Determining the stoichiometry and interactions of macromolecular assemblies from mass spectrometry

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Published online 22 March 2007; doi:10.1038/nprot.2007.73

The growing number of applications to determine the stoichiometry, interactions and even subunit architecture of protein complexes from mass spectra suggests that some general guidelines can now be proposed. In this protocol, we describe the necessary steps required to maintain interactions between subunits in the gas phase. We begin with the preparation of suitable solutions for electrospray (ES) and then consider the transmission of complexes through the various stages of the mass spectrometer until their detection. Subsequent steps are also described, including the dissociation of these complexes into multiple subcomplexes for generation of interaction networks. Throughout we highlight the critical experimental factors that determine success. Overall, we develop a generic protocol that can be carried out using commercially available ES mass spectrometers without extensive modification.

INTRODUCTION

The essential role of mass spectrometry (MS) coupled with the soft ionization processes of either matrix-assisted laser desorption (MALDI) or electrospray (ES) ionization in the field of proteomics is well established[1]. Alongside these developments for proteomics, ES has been developed for studying intact protein complexes, primarily to elucidate their stoichiometry and protein interactions and also to complement existing approaches[2]. As the methodology has progressed, with the introduction of nanoflow[3], a miniaturized version of ES, and with the development of instruments optimized for high mass assemblies[4], the range of applications has continued to grow. Recent highlights include the extraction of endogenous, heterogeneous complexes, including the yeast exosome[5,6], the 19S proteasome lid from yeast[7] and 2.3 MDa ribosomes from *Thermus thermophilus*[8].

The vast array of protein complexes that has now been studied enables some general guidelines to be established. These guidelines, for obtaining ES data from intact protein complexes, are based primarily on experience gained over the last decade. The general protocol that we propose can be customized for different investigations through adaptation of the preparative steps. As far as possible, we have designed the protocol such that it is applicable to standard commercial mass spectrometers. To provide the background to our protocol, we pose the following fundamental questions: What information is attainable and what are the limitations? What type of MS instrumentation is required? How are complexes isolated? What are the sample requirements? What buffer conditions are compatible with nanoflow ES? What are the options for buffer exchange? How are the MS conditions optimized? How are nonspecific interactions distinguished from specific ones? How are subcomplexes generated? How is data processed?

What information is attainable?

Two pieces of information from MS underpin its application to protein complexes: the masses of individual subunits and the mass of the intact complex. This information is used in the following ways:

(i) To determine the stoichiometry of subunits within a complex. Given an intact mass, the composition of the complex is determined as the sum of individual subunit masses. This is the most common application of nanoflow ES to protein assemblies and can be particularly useful in resolving ambiguities in stoichiometry arising from, for example, gel-filtration data[9].

(ii) To determine the relative binding strength and topology. MS/MS experiments can indicate which subunits are on the periphery of a complex as they are in general the most readily dissociated[7].

(iii) To identify protein–protein contacts. By generating subcomplexes using chaotropes in solution and then MS/MS to determine their composition, interaction maps can be generated[5].

(iv) To investigate subunit exchange in solution. This experiment takes advantage of a mass difference (from changes in the amino acid sequence or isotope labeling) to follow the dynamics of subunit exchange in solution[10,11].

(v) To investigate the assembly of complexes in solution. The formation of an intact complex from its component subunits can be monitored directly by following time-dependent changes in the mass spectrum[12].

What are the limitations?

The limitations are related to the following features of the method:

(i) Mass and heterogeneity of the complex. As the mass of a complex increases many of the processes in the mass spectrometer become less efficient, including desolvation, ion transmission and detection. For megadalton complexes, modifications to standard instrumentation are required[3]. Heterogeneous complexes present additional challenges. Many possible complexes may be close in mass (and m/z) such that a unique assignment of the subunit composition is not possible.

(ii) Qualitative nature of the data. The complexes and subcomplexes of protein assemblies are likely to contain a wide range of subunits and m/z values which means that intensity ratios (relative abundances) in their ES spectra are not quantitative owing to differences in ionization efficiency, charging and transmission across the mass range.

(iii) Gas-phase detection of solution-phase species. MS has a bias toward detecting electrostatic interactions as hydrophobic interfaces are weakened in the gas phase[13]. In addition to differences in their transmission, intensity ratios for different complexes may not reflect the ratios in solution as a result of this bias. In some cases, it may not be possible to maintain/detect an intact complex if its associations are particularly hydrophobic. The magnitude of the effect will depend on the range and number of interactions. For large protein assemblies, both hydrophobic and electrostatic interactions are

involved. As a consequence, it is more likely that there are sufficient interactions to maintain the complex intact in the gas phase.

(iv) Concentration and solubility of the complex. ES is sensitive to many solution additives and consequently complexes generally have to be electrosprayed from a buffer that differs from their purification or storage buffer (see later discussion).

What type of MS instrumentation is required?

There are three main considerations:

(i) ES flow rate. Standard ES probes operate in the microliter per minute range and employ nebulizing gas and heated desolvation gas. Although it is possible to obtain spectra of noncovalent protein complexes with a standard ES interface (many examples of which are included in an early review[14]), the majority of recent applications have used nanoflow ES[3]. The decrease in flow rate (approximately 20–50 nl min^{-1}) improves sensitivity and tolerance to buffer salts, facilitates spraying of aqueous buffers and removes the need for heating the source block or desolvation gas. The volume of sample required is reduced compared with standard ES as nanoflow capillaries are loaded with 1–2 µl. Capillaries are available ready-to-use from several commercial sources; however, it can be more cost effective to prepare and gold-coat the capillaries in-house (see MATERIALS). Electrical connection in the nano-ES probe is made through a conductive elastomer ferrule that contacts the gold-coated capillary.

(ii) Mass range. The spectra of proteins and complexes sprayed from 100% aqueous buffer solution at neutral pHs have lower charge states compared with those obtained from single proteins of equivalent mass in denaturing solvents at low pH. The maximum charge state can be estimated from the Rayleigh charge Z_R[15]: roughly this means that mass spectrometers with *m/z* ranges of 4,000, 8,000 and 20,000 *m/z* have corresponding mass limits of 100, 400 and 2,400 kDa. The majority of studies use ES time of flight (ToF) or quadrupole time of flight (QToF) mass spectrometers to achieve these higher mass ranges.

(iii) Pressure control. Multiprotein complexes, in general, require an increase in pressure in the transfer region between the source and analyzer (see later discussion). The simplest way to do this with an LCT or QToF instrument (Waters, Manchester, UK) is to reduce the conductance of the source vacuum line to the roughing pump by partially closing the isolation valve (SpeediValve). Depending on the vacuum system on other instruments, it may be necessary to install or change the position of the isolation valve to allow variation of the pressure in the source/transfer region. Other alternatives include increasing the orifice size of the sampling cone, fitting a sleeve around the front section of the first ion guide to cause a local increase in pressure (QSTAR Elite, Applied Biosystems), trapping ions in the ion guide or introducing gas to increase pressure[16].

We have several modified instruments[3] in our laboratory that extend the useable mass range/sensitivity of the ToF analyzer and allow MS/MS isolation of ions up to *m/z* 30,000. The main modifications are (i) a decrease in quadrupole R_f frequency; (ii) addition of gas inlets or sleeves in the first ion guide region; (iii) an increase in maximum collision cell pressure; and (iv) an increase in cone/extractor/collision cell voltage range. It is possible, however, to use standard ToF or QToF configurations, such as LCT or QToF1 instruments, to acquire mass spectra for complexes up to 1 MDa without the requirement for hardware modifications (A.A. Rostom and C.V. Robinson, unpublished data; ref. 17). The current practical mass range limit for modified instruments is 2–3 MDa.

How are complexes isolated?

Originally protein complexes were purified using classical biochemical methods, a time-consuming process for low-abundance protein complexes, and they required purification steps to be tailored to individual complexes. The development of epitope tagging and affinity purification techniques, performed under native conditions, has produced vast networks of protein interactions[18–20]. We have used the TAPtag purification strategy for isolation of protein complexes at natural expression levels. This protocol is described in detail elsewhere[21].

What are the sample requirements?

Low micromolar concentrations are required (1–20 µM) of complex as a final concentration for ES. Although it is possible to record spectra for solutions of complexes at concentrations of 100 nM or less, for example, the tetramer of alcohol dehydrogenase (ADH; data not shown), it is noteworthy that this is a commercially available complex. In this case, it is possible to optimize the conditions using a more concentrated solution. For a 'real' complex, we aim for a minimum concentration of approximately 1 µM. Endogenous complexes often have to be concentrated using a buffer exchange procedure to achieve this concentration. Volume requirements are 1–2 µl per nanoflow capillary: for a *de novo* investigation we suggest 5–10 µl as a minimum final volume to allow MS conditions to be optimized. With a 1 µM concentration a *minimum* of approximately 5 pmol protein complex will be available. Higher concentrations and volumes are of course desirable as they provide greater opportunity for optimization.

What buffer conditions are compatible with nanoflow ES?

Most common buffers used during isolation or storage of protein complexes contain salts that are largely nonvolatile and during ES cause suppression of ionization and/or extensive adduct formation. Detergents and large amounts of glycerol are also often present in the final buffer and are detrimental to ES. The majority of protein solutions therefore need to be buffer exchanged to ES-compatible buffers. The buffer exchange step that leads to the final solution conditions is probably the most critical process that determines success or failure. For many small molecule or proteomics applications, samples are usually buffer exchanged to 1:1 water:acetonitrile or methanol with 0.1–2% organic acid. These conditions typically denature proteins and cannot be used to detect intact complexes. To maintain complexes intact in solution, aqueous ammonium acetate solution with a pH range of 6–8 is most commonly used as both ammonia and acetic acid are volatile and evaporate readily during ES[22]. High concentrations of ammonium acetate also aid in reducing the effect of nonvolatile buffer components[22,23], presumably through displacement of nonvolatile adduct ions. The effect of increasing ammonium acetate concentration on the spectrum of ADH tetramer in the presence of 10 mM Tris–HCl is shown in **Figure 1a–c** These solution conditions mimic a 'worst case' scenario in which the involatile buffer, Tris–HCl, has not been removed through buffer exchange. At 50 and 250 mM ammonium acetate concentrations, only unresolved or partially resolved ADH charge states and/or Tris–HCl adducts are observed whereas at a concentration of 1 M ammonium acetate the charge states are resolved. The ADH spectrum acquired using the same MS conditions and 50 mM ammonium acetate in solution in the absence of contaminating buffer salts is shown in the inset **Figure 1d**; tetramer

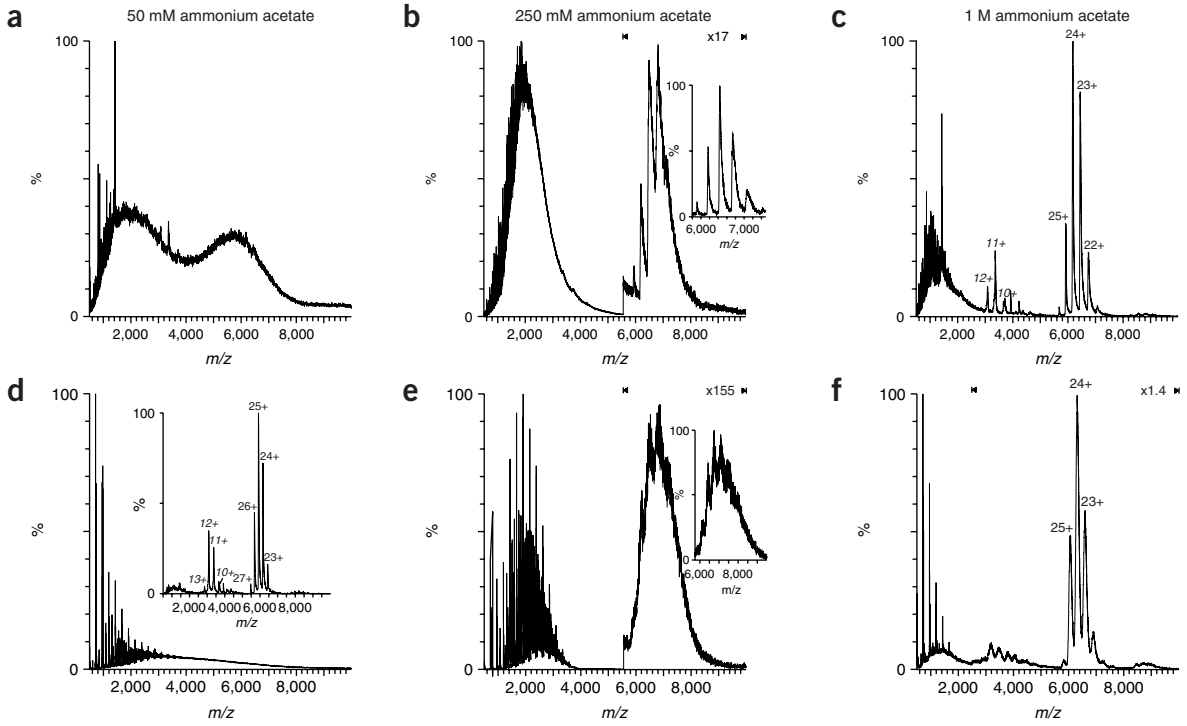

Figure 1 | Effect of ammonium acetate concentration and added buffer salts on the spectra of alcohol dehydrogenase (ADH). Main panels show the spectra obtained from a 5 μM solution of ADH tetramer solution containing either 10 mM Tris–HCl (**a–c**) or 10 mM HEPES (**d–f**) and with the ammonium acetate concentration indicated. Insets **b** and **e** show the effect of increasing the extractor voltage on the ADH spectra from 250 mM ammonium acetate solutions: with 10 mM Tris–HCl present, resolved charged states can be obtained but with HEPES present the spectrum is not improved significantly, providing further evidence that HEPES is more difficult to remove from the complex by gas-phase dissociation than Tris–HCl. All spectra were acquired in triplicate using identical MS conditions on a QToF1 instrument (capillary: 1.5 kV, cone 200 V extractor 0 V main panels, 100 V insets **b** and **e**, pressure readbacks: analyzer 1.5×10^{-5} mbar, ToF 1.5×10^{-7} mbar) and a new capillary for each replicate. One replicate was selected as 'average' from the three based on resolution and signal intensity. Solutions of ADH without added Tris–HCl or HEPES gave similar baseline-resolved charge states at all three ammonium acetate concentrations; inset **d** shows the ADH spectrum obtained from 50 mM ammonium acetate solution. Peaks in the range *m/z* 6,000–7,000 are labeled with the charge states of the tetramer. In **c** and **d**, charge states labeled in italics are assigned to the ADH monomer; unlabeled charge states in the region *m/z* 3,500–5,000 correspond to a 55 kDa species, assumed to be an impurity.

and monomer charge states are clearly resolved and the relative intensity of broad peaks assigned to clusters of buffer molecules at low *m/z* is lower than when Tris–HCl present. This ability to overcome the presence of contaminating buffer salts has led to routine use of up to 1 M ammonium acetate; occasionally up to 3 M is advantageous. A starting range of 100 mM to 1 M ammonium acetate is typical, although it is possible to use lower concentrations (or only water) if required. Factors to consider when selecting the ammonium acetate concentration are

(i) Knowledge of the salt tolerance of the complex. For example, protein–RNA or protein–DNA complexes may dissociate in high ammonium acetate concentration[24].

(ii) Certain buffer components are more problematic than others and in these cases higher ammonium acetate concentrations are preferable. For example, typical concentrations of HEPES, CHAPS or EGTA used extensively in buffers are more detrimental to spectra of protein complexes than, for example, Tris–HCl. **Figure 1d–f** (main panels) show ADH spectra at three concentrations of ammonium acetate with 10 mM HEPES present in the solutions. At the lowest ammonium acetate concentration (50 mM), no signal is detected for the complex whereas at 250 mM ammonium acetate, peaks assigned to the tetramer are observed but with insufficient resolution of the charge states to allow mass measurement. It is possible to resolve the tetramer charge states from 1 M ammonium acetate/10 mM HEPES solution but not to the same extent as the charge states observed in the presence of 10 mM Tris–HCl (**Figure 1a–c**). These data suggest that HEPES is more difficult to dissociate from the complex than Tris–HCl.

(iii) A requirement for other buffer components. If a complex or experiment requires the presence of specific metal ions, cofactors, reducing agents (DTT or β-mercaptoethanol) or other components, they can be added to the ammonium acetate buffer up to a concentration of approximately 1 mM per component. However, as low a concentration as possible, is preferable. The concentration that can be tolerated depends on the buffer component; for example, DTT can be present at 1–2 mM whereas in the case of the chelating agents EDTA and EGTA, concentrations above approximately 250 μM lead to extensive adduct formation. Depending on the experiment, these additional components can be added to the buffer directly or to the complex-containing solution after buffer exchange. Using a higher ammonium acetate concentration to reduce extent of adduct formation is preferable when cofactors are present.

What are the options for buffer exchange?

In some instances (usually a recombinant protein complex), it may be possible to acquire useable spectra simply by diluting the protein complex solution with ammonium acetate. The dilution factor required is dependent on the composition of the original buffer and may range from 20× to greater than or equal to 500×. For endogenous isolations of complexes, the protein concentration is likely to be too low for dilution and buffer exchange will be necessary. Selection of a buffer exchange protocol is based primarily on the concentration of the complex. For concentrations greater than or equal to 5 μM of the complex, microcentrifuge gel filtration columns with load volumes of 20–70 μl are most commonly used in our laboratory as they are rapid and result in minimal dilution (less than a factor of 1.3). Complex-containing solutions are loaded after pre-equilibrating the column with ammonium acetate solution at the required concentration. Depending on the composition of the buffer in the original solution (in particular greater than or equal to 5% glycerol or other components), it may be necessary to pass the complex-containing solution sequentially through two or three columns, although this will decrease the overall recovery of the complex. In the concentration range 1–5 μM, complexes can be buffer-exchanged using microcentrifuge gel filtration columns but for these lower concentrations we generally prefer to use 500 μl centrifugal ultrafiltration devices as, in addition to buffer exchange, the complex-containing solution can be concentrated to a volume of 5–25 μl (depending on the device). Initial complex-containing solutions below 1 μM usually require further concentration; centrifugal ultrafiltration allows concentration and buffer exchange in one step, although the lower molecular weight cut-off (MWCO) devices (3–10 kDa MWCO) can take several hours to achieve the required buffer dilution (usually greater than or equal to 10^4). Dialysis using low volume devices is an alternative to gel filtration spin columns or centrifugal ultrafiltration, although it is much slower and sample dilution can be problematic. In general, for concentrations of less than 1 μM all the buffer exchange protocols we have examined give variable results, presumably owing to differential adsorption of the complexes. Details of several different buffer-exchange devices are given in the MATERIALS section.

How are the MS conditions optimized?

Most charge states for protein complexes give rise to higher molecular masses than anticipated based on the calculated mass. We attribute these additional masses to water/adducts that remain attached to the complex in the gas phase. As a result, the aim is to find optimal desolvation conditions that strip away residual water and buffer components by collisions with gas molecules, without causing proteins to dissociate from the complex. The energy and frequency of the collisions within the source and transfer regions determine this balance[25]. In addition to desolvation, collisions in the transfer ion guide also effect the focusing of ions and bring them on-axis by reducing their radial and axial velocity (referred to as collisional cooling or focusing), which improves their transmission to the analyzer[16,26]. The combined effect of these collisions on the ES spectra obtained from protein assemblies is to generate higher ion intensities and narrower peak widths as the complex signal is transmitted with greater efficiency and distributed over fewer species. In addition, a reduced number of adducts per charge state brings the *m/z* of the observed charge closer to the theoretical value.

(i) Which parameters are important? Voltage and pressure settings in the ES source and collision cell are critical. Compared with ES of small molecules, higher accelerating voltages are required for complexes. For focusing devices (hexapoles and quadrupoles), transmission windows should be set for the *m/z* range of interest. Other parameters can be optimized for resolution and sensitivity, ideally using the target complex or with a 'standard' protein complex of similar mass. These complexes are also useful for practicing nano-ES, specifically cutting capillaries and optimizing the MS conditions (see MATERIALS). Typical spectra for ADH and pyruvate kinase are reported here; representative spectra of concanavalin A, avidin[27] and GroEL have been reported previously[28].

(ii) How is the ES optimized? A number of factors determine the quality of the spectra through their effect on the ES. These are capillary inner diameter (i.d.), capillary voltage, backing pressure, position of the capillary relative to the cone and the flow of desolvation gas. Optimum ES parameters are interdependent as well as dependent on the complex in solution (see **Table 1**). Capillary i.d. has a major influence, as under ideal conditions (no backing pressure) this determines the flow rate. **Figure 2** shows a typical capillary before and after cutting using tweezers; we estimate an i.d. of 1–10 μm for the cut capillary. It should be noted, however, that there is considerable variation between nanoflow needles whether they are prepared in-house or purchased[29]. The capillary tip is positioned 1–10 mm from the cone orifice with short distances usually being optimal at a lower capillary voltage. For the most part, the capillary voltage optimizes between 1,000 and 1,800 V and the flow of desolvation gas between 80 and 150 l h^{-1}. A backing pressure (0–2 bar) can be applied to initiate flow and then reduced once the ES is stable as higher quality spectra are usually obtained without any backing pressure. Under these conditions, the spray may not be visible with a magnifying lens. In some cases, however, a stable spray cannot be maintained without a backing flow and higher capillary voltage (1,800–2,000 V). In addition, it may be necessary to ES the sample solution for several minutes before a stable signal is obtained from a protein complex (presumably due to electrochemical/capillary effects[30,31]).

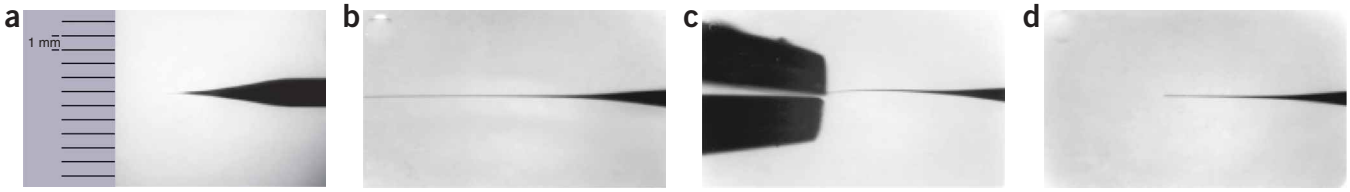

Figure 2 | Preparation of an open nanoflow electrospray capillary (**a**) nanoflow capillary before cutting, bars represent millimeter intervals (**b**) further magnification reveals the flexible extended tip (**c**) cutting the tip with AA tweezers and (**d**) the cut tip ready for introduction of the complex-containing solution.

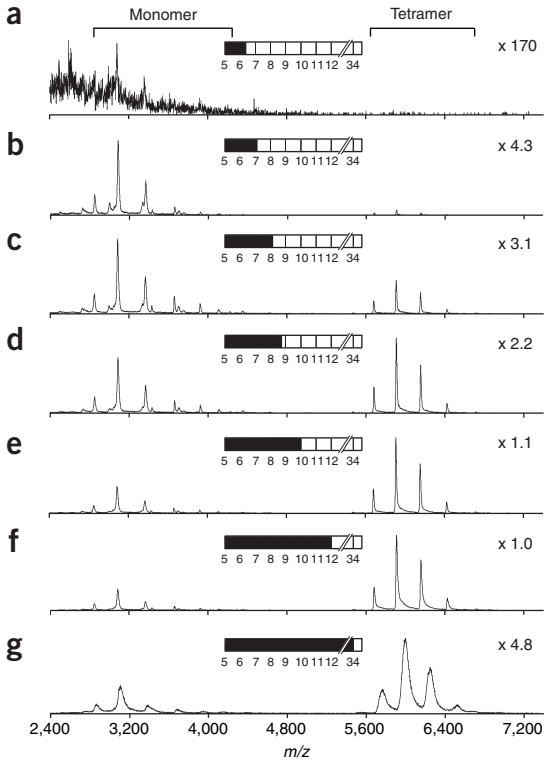

Figure 3 | Effect of pressure on the monomer:tetramer ratio of alcohol dehydrogenase (ADH). Spectra (sum of ten scans) were acquired from a solution of 5 μM ADH tetramer in 100 mM ammonium acetate pH 7.0 using a QToF1 instrument and the following settings: capillary: 1.5 kV, cone: 200 V, extractor: 0 V, collision cell voltage: 4 V. Pressure was increased by closing (stepwise) the isolation valve (SpeediValve) in the source roughing line. The bar indicates analyzer pressure readbacks ($\times 10^6$ mbar): (**a**) 6.3, SpeediValve fully open (**b**) 7.2 (**c**) 8.2 (**d**) 8.8 (**e**) 10 (**f**) 12 (**g**) 34. Magnification factors indicate the base peak signal intensity relative to spectrum (f).

(iii) How are acceleration voltages and pressures optimized?
Initially, complex-containing solutions are electrosprayed with intermediate voltages and the pressure in the source/transfer region increased until charge states from the complex are detected (on our instruments, cone 100–150 V and extractor 0–20 V). The charge states may not be resolved initially and often a broad peak distributed over a thousand or more *m/z* units is observed. Options for further optimization depend on the instrumental configuration. A general approach is to vary the pressures in the rough-pumped/intermediate vacuum region at fixed cone/extractor voltages to gauge the effect on the intensity of the charge states for the complex of interest (**Fig. 3**). An alternative strategy is to vary cone/extractor voltages at several fixed pressures. Similar spectra can be obtained from different combinations of voltages and pressures and a trial-and-error approach is needed as each complex will optimize under slightly different conditions. For a QToF-type instrument, the collision cell pressure and voltage are additional factors that come into play. Increasing these two parameters often improves the spectra of larger complexes (greater than 300 kDa) and they can also be used to increase the extent of desolvation for complexes that are poorly resolved. For low intensity, unresolved complexes, MS/MS with a wide isolation window can improve the transmission over a limited *m/z* range; combined with an increase in collision cell voltage and pressure, this may allow resolution of charge states.

How are nonspecific interactions distinguished from specific ones?
For many complexes, the spectra provide convincing evidence of a specific interaction as only one species is observed with a unique stoichiometry. Often a very low intensity dimer (less than 5% relative intensity) of the major oligomer is also present. At this level, we consider the dimer an artifact of the ES process. Low micromolar protein concentrations are preferable to reduce the possibility of nonspecific associations (**Fig. 4**). As a rough guide, evidence for specific oligomers comes not only from the relative intensities of the species, as described above, but also from the related stoichiometries (i.e., a dimer and a tetramer). Nonspecific interactions often show a continuum of oligomers, say, from monomers to heptamers, with relative intensities that decrease with mass. This simple analysis holds in many instances; however, both solution and MS conditions can affect the apparent equilibrium between oligomers: for example, the ammonium acetate concentration may influence the distribution of oligomers[24,32].

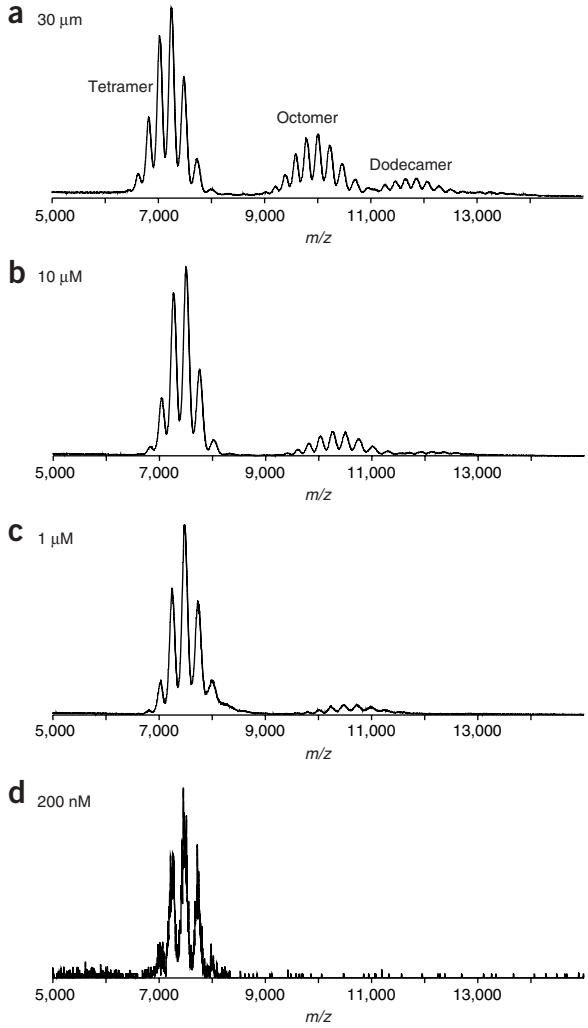

Figure 4 | Effect of concentration on the relative intensity of nonspecific oligomers of the tetramer of pyruvate kinase (**a**) 30 μM (**b**) 10 μM (**c**) 1 μM (**d**) 200 nM. Conditions: concentration of the tetramer as indicated, samples in 100 mM ammonium acetate pH 7.0 LCT settings: capillary: 1.5 kV, cone 150 V, extractor 0 V, pressure readbacks, inlets 8.6 mbar, ToF 2.4 $\times$ 10^{-6} mbar.

How are subcomplexes generated?

Subcomplexes provide information on protein–protein contacts and relative binding strengths of subunits. For homo-oligomers, these data allow subassemblies to be inferred (e.g., a hexamer yielding dimers but not trimers). In the case of complexes composed of more than one subunit, generating subassemblies can enable protein contacts to be determined.

Complexes can be disrupted in solution with changes in ionic strength[24,32], pH[27] or adding organic solvents to the solution[33]. Combined with MS/MS to determine the composition of smaller subcomplexes, this approach can allow determination of an interaction map for large, heterogeneous complexes[5]. Subcomplexes can also be formed in the gas phase via collision-induced dissociation (CID). In-source CID is brought about by increasing the accelerating voltages and/or decreasing the pressure. With the ability to carry out MS/MS experiments, CID is also possible in the collision cell and can be applied to either all charge states (as in-source CID) or only on selected charge states isolated over a narrow *m/z* range. The CID process generates highly charged monomers/subunits and the corresponding high *m/z* 'stripped' complexes[27,34,35]. Almost without exception, subunits are lost individually from a complex (loss of intact dimers, trimers, etc. is rarely observed). 'Stripped' complexes can go on to expel further subunits as the collision energy is increased. Although the factors that determine dissociation behavior are not fully understood for a heterogeneous complex, increasing the collision energy generates many 'stripped' complexes formed as a result of loss of different subunits. The order by which subunits are expelled may reflect their peripheral location and/or their ease of unfolding (see ANTICIPATED RESULTS).

How is data processed?

The extent of desolvation and/or adduct formation is the main determinant in obtaining an accurate molecular mass for a protein complex. Heterogeneity caused by, for example, post-translational modifications and different isoforms will also contribute to overlapping *m/z* values in a particular charge state. The centroid *m/z* value of a given charge state peak invariably leads to a measured mass greater than the mass of the 'naked' protein complex. A value closer to the true mass can be obtained by selecting an *m/z* value on the leading edge of the peak (fewer adducts attached). For many complexes, it is apparent that *m/z* values selected in this way occur at the same point on the leading edge of each charge state and consequently form a consistent series. As complexes get larger and more heterogeneous however, it becomes increasingly difficult to assign charge states with confidence. A formal approach is to allocate the charge states based on the series that gives the lowest S.D. for the average mass[36]. Recently, this approach has been extended and a correction factor derived that relates the peak width to a mass error that can then be applied to correct for adducts[37]. This was employed to mass measure ribosomal complexes with masses up to 2.3 MDa, at the current limit of methodology and instrumentation[37]. Where possible for smaller complexes (50 kDa–1 MDa), investing the time and effort in optimizing the solution and MS conditions is the preferred route.

MATERIALS

REAGENTS

- Ammonium acetate (greater than 98%; SigmaUltra, Sigma-Aldrich, cat. no. A7330)
- 7.5 M ammonium acetate solution (Sigma, Sigma-Aldrich, cat. no. A2706)
- Acetic acid (certified AR glacial 99.7+%; Fisher Scientific, cat. no. A/0400/PB08)
- Formic acid (certified AR 98.6+%; Fisher Scientific, cat. no. F/1900/PB08)
- Ammonium hydroxide solution (99.99%; Aldrich, Sigma-Aldrich, cat. no. 338818)
- Methanol (HPLC grade; Fisher Scientific)
- Ethanol (absolute, >99% Normapur; VWR)
- Iso-propanol (HPLC grade; Fisher Scientific)
- Dimethylsulfoxide (greater than 99.9%; Sigma, Sigma-Aldrich, cat. no. D8418)
- Acetronitrile (HPLC grade; Fisher Scientific)
- Cesium iodide (99.999%; Aldrich, Sigma-Aldrich, cat. no. 203033) at 100 mg ml^{-1} in water
- Cation exchange beads (AG 50W-W* resin, 100–200 mesh, hydrogen form; Bio-Rad, cat. no. 143-5441)
- Metal cations as acetate salts (if required)
- EDTA (99.995%; Aldrich, Sigma-Aldrich, cat. no. 431788) (if required)
- EGTA (greater than 99% BioChimikaUltra; Fluka; Sigma-Aldrich, cat. no. 03778) (if required)
- DL-DTT (greater than 99%; SigmaUltra, cat. no. D5545) (if required)
- β-Mercaptoethanol (greater than or equal to 99.0%; Sigma, cat. no. M6250) (if required)
- Cofactors ideally as ammonium or acetate salts (if required)
- Avidin (chicken egg white, 71 kDa tetramer; Calbiochem, Merck Biosciences Ltd., cat. no. 189725) at 5 μM tetramer in 100 mM ammonium acetate (if required)
- Concanavalin A (jack bean, 103 kDa tetramer; Sigma-Aldrich, cat. no. A3263) at 5 μM tetramer in 100 mM ammonium acetate (if required)
- ADH (*Saccharomyces cerevisiae*, 147 kDa tetramer; Sigma-Aldrich, cat. no. A3263) at 5 μM tetramer in 100 mM ammonium acetate (if required)
- Aldolase (rabbit muscle, 157 kDa tetramer; Sigma-Aldrich, cat. no. A2714) at 5 μM tetramer in 100 mM ammonium acetate (if required)
- Phosphorylase b (rabbit muscle, 196 kDa dimer; Sigma-Aldrich, cat. no. P6635) at 5 μM dimer in 100 mM ammonium acetate (if required)
- Pyruvate kinase (rabbit muscle, 232 kDa tetramer; Sigma-Aldrich, cat. no. P9136) at 5 μM tetramer in 100 mM ammonium acetate (if required)
- GroEL (*Escherichia coli*, 800 kDa 14-mer; Sigma-Aldrich, cat. no. C7688) at 2 μM 14 mer in 200 mM ammonium acetate (if required)

REAGENT SETUP

Commercially available protein complexes as test samples With the exception of GroEL, complexes listed in REAGENTS are prepared by making a solution of the standard material in 100 mM ammonium acetate at an estimated concentration of at least 10 μM for the complex, followed by a desalt step using a Micro Bio-Spin 6 chromatography column and 100 mM ammonium acetate. The recovered complex concentration can be checked through UV at 280 nm using calculated extinction coefficients.

The desalt procedure for GroEL is as follows: (i) dissolve the lyophilized protein in 80% buffer A/20% methanol where buffer A is: 20 mM Tris acetate, 50 mM KCl, 0.5 mM EDTA, 1 mM ATP and 5 mM $MgCl_2$, (ii) equilibrate by vortexing slowly for 2 h at room temperature, (iii) precipitate with 50% acetone (vol/vol), (iv) remove the supernatant and resuspend the precipitate in buffer A, (v) buffer exchange into 200 mM ammonium acetate through centrifugal ultrafiltration.

EQUIPMENT

- Zip-Tip_{C4} (Millipore, cat. no. ZTC04S024)
- Conductive elastomer for nanospray probe (Waters, cat. no. 6028626)
- Ready-made capillaries (Proxeon, Odense, Denmark; New Objective, Inc., Woburn, MA and other suppliers)
- Buffer exchange devices (see EQUIPMENT SETUP)
- In-house capillaries (see **Box 1**, **Fig. 2**)
- Borosilicate glass capillaries (packs of 500, Harvard Apparatus) 1.0 mm OD × 0.78 mm i.d. (cat. no. GC100TF-10)

BOX 1 | IN-HOUSE CAPILLARY PREPARATION

● TIMING: 40 min to make approximately 30 capillaries.

If you are using a capillary puller with a heated filament such as a Model P-97 (Sutter Instrument Co.), each new filament needs to be shaped and the puller program reset. The process of programming the puller is one of trial-and-error until an acceptable tip shape is obtained (**Fig. 2**). Apart from slight adjustment (if necessary) as the filament ages, the program can be used for the lifetime of the filament; typical lifetimes for filaments used for several hours a week are 4–6 months. Capillaries are prepared by performing the following steps:

1. Cut strips from doubled-sided adhesive pads and attach inside a glass Petri dish. Several strips can be used to support the pulled capillaries on the plate.
2. Pull the capillary and use 2a tweezers to transfer the capillary to the adhesive pad/Petri dish.
3. Once the dish/strip is full (approximately 30 capillary tips), coat the tips with gold using a sputter coater. We use a Polaron range sputter coater with Ar gas typically with the following conditions: vacuum pressure: 7×10^{-2} mbar, voltage: 1.8 kV, current: 35 mA, coat time: 1 min (1–2 coats required).

1.0 mm OD × 0.50 mm i.d. with filament (cat. no. GC100FS-10)
1.0 mm OD × 0.58 mm i.d. (cat. no. GC100-10)
- AA tweezers (Dumont, cat. no. 0302-AA-PO)
- 2a tweezers (Dumont, cat. no. 0302-2A-PO)
- Ceramic cutter (all-scribe FSOT cutter; Alltech Associates Inc., cat. no. 3194)
- Glass Petri dish (greater than 9 cm diameter)
- Double-sided adhesive pads
- 0.5–20 µl Eppendorf geLoader tips (Fisher Scientific, cat. no. 0030 001.222)
- Model P-97 flaming/brown micropipette puller (Sutter Instrument Co., Novato, CA)
- Polaron range model SC7680 sputter coater (Quorum Technologies, Newhaven, East Sussex, UK)
- Mass spectrometer

EQUIPMENT SETUP

Buffer exchange devices Micro Bio-Spin 6 chromatography columns, MW exclusion limit 6 kDa (Tris buffer, Bio-Rad, cat. no. 732-6221); NanoSep centrifugal devices (various MWCO, Pall Life Sciences) and Vivaspin 500 µl concentrators (various MWCO, Sartorius) are the buffer exchange devices we use most commonly. Other similar products are:

Centrifugal ultrafiltration Ultrafree-0.5 and Microcon centrifugal devices (Millipore).

Gel filtration Zeba desalt and microdesalt spin columns, MW exclusion limit 7 kDa (Pierce, Perbio Science), MicroSpin columns, MicroTip columns and Ultra-MicroTip columns (Harvard Apparatus).

Dialysis Micro and Ultra-Micro DispoDialyser (Harvard Apparatus), Slide-a-Lyzer dialysis cassette (0.1–0.5 ml), Slide-a-Lyzer mini dialysis units (Pierce, Perbio Science).

PROCEDURE

Preparation of complex-containing solutions: buffer exchange ● TIMING 20 min–24 h

1| Decide whether buffer exchange and/or concentration of the complex is required based on the guidelines given above. Follow the manufacturer's details for the selected buffer exchange/concentration device.

▲ CRITICAL STEP Most common buffer components form extensive adduct series and suppress ionization of protein complexes.

2| To vary the solution conditions after buffer exchange, aliquots of the complex-containing solution can be modified bearing in mind that 1–2 µl are required to load a capillary. To increase the ammonium acetate concentration, aliquots of a 7.5 M solution can be added to minimize dilution. To use organic solvents as chaotropic agents, add aliquots to give 5–50% vol/vol solutions starting with a low percentage (5–10%) and increase the organic content if necessary.

Nanoflow ES: intact complexes

3| Using 2a tweezers, take a coated capillary from the Petri dish and insert into the capillary holder down to the appropriate length for the nanoflow ES stage/source and tighten into position. The undrawn end of the capillary can be clipped using a ceramic cutter to facilitate the flow of the solution to the capillary tip.

! CAUTION Drawn capillaries are sharp.

4| Place the capillary/capillary holder on the microscope stage and cut the tip of the capillary using AA tweezers.

▲ CRITICAL STEP It takes some practice and experience to judge where to cut for particular capillary tip shapes. Leaving a longer tip means that, in addition to a lower flow rate, there is the option of further trimming if the capillary blocks or the gold coating gets stripped (see **Table 1** and **Figure 2**).

? TROUBLESHOOTING

5| Take up 1–2 µl complex-containing solution using a geLoader tip/10 µl pipette and insert the tip into the capillary. Start to withdraw the tip as the solution is dispensed. This step may be easier with the capillary holder secured on the bench, with Blu-Tak or double-sided adhesive tape. If preferred, the capillary can be loaded with the solution before cutting.

6| Attach the capillary holder to the *x–y–z* stage of the nanoflow ES interface. Apply a low backing pressure (if possible) to force a small drop of solution to the capillary tip before applying the capillary voltage. This ensures that the capillary is not blocked and facilitates initiating the spray. Once the spray is established, attempt to reduce the backing flow.

7| Adjust the capillary position, backing and desolvation gas flows and capillary voltage to get a spray (see optimization guidelines above and **Table 1**). Even if no signal is obtained initially for the complex, the solvent/buffer ions at less than 1,000 *m/z* can be used as an indicator of a flow/spray in the first instance.
▲ CRITICAL STEP A stable spray is essential for further optimization.
? TROUBLESHOOTING

8| Check for signals corresponding to the protein complex. If no charge states are apparent for a particular complex, set an intermediate cone voltage (100–150 V) and start to increase the pressure in the source/transfer region. The voltage range and pressure will depend on the instrumental setup and complex under study. Vary the pressures and voltages to maximize the ion counts and minimize the peak widths of the charge states of interest (see guidelines above). Check that changes in the spectra with respect to voltage and pressure are repeatable and not due to the instability of the spray. If no clear spectrum is observed, proceed to troubleshooting. It is quite possible, however, that a mass spectrum of suitable quality has been recorded by this stage, and if so, it may be advisable to carry out Step 9 to cross-check the overall mass and stoichiometry of the complex.
▲ CRITICAL STEP The voltages and pressures determine the transmission, desolvation and dissociation of a complex.
? TROUBLESHOOTING

9| To determine the masses of the subunits and their heterogeneity, take an aliquot (few microliters) of the sample and denature any proteins present. This can be carried out by (i) diluting the aliquot at least 1:1 with acetronitrile or methanol with 4% formic or acetic acid; (ii) using a Zip-Tip$_{C4}$ with 1:1 water:acetonitrile with 0.1% formic acid as the elution solvent; or (iii) adding cation exchange beads (previously rinsed in water) to a 5–10 µl sample aliquot to drop the pH. For (i), a high concentration (greater than 100 mM) of ammonium acetate and a low dilution with acidified organic solvent may prevent a large drop in the pH; higher percentages (10–30%) of acid may be used in this instance. In some cases, mass spectra are recorded for the intact complex but it is not possible to obtain denatured spectra using the methods described above.

Nanoflow ESI/MS/MS: dissociation of complexes

10| Adjust the spray, voltages and pressures as described for MS mode to give the optimum stable signal for the precursor ion/charge state of interest. Acquire a mass spectrum with these conditions.

11| Set the mass center and isolation width and acquire a few scans with the same voltage/pressure conditions as the MS spectrum. Select a scan range that is high enough to detect the high mass/lower charge dissociation products: it is preferable to set the maximum *m/z* range and then reduce the range once the highest *m/z* product has been determined (if signal is low).

12| Overlay the MS and MS/MS spectra of the precursor to check that the precursor mass and isolation window are correct.

13| To dissociate the complex, increase the collision cell voltage and pressure. Increasing collision cell voltage by 20–30 V increments at fixed pressure is usually the most straightforward way to determine the conditions that lead to dissociation. In general, the low *m/z* products (individual proteins and sometimes peptides) are more intense than high *m/z* products and the high *m/z* products are often only detected with higher collision gas pressures.

Calibration and data processing

14| Mass range and calibration: for ToF instruments, adjust the pusher frequency to match the *m/z* range of interest for the protein complex to maximize the number of pusher pulses per 'scan.'

15| Calibrate the instrument/spectra using a solution of CsI. A concentrated solution (100 mg ml^{-1} in water) will give ions greater than 10,000 *m/z* and will only require a short spray/acquisition time. The accelerating voltages and pressures may have to be altered slightly from those used for protein complexes.

16| For spectra that span a wide *m/z* range vary the smoothing functions and centroiding parameters in different *m/z* regions to reflect the resolution of the raw data. For Masslynx software, this may require processing the same averaged spectrum several times with different parameters or using the combine function to extract raw data over the *m/z* range of interest and then processing.

17| In some cases (low intensity MS/MS spectra in particular), it is easier to calculate the charge states and masses manually (using a simple Excel spreadsheet) from *m/z* values selected from processed spectra. Spectra of large complexes or mixtures of complexes are not trivial to process and it may be necessary to use a peak-fitting approach[37,38].

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Problem	Possible reason	Solution
No ions detected across *m/z* range	Blocked capillary	(1) Cut the tip of the capillary again[a] (2) Move the capillary closer to the cone to re-initiate the flow (higher field) and then withdraw rapidly. There is a chance that this will strip the gold coating (3) If capillaries are repeatedly blocked by a solution, dilute the solution. If this does not improve the spray, change the ammonium acetate concentration by addition, dilution, or a buffer exchange step (if possible)
	Spray not initiated	(1) Move the capillary closer to the cone (2) Increase the capillary voltage and/or backing flow to initiate flow (3) See blocked capillary (3)
	Stripped coating	(1) Remove the uncoated section[a] and re-initiate spray (2) If coating stripped frequently, reduce the capillary voltage and/or move the tip further from the cone
	Blocked cone/orifice	Clean the cone/orifice plate with 1:1 10% formic acid:isopropanol
Unstable spray and total ion intensity	Capillary position	Adjust capillary position (move tip closer to cone in the first instance)
	Capillary voltage	Increase/decrease
	Backing flow	Increase/decrease
	Capillary diameter	(1) If the backing flow needs to be high, the diameter is probably too small. Cut the tip again[a] (2) If the 'spray' is a jet or stream of droplets (even with an increased capillary voltage), the diameter is too large. Use another capillary
	Aggregation of the complex	(1) Dilute the solution to prevent partial blockage of the capillary (2) Change the solution conditions (see blocked capillary (3))
	Damaged tip coating	Remove the uncoated section[a] and re-initiate spray
No complex signal	Voltages and pressures not set correctly	Adjust voltages and pressures to give sufficient signal for optimization
	Capillary/electrochemical effects	Set voltages, pressures and 'average' spray conditions (see guidelines) and allow the sample to spray for 5–10 min. Some complexes yield a protein signal with time; this will gradually increase and level out over time
	High flow of buffer/solvent clusters	If there is a broad intense 'lump' between approximately 500–2,000 *m/z*: (1) move capillary further from cone (2) decrease capillary voltage (3) decrease backing flow (4) for water clusters, increase desolvation gas flow
	Solution conditions	(1) Increase/decrease ammonium acetate concentration and/or pH of sample solution (2) Buffer exchange using different pH or ammonium acetate concentration (if sufficient protein is available) (3) Consider other buffer components or cofactors that may be essential for the complex and add to solution or buffer for buffer exchange (4) See guidelines on unresolved/low signal intensity
	Low complex concentration	(1) For complexes in ammonium acetate, use a centrifugal vacuum concentrator (SpeedVac) to reduce the volume of the solution Note: Freeze-thawing may disrupt a complex and concentration of nonvolatile buffer components will occur (2) Use a centrifugal ultrafiltration device to concentrate the solution (see guidelines) (3) See guidelines on unresolved/low signal intensity

[a]The capillary tip can be cut either under a microscope using tweezers or by touching the tip against the cone/orifice plate with the capillary voltage set to 0 V and no backing flow.

No clear charge state series is observed (Step 8)

If no clear charge state series is observed, vary the voltages and pressures and watch for 'blocks' of signal over the *m/z* range of interest. If there is a limited *m/z* region giving a block for most scans, acquire for several minutes and average the spectra. A broad peak below approximately 2,000 *m/z* is very commonly observed and probably due to buffer/solvent clusters (see **Table 1**).

An unresolved block that occurs over a broad *m/z* range and jumps to lower *m/z* as voltages are increased or pressure decreased is possibly a solvent/buffer cluster. To confirm, acquire a spectrum of the buffer under the same conditions. Alternatively, with QToF-type instruments, introduce collision gas into the cell and increase the collision cell voltage by 10–30 V. A solvent/buffer cluster will usually dissociate to give an intense signal below approximately 1,000 *m/z* and the cluster will move to lower *m/z*. A protein complex might also give an intense low *m/z* signal, as adducts are dissociated. The block of signal at higher *m/z* will not continue to move significantly on the *m/z* scale and charge states may start to resolve making further optimization easier. If MS/MS is an option, this can be used to increase transmission of low intensity species. Use a wide isolation window (up to 200–300 *m/z*) to span the unresolved signal. Increase the collision cell voltage and gas cell pressure; if a cluster is present it will progress to lower *m/z* values as in MS mode (see above). A protein complex may start to resolve two or three charge states. If so, repeat the acquisition using different overlapping isolation windows to resolve more charge states. Combine the charge states from several isolations to measure the mass of the complex. This value may be an approximation but can be used to confirm that a protein complex is present.

If there is no discernible protein signal after a range of voltages, pressures and MS/MS conditions have been assessed with long acquisition times, check that protein is present in the solution (see Step 9). If charge states of free proteins are not observed from denaturing solution conditions, it is likely that the concentration of complex is not high enough for detection or that the buffer exchange process was inadequate. If available, tryptic digestion followed by MALDI/MS/MS can be used to confirm that proteins are present at very low concentration.

ANTICIPATED RESULTS

When optimizing both solution conditions and MS parameters, it is usual at the outset to observe spectra similar to those shown in **Figure 1a**. Two unresolved, broad 'peaks' are observed at approximately 2,000 *m/z* and approximately 6,000 *m/z*, the latter corresponding to the expected *m/z* range of the ADH tetramer. An increase in cone and/or extractor voltages will often increase the level of desolvation as shown in **Figure 1b** where an increase in the extractor voltage from 0 (main panel) to 100 V (inset) significantly improves resolution of the charge states. Alternatively, a decrease in pressure could be tried as a similar improvement in resolution would be anticipated. In some instances, most commonly when buffer exchange has not been sufficient, increasing the accelerating voltages or decreasing the pressure will not be effective: **Figure 1e** illustrates this point. For the ADH tetramer with a high level of HEPES present in solution a 100 V increase in extractor voltage has very little effect

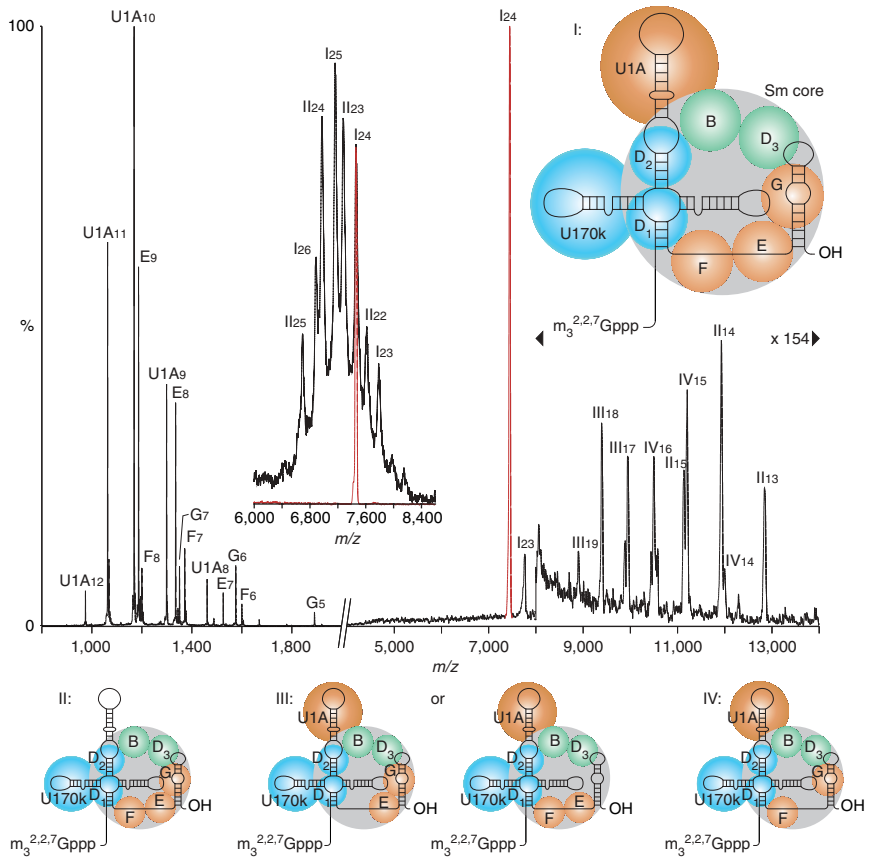

Figure 5 | Mass spectrometry (MS) and MS/MS spectra of a protein–RNA complex, human U1snRNP assembled *in vitro* (comprising seven Sm core proteins: D_1, D_2, D_3, B, E, F and G, two further proteins U170k and U1A, and synthetic U1snRNA). Inset shows the MS spectrum of the complex: two series of charge states (I and II) are evident corresponding to the complex with and without the U1A protein. The 24+ charge state from the U1 complex with U1A present was isolated in an MS/MS experiment (I24, red trace in inset). Four proteins (U1A, and the Sm core proteins E, F and G shown in orange in the schematic) were readily dissociated, followed by proteins B and D_3 (green) implying that the remaining three proteins (U170K, D_1 and D_2) (blue) are more closely associated with U1snRNA (main panel). Peaks are labeled with the protein or complex identity and charge state. U1A: 11,680 ± 0.3 Da; E: 10,671 ± 0.4 Da; F: 9,593 ± 0.2 Da; G: 9,447 ± 0.3 Da; B: 17,846 ± 1.5 Da; D_3: 13,959 ± 0.3 Da; I: 178,356 ± 46 Da, [Sm core+U1A+U170K+U1snRNA]; II: 166,657 ± 19 Da, [Sm core+U170K+U1snRNA]; III: 169,105 ± 44 Da, [complex I–F or G]; IV: 167,956 ± 27 Da, [complex I–E]. Conditions: 10 μM complex in 500 mM ammonium acetate. Modified QToF2 settings (main panel): capillary: 1.5 kV, cone 200 V, extractor 50 V, collision cell voltage: 110 V, pressure readbacks, source 9.3 × 10^{-3} mbar, analyzer 7.0 × 10^{-4} mbar, 1.3 × 10^{-6} mbar. Inset settings as main panel except collision cell voltage: 4 V.

on the charge state resolution (**Fig. 1e** inset). In situations where the complex concentration is too low or the solution conditions are not optimized for either the complex (solubility) or buffer composition (ES-compatible), the spectra may resemble **Figure 1d** (main panel) with no apparent signal for a complex. A check on the protein concentration in solution is then necessary (see TROUBLESHOOTING). Note in **Figure 1** that the buffer clusters can extend up to *m/z* 4,000–5,000 due to the high level of added buffer salts in this case; up to *m/z* 1,000–2,000 is more typical and the intensity of the clusters can often be reduced by decreasing the flow rate of the solution (see **Table 1**).

In optimizing the voltages and pressures, a compromise between desolvation, signal intensity and dissociation is necessary. For example, an analyzer pressure of 1.2×10^{-5} mbar (**Fig. 3f**) gives the highest tetramer intensity but the resolution of the charge states is improved at lower pressure (8.2×10^{-6} mbar), **Figure 3c**. These lower pressure conditions also increase the extent of tetramer dissociation as a greater proportion of monomeric ADH is observed (**Fig. 3c**,**f**). The pressure can also have an effect on the appearance of nonspecific oligomers in spectra, especially if the concentration of proteins is high. Spectra of pyruvate kinase were acquired at pressure settings that were intentionally high to maintain the nonspecific octomer and dodecamer (**Fig. 4**). Between concentrations of 200 nM and 10 μM of the pyruvate kinase tetramer, the major charge state series is assigned to the tetramer but at 30 μM the octomer is present at significant relative intensity. These spectra illustrate therefore that reducing the concentration of the complex in solution can allow specific interactions to be distinguished from those that form as a result of aggregation in the ES droplet (**Fig. 4a–d**).

To demonstrate anticipated results from a heterogeneous protein–RNA complex we have used human U1snRNP, assembled from nine recombinant proteins and U1snRNA. The mass spectrum contains two series of charge states corresponding to two complexes of 178.4 and 166.7 kDa (**Fig. 5**). The mass difference between these two complexes corresponds to one of the proteins anticipated in the complex (U1A). An MS/MS experiment, in which one charge state from the 178.4 kDa complex was isolated using the extended mass quadrupole on a modified QToF2 instrument and dissociated in the collision cell, confirmed that U1A was readily lost from the complex. Three further proteins (E, F and G) were also dissociated and at higher collision cell voltages, proteins B and D_3 are expelled from the complex (**Fig. 5** main panel). The order of dissociation of protein subunits in the gas phase is consistent with the Sm ring comprising of two dimers (D_1:D_2 and B:D_3) and a trimer (E:F:G) as suggested previously[39]. The data also suggest that the proteins that are not observed (D_1, D_2 and U170k) are more closely associated with the U1snRNA. Loss of E, F and G at lower energy than the other Sm proteins may indicate that the trimer is more exposed or has fewer contacts in the complex.

ACKNOWLEDGMENTS We thank D. Matak-Vinkovic, A. Sandercock, M. Sharon and J. Benesch for critical reading of the manuscript and L. Lane for evaluating 'standard' protein complexes. The human U1snRNP sample was kindly donated by D. Pomeranz-Krummel in the group of K. Nagai, MRC Laboratory of Molecular Biology, Cambridge, UK. We thank the Biotechnology and Biological Sciences Research Council (BBSRC) for funding.

COMPETING INTERESTS STATEMENT The authors declare no competing financial interests.

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