Supporting Information to:

Defining the stoichiometry and cargo load of viral and bacterial nanoparticles by Orbitrap mass spectrometry.

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Limiting factors in transmission efficiency of high mass ions.

Before setting out to make further modifications, in order to extend the accessible mass range beyond what we reported previously, we investigated which factors would influence high mass ion transmission and detection on the Orbitrap mass analyzer. As the effective potential well in the Orbitrap analyzer is independent of mass, there is no fundamental limit on m/z to be detected. However, ion transport from the ion source as well as trapping of ions prior to injection into the analyzer, utilizes RF-only multipoles, which provide focusing in highly m/z-dependent RF quasi-potential wells. For example, in a quadrupole of inscribed radius \( r_0 \) with an RF voltage of amplitude \( V_{RF} \) applied to the rods (0-peak), the quasi-potential (in Volts) is given by

\[
\phi(r) \approx \frac{eV_{RF}^2}{\left(\frac{m}{z}\right)(2\pi)^2 r_0^2} \left(\frac{r}{r_0}\right)^2
\]

and therefore is inversely proportional to m/z. A similar formula is also applicable for other types of RF-only devices, such as multipoles, ion tunnels, funnels, carpets, etc.- just the value of \( r_0 \) changes. For typical \( f \approx 3.2 \) MHz, \( r_0 \approx 2.87 \) mm (which corresponds to the standard C-trap settings in this instrument), \( V_{RF} = 500 \) V (0-p), m/z = 10000, the RF pseudo-potential wall that prevents the ions from impinging upon the quadrupole electrodes, amounts approximately to 0.7 V. Therefore, any process that supplies ions with radial energy in excess of this amount will simultaneously doom these ions to be lost for analysis. For example, if thermalized ions (with a radial kinetic energy at room temperature of about \( kT \approx 0.025 \) eV) enter as a parallel beam of radius \( R \) into an ion-optical lens with focal distance \( F \) followed by an RF-multipole, then after the focal point they will form a beam with angular divergence \( \gamma = R/F \). If \( R \) is large enough so that for a final acceleration \( U \) of ions into a multipole

\[
\phi(r_0) < U \cdot \left(\frac{R}{F}\right)^2
\]

then such ions are lost from the multipole. Even for slightly smaller \( R \), with the beam approaching the RF rods, efficiency of ion transport is already drastically reduced. Typically, such lenses are just thin apertures that are used to separate differentially pumped regions and have RF-multipoles on one or both sides. Generally, we could represent their focal distance as

\[
F = r_0 \cdot C(U_0, U)
\]

where \( U_0 \) is the ion energy per elementary charge prior to the lens and coefficient \( C \) is determined by the particular geometry. Typically, \( U_0 \ll U \) and then \( C \approx C(U) \).

The most efficient countermeasure to loss of ions would be to establish a sufficiently high pressure of residual gas in the corresponding multipole, so that any excess radial energy is damped in collisions, preferably over the length comparable to or smaller than \( F \). In addition to this, an
increase of RF amplitude or a decrease of frequency or radius of the multipole could be used for more efficient focusing of ions. It should be noted that an excessive increase of pressure becomes detrimental for RF focusing. Substantial damping by collisions takes place in the injection flatapole of the instrument used in this work (Fig. 1), and collisional cooling is also the likely reason why the pressure in the HCD cell needs to be raised to >0.1 mbar for trapping of high-mass ions and their fragments (see below). However, the pressure remains significantly lower in other parts of the instrument: i.e. the bent flatapole, transport octapole and the C-trap. While the pressure in the first two RF multipoles could be elevated relatively easily, it would be more difficult for the C-trap because it directly relates to the residual pressure in the Orbitrap analyzer (with a coefficient of approximately 3*10^-6) and the stopping length always exceeds $F$ in the C-trap.

To estimate the limit on $m/z$, the best-case assumption for an incoming ion beam would include its complete thermalization, so that most of the beam ($\approx 68\%$) is contained within radius $R_T$ where

$$\phi(R_T) \approx kT$$  \hspace{1cm} (4)

Substituting $R_T$ from (4) into (2)-(3), we obtain the upper limit for transmitted $m/z$:

$$\left( \frac{m}{z} \right)_{\text{max}} \leq \frac{C(U_0,U)}{(2\pi f)^2 r_0^2 \sqrt{U \cdot kT}} e^{V_{RF}^2 \ell^2 / (2U)}$$  \hspace{1cm} (5)

To estimate the $m/z$ limit for the C-trap, we need to take into account that capture of ions into the trap is accompanied by reflection in the retarding DC potential created by end apertures on both sides of the RF rods. Penetration of the DC potential into a multipole (see Supplementary Figure S1) depends on the distance from the aperture as $\exp(\pm x/h)$ where $1/h^2$ is the eigenvalue of the Laplace operator in the multipole’s cross-section. It could be shown that at a sufficient distance from the aperture (of about $r_0$) the focal distance of the exponential retarding field depends weakly on the ion energy and is $F\approx0.41\ h=0.2\ r_0$. Therefore, for this field it holds that $C(U,U_0)=0.2$. For a maximum RF voltage of $V_{RF}=1500\ V$ (0-p) and a residual energy from the original jet expansion corresponding to 5-8 V acceleration, this corresponds to $(m/z)_{\text{max}}\approx29000-36000$, which roughly fits with the highest $m/z$ experimentally observed so far in this mass spectrometer (see below). Due to the spread of initial velocities and coordinates, this cut-off is never sharp and well-defined, but rather manifests itself as an increasingly unstable and intensity-suppressed signal at higher $m/z$.

For acceleration lenses in the bent flatapole, transport octapole and on the entrance to the HCD cell, the focal distance $F$ is much greater, of the order of $1.5\ r_0$ or higher and therefore even lower RF amplitudes are sufficient to retain ions of such $m/z$ at energies up to 10-20 V. However, ions do need to lose almost all their kinetic energy by the time they reach the back of the HCD cell to avoid catastrophic scattering in the exponential retarding field at the turning point. Indeed, formula (5) could be re-written as a limit on maximum kinetic energy of ions as they approach the end of a multipole:
\[ U_{\text{max}} \leq \frac{C(U_0, U)}{(2\pi f)^4} \frac{e^2V_{RF}^4}{kT \cdot (m/z)^2} \]  

(6)

i.e. a change of frequency from e.g. 3.2 MHz to 2.8 MHz increases the allowed energy by a factor 1.7x, while in the exponential retarding field it plummets by a factor of 50x relatively to transmission lenses (see Supplementary Figure S2).

**SI experimental procedures**

The RF frequency of the injection flatapole, bent flatapole, transport octapole, C-trap and HCD cell were lowered by 20-25%. In particular, the RF frequency of the C-trap was lowered from 3.2 MHz to 2.8 MHz, which, according to Supplementary equation 5, should lead to an extension of the upper mass limit from 29000-36000 to 37000-47000 \( m/z \), and generally improve transmission of ions at high \( m/z \).

Tuning of some crucial instrument parameters was necessary for the analysis of mega Dalton assemblies. Mainly, whereas the instrument is normally operated at a source DC offset of 25 V, this did not allow transmission of the tested virus particles. Instead, no offset (0 V) resulted in base peak intensities of \( \sim 10^5 \) at 100 ms injection time. In addition, the required high xenon pressure in the HCD cell for efficient transmission of the high mass ions resulted in elevated pressures in the Orbitrap compartment as well (\( 10^{-9} \) mbar, compared to \( 10^{-11} \) in normal operating mode). This resulted in rather unstable and dampened transients, which gave rise to noisy spectra at longer transient times. Therefore, transient times were adjusted according to S/N to either 32 or 16 ms (compared to 64-256 ms in normal Orbitrap operating mode). For the same reason, transients of the same acquisition were averaged before FT as microscans, rather than combining spectra after FT. It should be noted that ions at high \( m/z \) are close in frequency (~50 kHz) to the high-pass filter of the pre-amplifier of the image current detection. To prevent erroneous eFT calibration at high \( m/z \), the pre-amplifier was replaced with a version equipped with a 22 kHz high-pass filter.
**SI Tables**

**Supplementary Table S1.** Peak assignments for HCD spectra of GroEL.

| m/z     | FWHM | M/ΔM, FWHM | z  | mass (Da) |
|---------|------|-------------|----|-----------|
| 10539.5 | 7.1  | 1478        | 76 | 800928    |
| 10679.9 | 7.8  | 1363        | 75 | 800921    |
| 10822.5 | 7.6  | 1433        | 74 | 800792    |
| 10973.4 | 7.4  | 1479        | 73 | 800982    |
| 11124.3 | 7.4  | 1495        | 72 | 800878    |
| 11281.0 | 8.1  | 1392        | 71 | 800882    |
| 11442.0 | 6.5  | 1760        | 70 | 800871    |

|                  | average | standard deviation |
|------------------|---------|--------------------|
|                  | 800893  | 59                 |
| m/z     | FWHM | M/ΔM, FWHM | z  | mass (Da) |
|---------|------|-------------|----|-----------|
| 13047.1 | 8.7  | 1501        | 57 | 743628    |
| 13278.9 | 9.2  | 1451        | 56 | 743562    |
| 13522.3 | 9.5  | 1422        | 55 | 743674    |
| 13772.2 | 10.3 | 1332        | 54 | 743647    |
| 14032.5 | 9.8  | 1427        | 53 | 743670    |
| 14302.0 | 10.1 | 1414        | 52 | 743651    |
| 14583.0 | 10.2 | 1426        | 51 | 743683    |
| 14874.8 | 10.5 | 1422        | 50 | 743692    |
| 15179.1 | 10.9 | 1391        | 49 | 743727    |
| 15494.8 | 11.6 | 1335        | 48 | 743702    |
| 15825.0 | 11.8 | 1335        | 47 | 743726    |
| 16168.5 | 12.2 | 1329        | 46 | 743705    |
| 16527.8 | 12.5 | 1324        | 45 | 743705    |
| 16904.3 | 12.3 | 1374        | 44 | 743745    |
| 17297.0 | 12.8 | 1347        | 43 | 743728    |
| 17709.4 | 13.0 | 1361        | 42 | 743754    |
| 18141.9 | 13.3 | 1360        | 41 | 743778    |
| 18595.4 | 13.9 | 1341        | 40 | 743777    |
| 19071.8 | 14.4 | 1325        | 39 | 743760    |
| 19574.8 | 14.5 | 1351        | 38 | 743804    |
| 20102.9 | 16.0 | 1258        | 37 | 743770    |
| 20662.3 | 16.1 | 1281        | 36 | 743808    |
| 21256.4 | 19.8 | 1074        | 35 | 743939    |

| average | standard deviation |
|---------|-------------------|
| 743723  | 76                |
| m/z     | FWHM | $M/\Delta M$, FWHM | z  | mass (Da) |
|---------|------|---------------------|----|-----------|
| 19612.8 | 16.7 | 1171                | 35 | 686414    |
| 20190.0 | 17.4 | 1160                | 34 | 686427    |
| 20802.2 | 18.6 | 1120                | 33 | 686439    |
| 21453.6 | 18.3 | 1172                | 32 | 686484    |
| 22144.3 | 18.8 | 1175                | 31 | 686442    |
| 22883.2 | 20.2 | 1135                | 30 | 686466    |
| 23671.8 | 21.2 | 1119                | 29 | 686453    |
| 24516.7 | 22.0 | 1116                | 28 | 686439    |
| 25425.4 | 24.0 | 1058                | 27 | 686458    |
| 26403.2 | 25.3 | 1042                | 26 | 686456    |
| 27460.3 | 27.5 | 997                 | 25 | 686481    |
| 28606.6 | 28.9 | 989                 | 24 | 686533    |
| 29850.4 | 30.3 | 984                 | 23 | 686535    |
| 31208.5 | 31.4 | 993                 | 22 | 686566    |
| 32693.2 | 34.1 | 958                 | 21 | 686536    |
| 34327.8 | 36.1 | 952                 | 20 | 686536    |
| 36134.3 | 36.5 | 991                 | 19 | 686533    |
| 38143.8 | 40.9 | 934                 | 18 | 686571    |

| average | standard deviation |
|---------|---------------------|
| 686487  | 51                  |
### Supplementary Table S2. Peak assignments of encapsulin, Dd, AAV1 and CCMV spectra.

| m/z     | FWHM | $M/\Delta M$, FWHM | $z$ | mass (Da) |
|---------|------|---------------------|-----|-----------|
| 16144.1 | 46.3 | 349                 | 127 | 2050179   |
| 16277.2 | 46.5 | 350                 | 126 | 2050800   |
| 16404.6 | 44.4 | 369                 | 125 | 2050448   |
| 16540.0 | 43.4 | 381                 | 124 | 2050836   |
| 16675.3 | 41.8 | 399                 | 123 | 2050944   |
| 16813.6 | 40.4 | 416                 | 122 | 2051136   |
| 16951.3 | 39.6 | 428                 | 121 | 2050990   |
| 17092.1 | 39.5 | 433                 | 120 | 2050931   |
| 17235.2 | 39.1 | 440                 | 119 | 2050872   |
| 17378.7 | 39.6 | 438                 | 118 | 2050565   |
| 17522.7 | 40.7 | 431                 | 117 | 2050033   |
| 17666.6 | 42.4 | 417                 | 116 | 2049211   |
| 17814.8 | 42.7 | 417                 | 115 | 2048587   |

| average | standard deviation |
|---------|---------------------|
| 2050425 | 761                 |
| m/z     | FWHM | $M/\Delta M, \text{FWHM}$ | $z$  | mass (Da)  |
|---------|------|------------------------|------|------------|
| 20038.2 | 43.7 | 459                   | 176  | 3526545    |
| 20149.5 | 40.8 | 493                   | 175  | 3525989    |
| 20263.2 | 47.9 | 423                   | 174  | 3525623    |
| 20382.3 | 45.7 | 446                   | 173  | 3525956    |
| 20501.1 | 45.2 | 454                   | 172  | 3526009    |
| 20623.5 | 49.1 | 420                   | 171  | 3526453    |
| 20745.0 | 48.3 | 429                   | 170  | 3526480    |
| 20868.7 | 48.9 | 426                   | 169  | 3526636    |
| 20992.9 | 48.0 | 437                   | 168  | 3526639    |
| 21122.1 | 50.9 | 415                   | 167  | 3527229    |
| 21246.6 | 50.9 | 417                   | 166  | 3526776    |
| 21377.3 | 49.6 | 431                   | 165  | 3527093    |
| 21509.8 | 54.1 | 397                   | 164  | 3527446    |
| 21643.9 | 51.4 | 421                   | 163  | 3527794    |
| 21779.3 | 54.2 | 402                   | 162  | 3528077    |
| 21920.3 | 48.4 | 453                   | 161  | 3529009    |
| 22056.0 | 51.2 | 431                   | 160  | 3528803    |
| 22200.9 | 55.1 | 403                   | 159  | 3529776    |

| average | standard deviation |
|---------|-------------------|
| 3527130 | 1159              |

| m/z     | FWHM | $M/\Delta M, \text{FWHM}$ | $z$  | mass (Da)  |
|---------|------|------------------------|------|------------|
| 20578.6 | 15.4 | 1336                   | 178  | 3662809    |
| 20702.4 | 20.8 | 995                    | 177  | 3664151    |
| 20824.0 | 18.9 | 1102                   | 176  | 3664844    |
| 20943.5 | 21.5 | 975                    | 175  | 3664936    |
| 21062.4 | 21.9 | 960                    | 174  | 3664684    |
| 21185.8 | 23.4 | 904                    | 173  | 3664972    |
| 21308.7 | 23.3 | 913                    | 172  | 3664923    |
| 21433.1 | 23.1 | 929                    | 171  | 3664891    |
| 21559.8 | 20.6 | 1048                   | 170  | 3665001    |
| 21686.5 | 21.0 | 1035                   | 169  | 3664846    |
| 21812.8 | 22.3 | 976                    | 168  | 3664384    |
| 21946.8 | 23.4 | 939                    | 167  | 3664944    |
| 22073.1 | 20.5 | 1079                   | 166  | 3663962    |
| 22210.8 | 24.1 | 922                    | 165  | 3664624    |

| average | standard deviation |
|---------|-------------------|
| 3664569 | 601               |
### Adeno-Associated Virus Serotype 1, series2

| m/z  | FWHM | M/ΔM, FWHM | z | mass (Da) |
|------|------|-------------|---|-----------|
| 20621.4 | 24.9 | 829 | 178 | 3670429 |
| 20742.5 | 21.2 | 978 | 177 | 3671247 |
| 20861.5 | 21.4 | 976 | 176 | 3671439 |
| 20980.4 | 19.7 | 1063 | 175 | 3671388 |
| 21103.9 | 21.7 | 974 | 174 | 3671906 |
| 21226.7 | 22.2 | 957 | 173 | 3672048 |
| 21348.0 | 20.6 | 1037 | 172 | 3671682 |
| 21475.8 | 21.7 | 990 | 171 | 3672186 |
| 21601.4 | 21.6 | 1001 | 170 | 3672068 |
| 21727.6 | 22.0 | 987 | 169 | 3671795 |
| 21856.0 | 22.2 | 986 | 168 | 3671645 |
| 21989.5 | 20.0 | 1102 | 167 | 3672078 |
| 22120.9 | 21.7 | 1022 | 166 | 3671895 |
| 22249.7 | 19.6 | 1134 | 165 | 3671027 |

Average standard deviation: 488

### Adeno-Associated Virus Serotype 1, series3

| m/z  | FWHM | M/ΔM, FWHM | z | mass (Da) |
|------|------|-------------|---|-----------|
| 20658.9 | 17.8 | 1163 | 177 | 3656452 |
| 20783.7 | 19.1 | 1088 | 176 | 3657755 |
| 20900.5 | 20.7 | 1011 | 175 | 3657409 |
| 21022.0 | 21.0 | 1001 | 174 | 3657656 |
| 21143.6 | 20.4 | 1035 | 173 | 3657666 |
| 21267.1 | 20.6 | 1035 | 172 | 3657762 |
| 21391.8 | 21.8 | 982 | 171 | 3657830 |
| 21518.6 | 22.2 | 969 | 170 | 3657985 |
| 21643.1 | 22.9 | 943 | 169 | 3657512 |
| 21771.5 | 21.8 | 997 | 168 | 3657444 |
| 21903.9 | 21.6 | 1016 | 167 | 3657781 |
| 22030.2 | 24.3 | 906 | 166 | 3656851 |
| 22162.5 | 26.5 | 835 | 165 | 3656652 |

Average standard deviation: 485
### Cowpea Chlorotic Mottle virus

| m/z      | FWHM | $M/\Delta M$, FWHM | $z$ | mass (Da) |
|----------|------|--------------------|-----|-----------|
| 22623.7  | 35.9 | 631                | 197 | 4456672   |
| 22737.7  | 41.7 | 545                | 196 | 4456393   |
| 22850.2  | 44.5 | 514                | 195 | 4455594   |
| 22972.6  | 47.9 | 479                | 194 | 4456490   |
| 23089.1  | 49.7 | 464                | 193 | 4456003   |
| 23208.6  | 52.5 | 442                | 192 | 4455859   |
| 23331.6  | 54.6 | 428                | 191 | 4456145   |
| 23452.5  | 57.8 | 406                | 190 | 4455785   |
| 23578.0  | 61.5 | 383                | 189 | 4456053   |
| 23705.5  | 61.2 | 387                | 188 | 4456446   |
| 23835.4  | 61.8 | 386                | 187 | 4457033   |
| 23967.3  | 60.0 | 399                | 186 | 4457732   |
| 24094.4  | 57.4 | 420                | 185 | 4457279   |
| 24223.6  | 50.2 | 482                | 184 | 4456958   |
| 24349.7  | 50.3 | 484                | 183 | 4455812   |

|          | average mass | standard deviation |
|----------|--------------|--------------------|
|          | 4456417      | 618                |
Supplementary Table S3. Quantification of TFP loading in encapsulin from HCD spectra of the loaded nanocompartment. The TFP-mass is calculated from the average determined mass, minus 59 (1st product ion) or 58 (2nd product ion) times the encapsulin monomer mass (28594 Da). The number of TFP (#TFP) is calculated by dividing the TFP-mass by the mass of one TFP monomer (34667 Da).

| assignment (#TFP) | Mass (Da) | TFP-component |
|------------------|-----------|---------------|
|                  | average   | standard      | TFP-mass | #TFP |
|                  | deviation |              |          |      |
| 1st product ion  |           |               |          |      |
| 8                | 1964580   | 626           | 277534   | 8.01 |
| 9                | 1999406   | 197           | 312360   | 9.01 |
| 10               | 2034081   | 232           | 347035   | 10.01|
| 11               | 2068801   | 200           | 381755   | 11.01|
| 12               | 2103584   | 378           | 416538   | 12.02|

| 2nd product ion  |           |               |          |      |
|                  | average   | standard      | TFP-mass | #TFP |
|                  | deviation |              |          |      |
| 9                | 1970968   | 161           | 312516   | 9.01 |
| 10               | 2005560   | 391           | 347108   | 10.01|
| 11               | 2040093   | 275           | 381641   | 11.01|
| 12               | 2074680   | 495           | 416228   | 12.01|

Supplementary Table S4. Stoichiometry of AAV1 capsids. Using theoretical VP1/VP2/VP3 masses, all stoichiometry's with theoretical masses that are within two standard deviations of the experimental mass are listed.

| peak series | mass (kDa) | stddev | VP1 | VP2 | VP3 |
|-------------|------------|--------|-----|-----|-----|
| 1           | 3643       | 0.7    | 0   | 10  | 50  |
| 2           | 3650       | 0.5    | 0   | 11  | 49  |
| 3           | 3657       | 0.5    | 1   | 9   | 50  |
Supplementary Figure S1. Ion instability near the end aperture of a RF-only field occurs when diverging radial force created by DC potential $\psi$ overpowers focusing by RF quasi-potential $\varphi$. 
**Supplementary Figure S2.** The maximum allowed acceleration of ions (for efficient transmission) as a function of $m/z$ for different $f$, $V_{RF}$ and $C$, according to equation 6 of the main text. The curves illustrate how reduced RF frequency ($f$), higher RF amplitude ($V_{RF}$) and increased focal distance (via increase in $C$) are beneficial for transmission of higher $m/z$ ions.

**Supplementary Figure S3.** Effect of reduced RF frequency on the injection flatapole and bent flatapole on the transmission of CsI clusters at 20000 $m/z$. CsI spectra were acquired starting with the lowered RF frequency (2.8 MHz, highlighted in blue). Mid-acquisition, while maintaining flow through the nanoelectrospray capillary, we switched back to the original RF frequency of the ion guides (3.3 MHz, highlighted in red), thereby demonstrating a 4-5 fold gain in transmission due to the lowered RF frequency on these two particular ion guides.
Supplementary Figure S4. Effect of reduced RF frequency on HCD spectrum of GroEL. a) HCD spectrum of GroEL. Signals are normalized to the most intense 14-mer charge state precursor ion. The acquired spectrum before the modification is shown in green, that after modification in red. b) Zoom-in of the blue highlighted m/z region in "a)" where 13-mer and 12-mer ions overlap. The closely spaced 13/12-mer peaks are still well resolved. For detailed peak assignments see Supplementary Table S1.

Supplementary Figure S5. Effective resolution as a function of m/z for intact GroEL 14-mer and 13-/12-mer product ions on the modified Exactive Plus with lowered RF frequency.
**Supplementary Figure S6.** Validating quantitation of cargo encapsulation in encapsulin VLP. a) Experimental spectrum of intact encapsulin VLP. b) Simulated spectra of the identified encapsulin-cargo complexes. Note that each consecutive stoichiometry is offset in y. c)Overlay of the experimental spectrum with the sum of spectra in “b)”.

**Supplementary Figure S7.** AAV1 capsid gross morphology and VP composition. A) 5µl of AAV1 capsids were stained with 2% uranyl acetate on holey carbon grids and imaged on a FEI spirit TEM. B) Coomassie stained SDS PAGE of denatured AAV1 capsids showing VP1, VP2 and VP3.