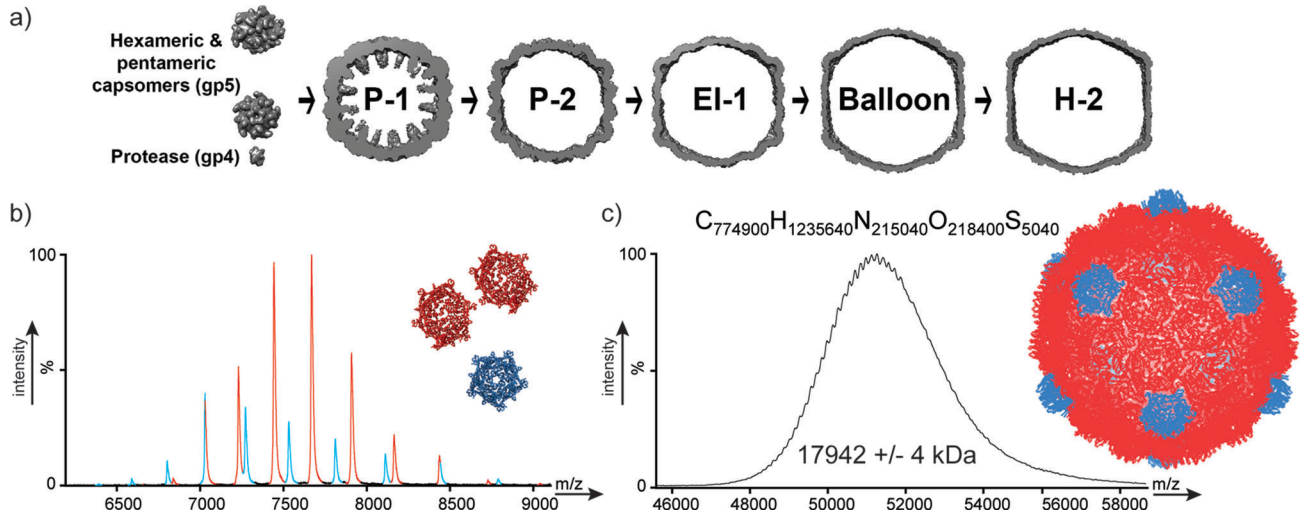

Figure 1. Monitoring the assembly of HK97 capsids with native ESI-MS. a) Assembly and maturation pathway of HK97. b) Free capsomers with penton signal in blue and hexon signal in red. c) Intact Prohead-1 particle. A well-resolved series of charge states is observed allowing the accurate mass calculation.

individual charge states. By combining a decreased buffer ionic strength (12.5 mM) with application of the maximum collision energy of 400 V using xenon as the collision gas, a series of charge states clearly became visible (Figure 1 c). Under these conditions, we achieved a base peak intensity of roughly 10 counts per 2 s scan, such that one spectrum required approximately 50 min of acquisition time.

As originally demonstrated by Fenn and Mann, charge states can be assigned from the mass-to-charge ratios of adjacent peaks in order to calculate a mass.[12] This is trivial for smaller protein complexes; however, the charge state assignment is increasingly ambiguous with larger, poorly desolvated complexes. The strategy employed here for charge state assignment was to systematically test a wide range of assignments and look for one that results in a minimum variation in calculated mass within the same spectrum (see Figure S3 in the Supporting Information). This is essentially the strategy that is employed in the recently described LeastMass software.[13] This procedure was repeated for three independent measurements and the charge state assignments were checked for consistency. This charge state assignment strategy yielded an average mass of 17942 ± 4 kDa across the triplicate experiments. This is a deviation of $+1.3\,\%$ compared to the theoretical mass of Prohead-1^{gp5} (17742 kDa) and we attribute this discrepancy to incomplete desolvation.

This result demonstrates that native MS can be used to study larger systems such as $T = 7$ capsids in the case of HK97. This opens up new avenues for studying a wider variety of capsid structures in their role as pathogens and nanotechnological platforms. This result also confirms that native MS on a QToF instrument has indeed a high upper mass limit for the analysis of protein complexes. As the individual charge states are just resolved in the spectrum of the Prohead-1^{gp5} particle, we now estimate that the upper mass limit is around 20 MDa. To further illustrate that desolvation, and not the instrument hardware, is the limiting factor for protein complex analysis with native MS, we calculated the theoretical peak width of capsomers and Prohead-1^{gp5}, taking into account the inherent isotope distribution of the assembly and the resolution of the mass analyzer.

The inherent width of a charge state in the mass-to-charge ratio dimension is proportional to the width of the isotope distribution and inversely proportional to the number of charges. The isotope distribution is binomial and hence scales to the square root of mass. The number of charges is also expected to scale to the square root of mass, assuming the charged residue model of electrospray ionization.[14] However, here we show that it scales slightly stronger empirically ($mass^{0.55}$), from which it follows that peaks get narrower with increasing size of the complex (see Figure S4 in the Supporting Information). The instrumental resolution of our QToF instrument was empirically determined from the width of singly charged, CsI clusters that do not contain natural isotope variants (see Figure S5). CsI clusters were generated by nESI and the determined resolution is approximately constant across the m/z range at a value of around 7000 at full-width at half-maximum (FWHM) on the QToF instrument employed here.

We compared the experimentally acquired spectra of free capsomers and Prohead-1^{gp5} with the theoretical peak widths defined by either the natural isotope distribution or the instrument resolution (Figure 2.). It is apparent from this comparison that both the capsomers and Prohead-1^{gp5} particle have much broader peaks than predicted based on instrument resolution or natural isotope distribution. The peaks of the capsomers are also clearly asymmetric, a feature that likely originates from superposed binomial distributions of buffer and salt adducts. The experimental resolution, expressed as the mass-to-charge ratio divided by the peak width (FHWM), for capsomers and Prohead-1^{gp5} are approximately 500 and 200, respectively. This difference between the two assemblies demonstrates how desolvation becomes less efficient with increasing size of the complex.

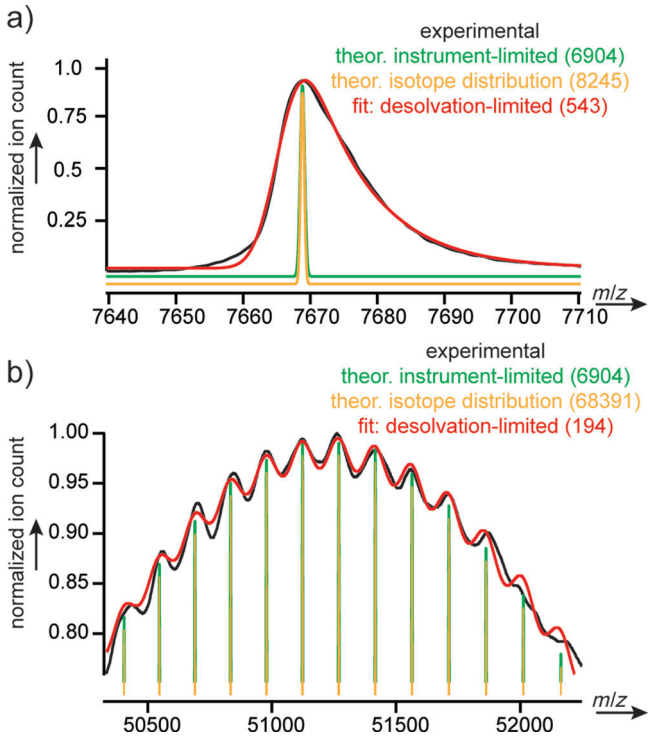

Figure 2. Incomplete desolvation is the limiting factor in attaining narrow peak width in native MS of protein complexes. Experimental peaks of hexons (a) and Prohead-1^{gp5} (b) are compared to theoretical peaks with widths defined by instrument resolution and the natural isotope distribution. The "incomplete desolvation limited" curves are simulated to fit the experimental data (*m*/*z* positions of the peaks are fixed and defined by the experimentally determined mass, the width is fitted to the signal). The numbers in parentheses indicate the effective resolution (FWHM).

These investigations indicate that native MS would benefit from more efficient desolvation. It would greatly increase the effective resolving power of the instrument as well as eliminate any practical upper mass limit other than transmission and detection efficiency (we argue that the superior resolving power of the Orbitrap Exactive platform that was recently adapted for native MS is also primarily attributable to more efficient desolvation).[15] This is illustrated in Figure 3, where we calculated the expected separation between consecutive charge states (as determined from the empirical charging behavior of protein complexes) and compare this with peak width at increasing effective resolution (FWHM). The intersection between these curves is essentially the point at which consecutive charge states are separated at half-maximum intensity. The peak width as determined from the isotope distribution is always smaller than the separation between consecutive charge states. From this it follows that nESI does not impose any inherent upper mass limit on protein assemblies that can be analyzed with charge state resolution using MS. The instrument resolution is not a limiting factor either. Even at the moderate resolution of 7000 exhibited by the modified QToF used in the above-described experiments, the point where peak separation and peak width start to overlap is around 40 GDa. Considering the decrease in effective "resolution" caused by incomplete

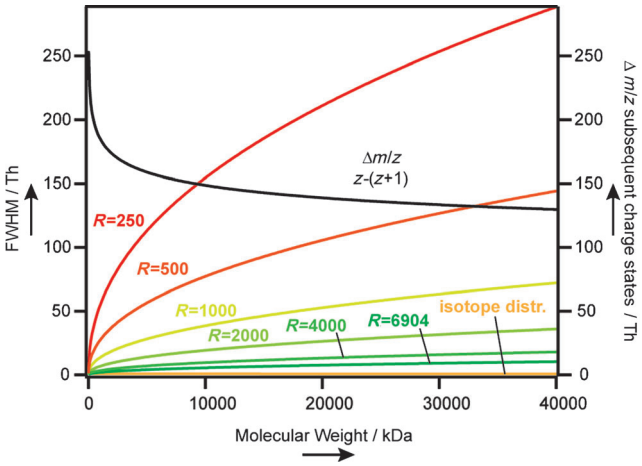

Figure 3. Theoretical upper mass limit of native MS studies of protein complexes as a function of effective resolution. Shown are the separation between subsequent charge states (black line, right-hand *y* axis) and the calculated peak width as a function of molecular weight (colored lines, left-hand *y* axis).

desolvation of the specimen, the limit drops from the GDa to the MDa range.

In conclusion, we demonstrate that native MS can be used to study protein complexes up to 20 MDa with charge state resolution on a QToF instrument. We applied this approach to characterize the first assembly intermediate of the bacteriophage HK97, Prohead-1. These preliminary data will allow the use of native MS to further our understanding of bacteriophage assembly and maturation. Our data also indicate that the performance of current native MS instrumentation could be greatly enhanced when more efficient desolvation can be achieved.

Experimental Section

Free capsomers were analyzed in 50 mM pH 7.4 ammonium acetate buffer, partially denatured capsomers in 50 mM pH 9.5, and intact Prohead-1^{gp5} in 12.5 mM pH 7.4. Aliquots of 1–2 μL were loaded into gold-coated borosilicate capillaries, prepared in-house, for nano-electrospray ionization. Samples were analyzed on a modified QToF II instrument optimized for high-mass protein analysis. The measurements were performed with xenon as the collision gas. The "steering" parameter was adjusted for Prohead-1^{gp5}, which resulted in better transmission of these large ions. The source conditions for both capsomers and Prohead-1^{gp5} were as follows: capillary 1300–1500 V, cone 160 V, extraction cone 0 V, and 10 mbar backing pressure. The pressure in the collision cell was 2×10^{-2} mbar, 60 V collision energy was applied to free capsomers, and 400 V to Prohead-1^{gp5}. Additional details on data analysis are available in the Supporting Information.

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