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Mass spectrometry-based studies of virus assembly

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The assembly of exact numbers of protein monomers into the distinct architectures of virus capsids has long been of intrigue. Despite the diseases associated with viruses, there is a paucity of anti-viral therapies; however, mapping virus capsid assembly at the molecular level may lead to the development of more therapeutics.

Native mass spectrometry is a powerful, versatile tool with which to monitor biomolecular assembly pathways and identify key intermediates. Recent highlights in this field in terms of MDa mass measurements, identification of capsid intermediates, and the effect of external parameters on assembly are discussed. Examples from ion mobility spectrometry-mass spectrometry, charge detection mass spectrometry, and gas-phase electrophoretic molecular analysis research are presented.

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Introduction

It was towards the end of the 19th century that the first virus, tobacco mosaic virus, was identified [1] and since then these complex and fascinating structures have been under continual investigation. Biologically, a virus can be described as a hostile invader which attacks living cells and coerces its unhappy hosts into becoming a production factory generating many replicate copies of the invading virus. Architecturally, the virus contains genetic material (RNA or DNA) protected within a protein shell, or capsid, which is released on making contact with the host cell. The consequences can range from unpleasant to fatal and currently there are few therapeutics to combat viruses.

The capsids associated with each virus are identical and represent a feat of nano-engineering, each capsid containing a specific number of protein monomers assembled in a defined 3D structure. Many such structures conform to the rules of icosahedral symmetry, being composed of fixed numbers of protein monomers for example 60, 120, 180, 240, and so on, corresponding to $T = 1, 2, 3, 4$, and so on, respectively, where T is the triangulation number defining the number of monomers (in steps of 60) per capsid [2].

Mass spectrometry (MS) is now a firmly established technique for the study of biomolecules and their complexes [3,4•,5,6•,7]. Using electrospray ionisation-ion mobility spectrometry-mass spectrometry (ESI-IMS-MS), non-covalent complexes can be maintained intact in the gas-phase and their masses measured, their composition and stoichiometries assessed using tandem MS dissociation techniques, and their shapes estimated in terms of their collision cross-sectional areas (CCSs) [4•,8–10]: all in a single experiment. The speed of these analyses allows the assembly and disassembly of biomolecular complexes to be monitored in real-time, and individual components to be identified from within these heterogeneous mixtures. Two earlier reviews have presented detailed accounts on the use of mass spectrometry applied to virus assembly [11,12]; this article focuses on selected highlights of recent literature.

Electrospray ionisation-ion mobility spectrometry-mass spectrometry

Until recently, ESI-IMS-MS employing instrumentation of quadrupole-time of flight (Q-Tof) geometry has been the most frequently used MS-based technique for analysing macro-biomolecules and monitoring their assembly processes, on account of its high mass capabilities, high sensitivity, in-built IMS 'shape sorter' separative powers, and commercial availability.

For example, the *in vitro* assembly pathway of the MS2 bacteriophage has been monitored, from the non-covalently bound homo-dimer of the coat protein (monomeric mass 13.7 kDa) and RNA starting materials into the intact $T = 3$ icosahedral capsid containing 180 copies of the coat protein monomer [13]. During capsid assembly, two distinct, transient, on-pathway intermediates were identified with masses of 182.9 and 304.8 kDa. The stoichiometries of the intermediates were determined from their mass measurements and confirmed by repeating the assembly reaction using isotopically labelled protein [14]. These data, together with the corresponding ESI-IMS-MS derived CCSs, were used to model potential

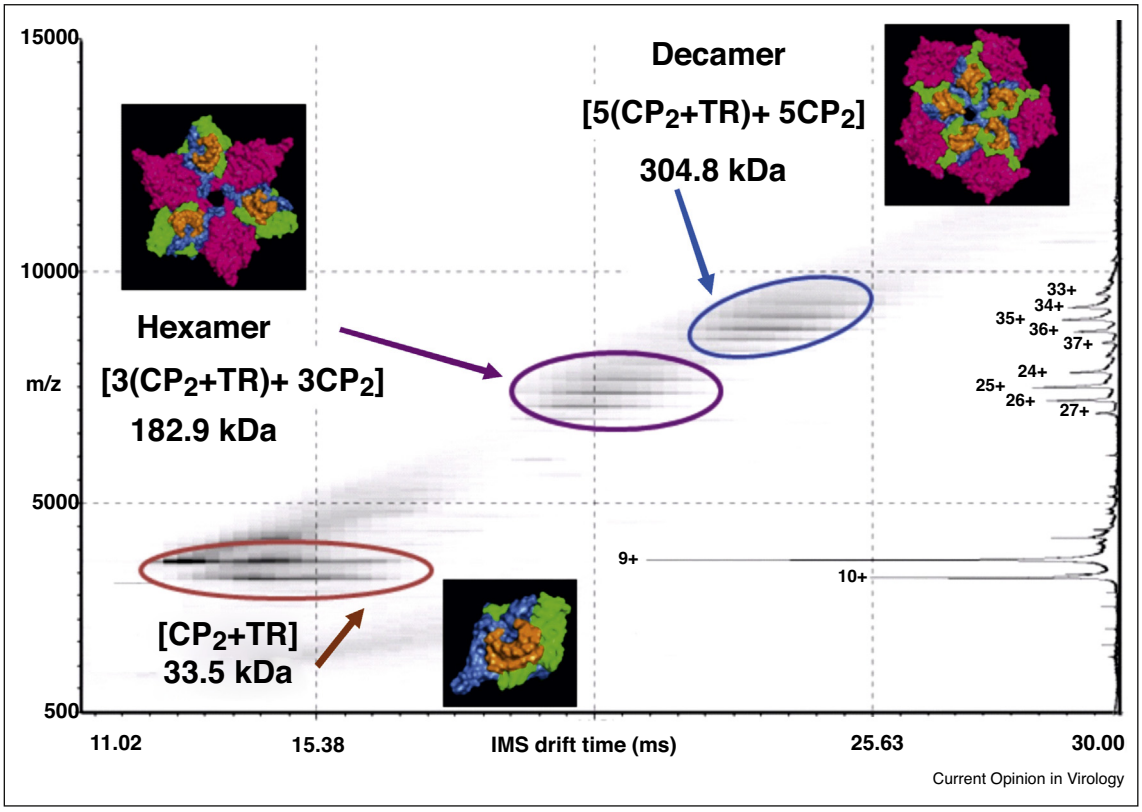
structures from which the architectures of the two intermediates (one hexameric, the other decameric) were ascertained. Using ESI-MS-collision induced dissociation (CID)-IMS-MS, the structures were postulated as being key building blocks for the threefold and fivefold axes of symmetry found in icosahedral capsids [13] (Figure 1).

Human norovirus has been studied in some depth by Heck and co-workers [15,16,17•]. Human norovirus is a single-stranded RNA virus with a $T = 3$ icosahedral capsid formed from 90 homo-dimers of the VP1 coat protein (monomeric mass 56 kDa). One outstanding example of this work focussed on a particular norovirus strain, namely the Norwalk virus, and ESI-IMS-MS was used to study the *in vitro* assembly and stability [15]. At pH 7, the mass spectrum showed a broad distribution of ions indicative of the intact capsid (mass ca 10.1 MDa) (Figure 2, $VP1_{180}$). The stability of the capsids was investigated under a range of pH conditions; for example, at pH 9 capsid disassembly was observed and a variety of VP1 oligomers detected, the most populated of which was the VP1 60-mer (mass *ca* 3.4 MDa) (Figure 2, $VP1_{60}$). These observations indicated that the intact capsids had disassembled, predominantly into VP1 dimers, the latter which then re-formed to produce $T = 1$ capsids. Spherical geometries for both the $T = 3$ (VPI_{180}) and $T = 1$ (VPI_{60}) capsids were proposed by use of atomic force microscopy (AFM).

More recently, two other variants of the human norovirus, West Chester GI.1 and GII.17, have been compared to the Norwalk strain using ESI-IMS-MS [18••]. In addition to the $T = 3$ capsids, both West Chester variants form smaller $T = 1$ capsids under certain conditions. The ESI–MS spectrum of GI.1 and GII.17 contained a broad distribution of ions centered at m/z 40 000 consistent with intact $T = 3$ capsids containing 180 copies of the coat protein monomer, as well as a further ion distribution at m/z 26 000 (indicating the presence of $T = 1$, 60-mer capsids), an intermediate signal consistent with 80 copies of the coat protein monomer, and a range of smaller oligomers.

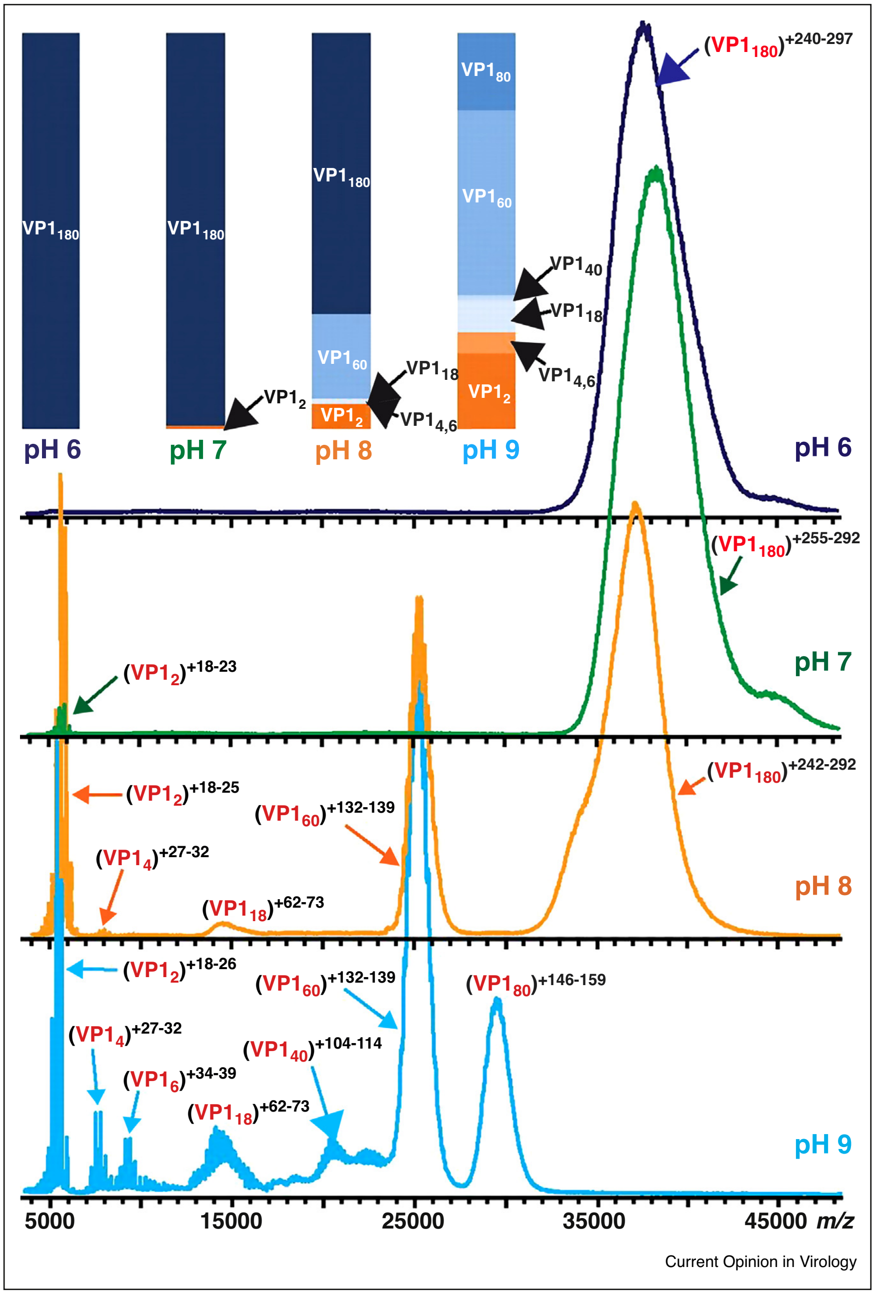
In a quest to discover cellular receptors of viral proteins, the binding properties of human noroviruses with

Figure 1



Monitoring MS2 $T = 3$ capsid assembly using ESI-IMS-MS. Driftscope plot (drift time (ms) versus m/z versus intensity) showing the initiation complex ($(CP_2 + TR)$, 33.5 kDa) together with two key intermediates: the hexamer ($(3(CP_2+TR)+3CP_2)$, 182.9 kDa) and the decamer ($(5(CP_2+TR)+5CP_2)$, 304.8 kDa), where CP = coat protein and TR = RNA assembly initiator. The models were constructed from the MS2 co-ordinates (PDB 2MS2).
Figure modified from [13] (permission not required).

Figure 2



Stability of rNVLPs (recombinant norovirus-like particles) over a pH range of 6–9 monitored by native ESI–MS. Mass spectra were obtained from aqueous solutions of the rNVLPs (30 μM VP1) in a 250 mM ammonium acetate buffer at the pH values indicated. The summed intensities for all observed VP1 oligomers at each pH are summarized in the bar graphs in the upper left corner.
(Figure 2; reproduced with permission from The American Society for Biochemistry and Molecular Biology [15]).

oligosaccharides have been quantified using ESI–MS [19] and interactions of the Tulane virus with sialic acids investigated [20].

The capsid assembly and interactions of Hepatitis B virus (HBV) have also been under scrutiny [16,21,22]. HBV capsids enclose a DNA genome and differ from most viruses as the monomer forms two distinct capsid morphologies: both $T=3$ (3 MDa) and $T=4$ (4 MDa) icosahedral structures. Using ESI-IMS-MS, both types of spherical capsids were detected, together with lower order oligomers ranging from dimers to 28-mers [16]. The

CCS measurements for these oligomers indicated a sheet-like structure, in comparison with the less compact measurements obtained for a range of globular proteins of similar molecular mass. From these data, the authors proposed that the capsid assembly process begins with a nucleus exhibiting a five-fold axis of symmetry which is augmented by the addition of coat protein dimers until the final capsid structure is completed.

HBV infection of humans triggers the production of antibodies directed against the invading coat protein. With this in mind, *in vitro* binding of the antigen-binding domain of monoclonal antibodies to HBV capsids has been investigated using hydrogen deuterium exchange (HDX)-ESI-MS [21]. The antigen-binding domains under scrutiny were Fabs E1 and 3120. Firstly, following in-line HDX, the HBV capsids underwent pepsin proteolysis *en route* to LC–MS/MS analysis of the resulting peptides, which produced 98% sequence coverage of the coat protein. The experiment was repeated in the presence of the two antibodies. Fab E1 was found to induce conformational changes in four regions of the capsid; in each case, increased HDX protection was observed in the presence of Fab E1, thus indicating a lessening of the conformational flexibility of the HBV capsid. Fab 3120 produced similar results.

The interaction of Fab 3120 with intact HBV capsids was explored further when it was shown that increasing the ratio of antibody to capsid resulted in changes to the mass spectrum, which typically shows signals consistent with the $T = 3$ and $T = 4$ capsids. The two charge state distributions centered at m/z 23 364 and 27 842, indicating $T = 3$ and $T = 4$ capsids of 3 and 4 MDa, respectively, were replaced with two distributions of ions at much higher m/z, indicating capsid-antibody binding. The $T = 3$ capsids decorated with Fab 3120 were estimated to have an average mass of 5.35 MDa (indicating an average of 49 bound Fabs), and the corresponding $T = 4$ capsids an average mass of 7.98 MDa (i.e. an average of 83 bound Fabs) [22].

The effect of the conformational properties of the HBV coat protein homo-dimer on capsid assembly has been studied [23,24]. In a similar vein, a comparison of assembly intermediates originating from the similar N protein monomers of the Bunyamwera and the Schmallenberg viruses, which form unique ranges of oligomers on binding RNA, has been made [25].

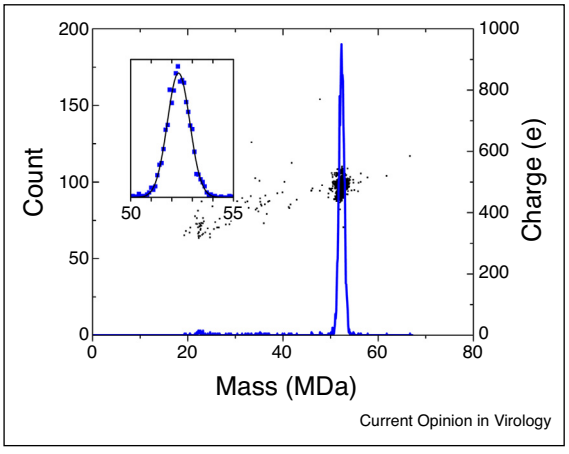
HK97, a double-stranded DNA $T = 7$ bacteriophage which assembles from a mixture of two different coat proteins, has also been investigated using ESI–MS [26,27]. The *in vitro* protein assembly process produces an icosahedral intermediate named Prohead-1, a physical enlargement of which generates the mature capsid. The molecular mass of Prohead-1 was measured as 17.942 MDa using a Q-Tof mass spectrometer.

More recently, the Orbitrap mass spectrometer [28] has emerged as a powerful high mass, high sensitivity instrument. The Orbitrap traps ions (typically ESI-generated) as they spin in an orbit around a spindle-like electrode and the current from the trapped ions is converted into a mass spectrum using Fourier transform technology. The mass analyses of cow pea chlorotic mottle virus (4.5 MDa), adeno-associated virus serotype 1 (3.7 MDa), and adenovirus dodecahedron (3.5 MDa) have all been reported with standard deviations of only ca 0.013% [29]. A comparison of Q-Tof and Orbitrap mass spectrometers has been made for two related viruses: Brome Mosaic virus (BMV) and Cowpea Chlorotic Mottle virus (CCMV). The masses of the $T = 3$ capsids of both viruses, which contain single-stranded RNA, are ca 4.6 MDa, which includes ca 1 MDa for the genomic content. BMV and CCMV are both produced as mixtures of three particles carrying different segments of the genome, varying by approximately 0.1 MDa in mass. Despite their large size and heterogeneity, native mass spectra with resolved series of charge states ions were obtained for both BMV and CCMV [30].

Charge detection mass spectrometry

One technique which has had a recent and high impact on the analysis of large biomolecular structures is that of charge detection mass spectrometry (CDMS) [31–33,34•]. Rather than just measuring the mass-to-charge ratio (m/z) of the analyte ions, CDMS is a single-particle technique where the mass of an ion (generated, for example, by ESI)

Figure 3

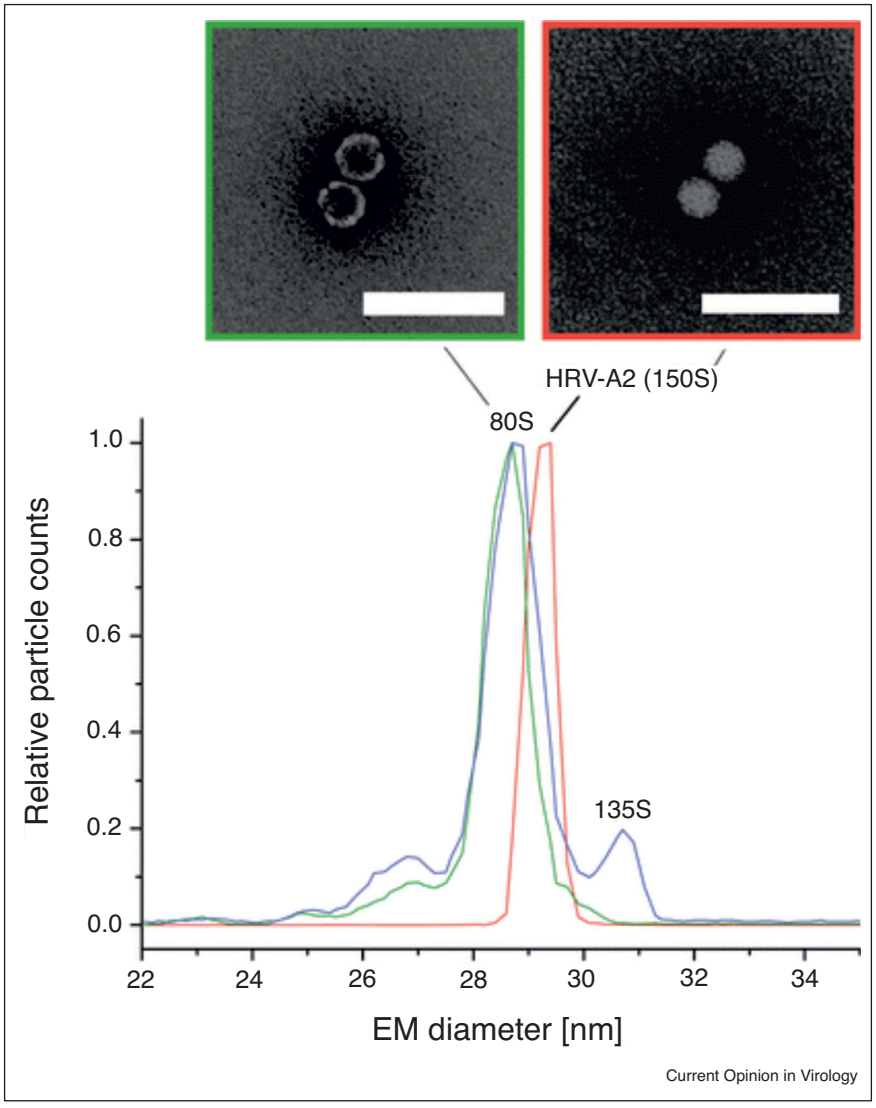


Mass spectrum of phage P22 obtained by use of CDMS. The spectrum is a histogram constructed with 100 kDa bins. The inset shows an expanded view of the peak at around 52 MDa. In the inset, the blue points are the measured values and the black line is a Gaussian fit to the experimental data. The black points overlaying the main spectrum are a charge versus mass scatter plot showing that the average charge of the phage is about 490 e.
(Figure 3; reproduced with permission from John Wiley & Sons [36••]).

is determined directly from the simultaneous m/z and z measurements of that ion [31].

Recent instrumental developments have led to detailed investigations on HBV using CDMS [32,33,34•]. Jarrold *et al.* assembled HBV capsids *in vitro* and generated a spectrum showing peaks consistent with both $T=3$ and $T=4$ capsids of masses 3.0 and 4.0 MDa, respectively, together with minor peaks of 3.5 and 3.7 MDa, consistent with assembly intermediates trapped under the high salt conditions used for the assembly process [34•]. Under milder assembly conditions, these intermediates reformed over a few days, leaving a spectrum indicating just the two intact capsids. These observations were consistent with transmission electron microscopy (TEM) results, which indicated the presence of incomplete $T=4$ capsids together with both $T=3$ and $T=4$ capsids. Early intermediates in the HBV capsid assembly process, including oligomers up to a pentamer of the coat protein dimers and a larger species of 1.1 MDa (ca 32 dimers) have also been identified using CDMS. Interestingly, the *in vitro* assembly of HBV capsids was found to follow different pathways depending on the precise salt conditions and protein monomer concentration [33]. Recently, it has been shown that the initial assembly process can produce overgrown particles with more a small number of extra subunits, which self-correct to a perfect $T=3$ capsid [35].

Figure 4



GEMMA analysis of viral and subviral particles on a custom-built instrument with high peak resolution allows for the separation of intact virions and sub viral A-particles and empty B-particles. Spectra of three different samples containing mostly native virions (red), a substantial fraction of A-particles (blue), and mostly B-particles (green) are shown. The insets show TEM images of corresponding particles (100 nm size bar). (Figure 3, reproduced from [40••], American Chemical Society; permission not required).

In terms of mass measurements of large biomolecular complexes, the CDMS analysis of the intact bacteriophage P22 is a highlight [36••]. P22 is a multi-component (containing a total of 521 copies of nine different proteins), tailed, double-stranded DNA phage with a theoretical mass of 51.6 MDa (although this stoichiometry is not completely proven) (Figure 3). Remarkably, a mass of 52.2 Da was measured using CDMS. The width of the P22 peak (although only ~0.7 MDa) was attributed to the presence of variable quantities of the DNA packaged within the phage.

With regard to P22 capsid assembly, CDMS has been employed to monitor the assembly products of two P22 coat protein variants: A285 T and A285Y [37]. P22 pro-capsids of $T = 7$ icosahedral symmetry and mass ca 24 MDa [38] assemble from the coat protein aided by a chaperone scaffolding protein, which exits the pro-capsids during their maturation into full capsids. Coat protein substitutions at position 285 are known to change the pro-capsid assembly pathway, and this study indicated that A285 T formed $T = 4$ and $T = 7$ pro-capsids (ca 12 and 25 MDa, respectively), whilst A285Y formed $T = 3$ and $T = 4$ pro-capsids (ca 8 and 12 MDa, respectively).

The woodchuck hepatitis virus (WHV) is closely related to HBV, the two viruses having some 65% sequence identity in their respective coat protein building blocks. Cryo-electron microscopy has shown both viruses to be predominantly $T = 4$ capsids ca 4 MDa in mass [39]. This observation was supported by CDMS analyses, which also indicated that the ca 5% of $T = 3$ capsids detected for HBV are extremely rare in the case of WHV.

Gas-phase electrophoretic mobility molecular analysis

Gas-phase electrophoretic mobility molecular analysis (GEMMA) is another approach which has been used successfully to measure the masses of virus capsids [40••,41–44]. GEMMA employs electrospray-generated ions which are charge-reduced using a ^{210}Po α-particle source to produce singly charged ions. Separation of these singly charged ions is achieved by use of a differential mobility analyser which sorts ions according to their electrophoretic mobility (EM) diameters and generates a particle size distribution pattern [45]. Using calibration standards of known mass, the mass of an analyte ion can be determined from its EM diameter. For example, the tick-borne encephalitis virus (TBEV), which is transmitted via ticks to humans, was analysed using this methodology and found to have an EM diameter of 46.8 ± 1.1 nm, which equated to a mass of 19.5 MDa. These findings were supported by TEM and AFM data [40••].

Human rhinovirus serotype 2 (HRV-A2), an icosahedral virus composed of 60 copies each of four viral proteins (VP1-4) encapsulating single-stranded RNA, has also been studied using GEMMA [40••]. As the RNA is transferred from the protein capsid into the infected cell, the capsid undergoes conformational changes which result in the production of sub viral particles, namely the A-particle which has lost VP4 but retains the RNA genome, and the nucleic acid-void capsid, the B-particle. After comprehensive purification of the virus, GEMMA was used to measure an EM diameter of 29.3 nm, which was extrapolated to produce a mass of 7.9 MDa, less than 1% difference from the theoretical mass of 8.0 MDa (Figure 4). *in vitro* release of the RNA was achieved by applying heat and the subsequent decline of the intact virus was monitored in addition to the emergence of the A-particles (30.6 nm EM diameter; these species are known to be ~4% expanded compared with the virion) and empty B-particles (28.7 nm EM diameter). The assignments of these components were confirmed by use of TEM, which produced images clearly differentiating between RNA-filled and empty capsids (Figure 4).

Conclusion

Over the past few years, high quality mass spectrometric measurements of intact, non-covalently bound, mega-Dalton virus capsids have become possible, which would have been proclaimed unachievable two decades ago. This is not to say that such measurements are easy. Both meticulous sample preparation and careful instrumental optimisation are required; even so, the presence of unspecified amounts of RNA or DNA can broaden peaks in the spectrum and eliminate the possibility of a useful mass measurement.

Not only can intact capsids be mass measured but also their assembly pathways can be monitored *in vitro* in real-time, and key on-pathway intermediates, often distinct icosahedral building blocks which dictate the unique axes of capsid symmetry, identified. Differences in assembly pathways as a result of changing the experimental conditions (e.g. pH and/or buffer solutions) and by using protein variants have been mapped in detail.

Armed with these data and exciting new technologies, the future looks promising. If effective anti-viral therapeutics are to be designed, it is vital to understand at the molecular level how functional biomolecular complexes are formed from their individual components. Not only does mass spectrometry show great promise in this area, it can also be employed as a screening tool for inhibitors of virus capsid assembly and, if used in conjunction with chemical labelling and cross-linking techniques, to probe the precise location of capsid:small molecule and capsid:antibody interactions.

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