Analytical Approaches for Size and Mass Analysis of Large Protein Assemblies

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Abstract

Analysis of the size and mass of nanoparticles, whether they are natural biomacromolecular or synthetic supramolecular assemblies, is an important step in the characterization of such molecular species. In recent years, electrospray ionization (ESI) has emerged as a technology through which particles with masses up to 100 MDa can be ionized and transferred into the gas phase, preparing them for accurate mass analysis. Here we review currently used methodologies, with a clear focus on native mass spectrometry (MS). Additional complementary methodologies are also covered, including ion-mobility analysis, nanomechanical mass sensors, and charge-detection MS. The literature discussed clearly demonstrates the great potential of ESI-based methodologies for the size and mass analysis of nanoparticles, including very large naturally occurring protein assemblies. The analytical approaches discussed are powerful tools in not only structural biology, but also nanotechnology.

1. INTRODUCTION

Various biomolecules coexist in the cell, such as DNA, RNA, proteins, lipids, and metabolites, and dynamically interact with each other transiently or in stable complexes (1, 2). These interactions largely regulate their cellular functioning. Some notable examples of natural supramolecular assemblies are nucleosomes (assemblies of histone octamers with DNA), the ribosome (assemblies of more than a dozen proteins with RNA), transcription complexes, GroEL, the proteasome, and viruses (composed often of DNA, proteins, and lipid bilayers). Smaller aggregates also exist, such as enzymatic homomultimers, amyloid aggregates, IgG and IgM antibodies, and noncovalent protein-lipid complexes. With the general feeling now being that a plethora of proteins, protein-DNA, protein-RNA, and protein–small molecule assemblies coexists in the cell, more biomolecular assemblies are awaiting discovery as well as structural and functional analysis. The size and mass analysis of newly discovered assemblies is the first prerequisite in determining the constituents, the stoichiometry, and possibly the shape and topology of these assemblies. Moreover, methods for size and mass analysis may be used to study the assembly and dissociation behavior of these assemblies as a function of time or under the influence of changing environmental conditions. Here we review emerging analytical methods to characterize supramolecular assemblies, ranging in mass from approximately 100 kDa to 100 MDa, spanning between 1 and 100 nm in diameter. A large portion of the work described focuses on protein assemblies, as they are important in a variety of biological processes and are relatively amenable to study. We focus on native mass spectrometry (native MS) in particular but cover a range of other electrospray ionization (ESI)-based methods as well. We also highlight a selected number of applications that are illustrative of the current state of these analytical approaches for the size and mass analysis of protein assemblies.

2. ANALYTICAL CHALLENGES IN THE MASS ANALYSIS OF PROTEIN ASSEMBLIES

Whereas it has become relatively straightforward to obtain a list of protein interaction partners with affinity-purification and MS (AP-MS)-based identification protocols, more precise structural analysis of a protein complex presents a much bigger analytical challenge (3–6). AP-MS methods do not generally yield information on the size of a protein complex or the stoichiometry of the coassembled subunits, and also often do not disclose important information on cointeracting DNA, RNA, and small molecules (nucleotides, lipids, etc.). It is also not usually possible to distinguish between different coexisting states of higher-order oligomers. To obtain a more complete picture of a supramolecular protein assembly and its composition, it is thus necessary to analyze the intact assembly. Moreover, if the analytical approach allows one to further dissect the purified protein complex from the top down, additional information on protein subunit arrangement and shape can be obtained.

There are several challenges associated with a comprehensive analysis of protein assemblies. For instance, protein complexes can range from a few kilodaltons to several tens of megadaltons in molecular weight (see **Figure 1**), which would ideally be covered by the same analytical approach. Whereas the core assembly of a protein complex is often formed through stable high-affinity interactions, accessory components may be weakly bound and transiently associated. The transient nature of the assembly requires that the analytical separation and detection are relatively fast, so as to prevent smearing as often observed in gel electrophoresis or size-exclusion chromatography. In other words, when analytically separating complexes of different size/mass, the mass information must be extracted before dissociation or reassociation can take place. As a result, many commonly

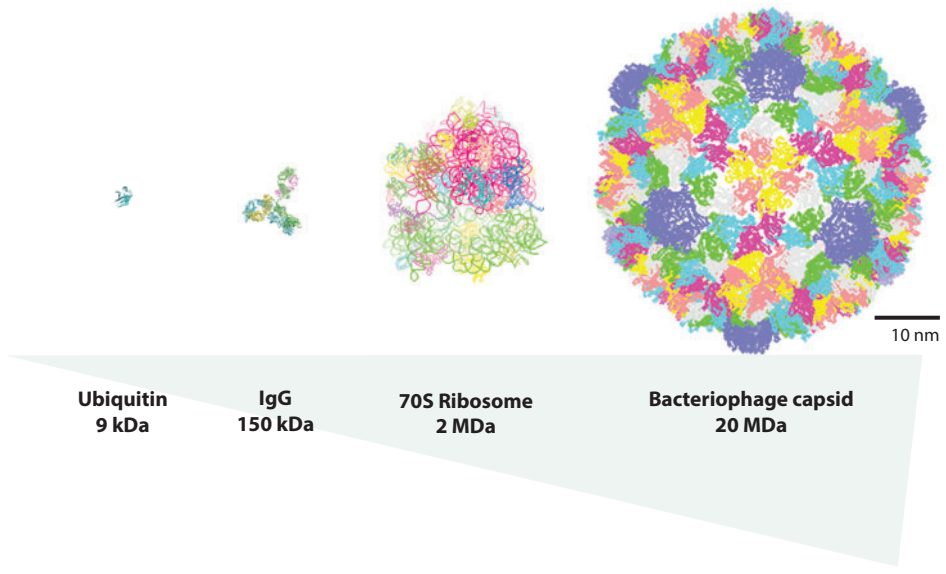


Figure 1

Structural models of ubiquitin, IgG, the ribosome, and a virus capsid illustrating the size and mass span of some biologically relevant protein complexes.

used techniques for determining protein size and mass, such as light scattering–based techniques, are unable to efficiently distinguish multiple coexisting states due to ensemble averaging or a lack of resolution. Many proteins within protein complexes are also often decorated by posttranslational modifications and can bind small molecule substrates or effectors such as nucleotides, lipids, or sugars, yielding very subtle variations in mass that can be uncovered only with high-mass resolution and precision. Generally speaking, the shorter the timescale of analytical separation and detection and the higher the precision and accuracy of the mass determination, the more information can be gained on the protein complex under study.

3. ELECTROSPRAY IONIZATION AS A SOURCE OF GAS-PHASE IONIZED PROTEIN ASSEMBLIES

Because most mass analyzers are operated under high vacuum, protein complexes need to be ionized and carefully transferred into the gas phase. In most approaches, ESI is the method of choice (7–10). In ESI, an analyte solution is transferred into a conductive capillary. A potential is applied between the capillary and the inlet of the mass spectrometer. This potential difference leads to the formation of a so-called Taylor cone at the tip of the capillary (11). Small charged droplets containing the analytes are formed at the tip of the Taylor cone by jet fission. Solvent evaporates from the droplets, which increases their charge density. Once the charge density reaches a certain limit, i.e., the Rayleigh limit, the droplets may undergo a Coulombic-driven explosion and jet fission. This process repeats until the droplets are so small that only a single analyte molecule is contained within them. As the remainder of the solvent evaporates, part of the excess charge on the droplet surface is carried over to the analyte, forming multiply charged (protonated) ions (according to the charge residue model of ESI) (9, 11, 12).

Whereas the solvent-free analysis of protein complexes does offer some advantage (i.e., interpretation of the obtained masses is not complicated by additional mass that originates from hydration layers, counterions, etc.), special care needs to be taken so as not to disrupt the native contacts of a protein complex or introduce gas phase–related artifacts to its structure. Several experiments and computational studies have pointed out that whereas some structural rearrangements do take place, much of the backbone solution-phase structure can be retained following complete desolvation (under carefully controlled conditions with minimal ion heating) (13–15). ESI is generally considered to be one of the most gentle ionization methods available and is the preferred ionization method in most MS studies of protein complexes. By using lower flow rates through the capillary, the initially formed droplets are smaller, and fewer fission events take place before effective ionization of the analyte, which makes ionization even more gentle (11, 16). Scaling down to flow rates of only a few tens of nanoliters per minute is known as nanoESI and is the preferred ionization method in most native MS studies (see Section 5). It offers the additional advantage of enhanced sensitivity.

The use of ESI in MS does pose a few restrictions on experimental design. It is for instance not feasible to analyze the protein complexes in buffers that are typically used in biochemistry and molecular biology (i.e., solutions containing high-salt concentrations, Tris, or HEPES, etc.). These buffer components ionize efficiently and have millimolar concentrations, thereby dominating the spectrum, and they may suppress ionization of the protein analyte, typically present at micromolar concentrations. Moreover, these buffer components may form adducts with the protein complex under study, which complicates interpretation of the obtained ion signals and distorts spectral quality. One is therefore restricted to the use of volatile buffer components such as ammonium acetate, ammonium bicarbonate, or the recently introduced ethylenediamine diacetate (EDDA). Of these examples, ammonium acetate is by far the most commonly used for the analysis of intact protein complexes, even though it has a very limited buffer capacity. Ammonium bicarbonate has a higher buffer capacity but may cause protein unfolding at the gas-water interface introduced by bubble formation of the CO_2 gas (17). EDDA was recently introduced and shown to be particularly suitable for the analysis of the ATP-binding GroEL chaperone, resulting in very good desolvation and high-mass resolving power (18).

There are also concentration limits for the analysis, especially when using nanoESI. The lower limit depends on the sensitivity of the mass analyzer (it is typically approximately 100 nanomolars in native MS studies employing nanoESI), whereas at higher concentrations (more than several tens of micromolars) the crowded solution starts to restrict flow through the ESI capillary. When studying protein-ligand interactions, high concentrations of ligands in ESI will generate protein ions with nonspecifically bound ligands (the higher the concentration, the more likely a free ligand will share a droplet with a free protein and adsorb onto the protein as desolvation proceeds).

ESI generally yields a distribution of multiply charged ions. The number of charges that is carried over to the protein complex depends to some extent on the conditions of the spray solution but is dominated by the solvent-accessible area of the analyte (19). The charge-state signature of a protein ion can thus be used to monitor conformational changes (that result in large changes in solvent accessibility) and is sometimes used to monitor protein folding (20, 21). Several supercharging and charge-reducing agents have been described that allow one to manipulate the average number of charges that is acquired by the analyte (22–26). As most MS approaches yield information on mass-to-charge ratio, charge-state assignment is crucial for calculating accurate masses and is rather straightforward for highly resolved spectra of relatively small protein complexes (charge states can be calculated from matched peaks) (27). Specialized software has been described to aid in the interpretation of more complicated spectra and a more elaborate strategy for

charge-state assignment is often required in the cases of complicated spectra and lesser-resolved peaks (28–31).

4. ALTERNATIVE IONIZATION METHODS

Although ESI is widely reported as the most suitable ionization technique for the analysis of noncovalent protein complexes, alternative ionization techniques have also been successfully applied, which we mention here briefly. In particular, large noncovalent assemblies have been studied using matrix-assisted laser desorption/ionization (MALDI) and laser-induced liquid bead ion desorption (LILBID) (32–34). In the case of MALDI, the analyte is embedded in a dry matrix that, upon laser excitation, ionizes, desorbs, and transfers charge to the analyte. In LILBID, microdroplets are excited at the absorption maximum of water, which, beyond a certain threshold laser intensity, disrupts the droplet through which the analyte is transferred into the gas phase. Most notably, the use of MALDI and LILBID has facilitated the analysis of intact IgG antibody, RNA polymerase, detergent-solubilized membrane proteins, amyloid assemblies, and virus particles.

5. NATIVE MASS SPECTROMETRY

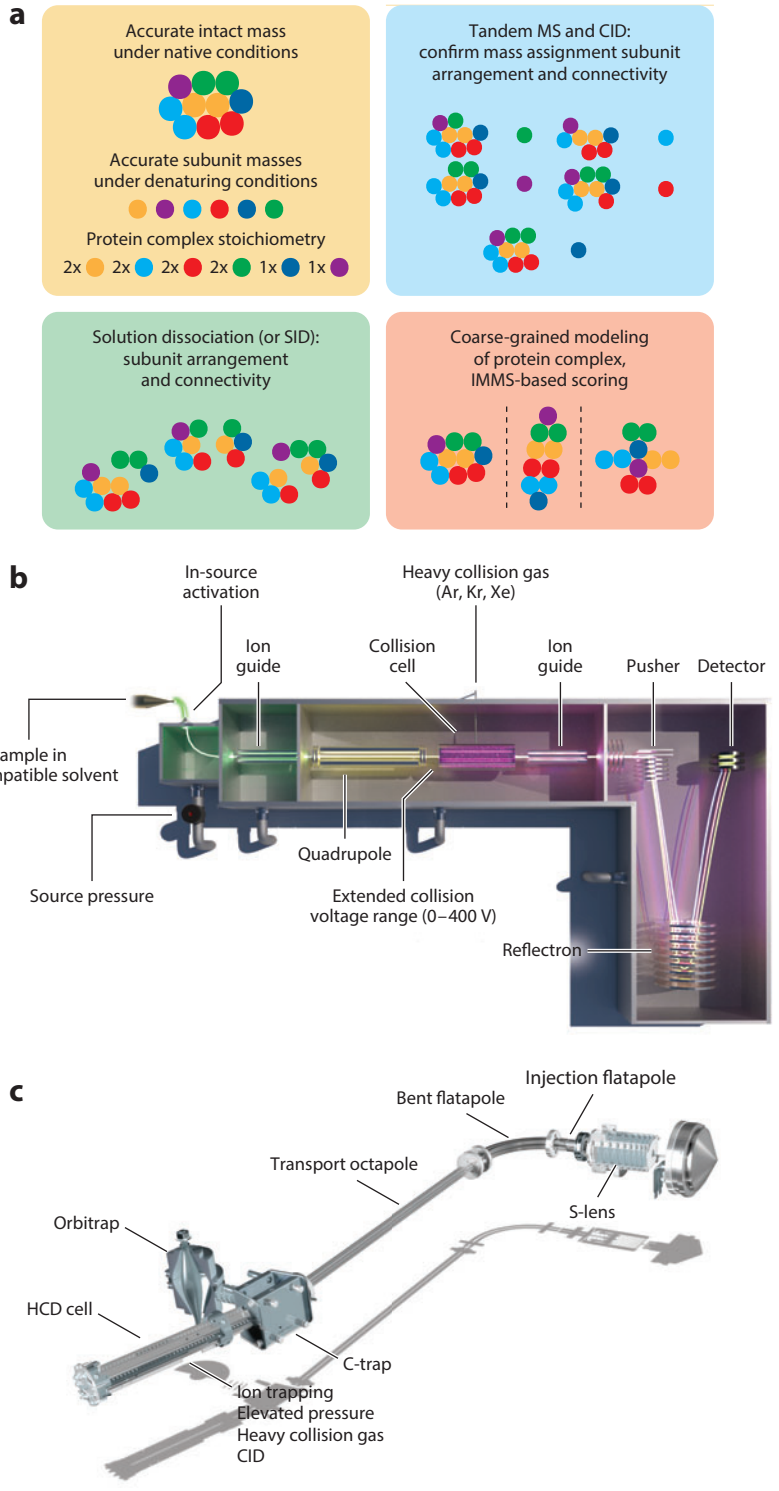
Native MS is a term coined to describe the mass analysis of noncovalent protein complexes under nondenaturing conditions, meaning that protein complexes are analyzed in buffered aqueous solution, as close to physiological conditions as is still compatible with ESI (35, 36). The information that native MS offers on protein complexes is outlined in **Figure 2*a*** and discussed in detail in this section. Most reported native MS studies are carried out with Time-of-Flight (ToF) mass analyzers because of their superior resolution and sensitivity for high mass, or m/z, ions. In ToF analyzers, the mass-to-charge ratio of an ion is determined by measuring its flight time along a defined path in the analyzer. These instruments are often slightly modified versions of commercially available hybrid quadrupole Time-of-Flight (QToF) instruments, where most modifications are aimed at optimizing transmission of high-mass ions (37–39). A schematic of a QToF for native MS is presented in **Figure 2*b***.

Whereas the standard commercial instruments are operated at a typical pressure of 1 millibar in the source region, the transmission of large ions is greatly improved by increasing the pressure to approximately 10 millibars (40, 41). The increased pressure focuses large ions by collisional cooling, and a similar approach is often also employed in the multipole ion guides of high-mass QToF instruments (42). This can be achieved by fitting a flow-restrictive sleeve on the ion guide or by feeding a gas line into the pumping stage. The use of heavier buffer gas (such as argon or xenon, compared to helium or nitrogen) further improves the transmission of high-mass ions. As with the source and transfer stages of the instrument, the collision cell is usually also operated at elevated pressures using a heavier inert collision gas (xenon, krypton) to further improve transmission. The heavy collision gas also aids in collisional activation of large ions as more energy is transferred per individual collision. This activation in the collision cell can also be used to improve spectral quality by stripping ions and/or buffer adducts from the analyte of interest (43). Sufficiently high activation leads to gas-phase dissociation, whereby the formed fragment ions can help confirm mass assignments and provide structural constraints on subunit arrangement (44–48). The ability for collisional activation has been further expanded in some instruments by extending the applicable DC collision potential to the collision cell (49).

Most ToF analyzers that are used in native MS have a nominal resolution ($M/\Delta M$) between 5,000 and 10,000. Compared to many other popular mass analyzers, there is only a shallow drop-off in resolution with increasing m/z, which is one reason why ToF analyzers are very suitable

Figure 2

(*a*) Illustration of different experiments used in native MS. (*b*) Schematic of a modified QToF instrument. Adapted from Reference 64, with permission. (c) Schematic of a native MS Orbitrap instrument. Abbreviations: CID, collision-induced dissociation; HCD, high-energy collisional dissociation; IMMS, ion mobility–mass spectrometry; MS, mass spectrometry; QToF, quadrupole Time-of-Flight.



for native MS studies. Despite the numbers for the mass resolution quoted above, the effective resolution on a large protein complex's charge state reported on these instruments is usually no higher than 1,500 and mostly only a few hundred. Some have argued that effusive expansion of the ion beam for large analytes (during transfer from high- to low-pressure ion guides) causes similar dispersion in the pusher region and therefore in the obtained flight-time profiles (50). However, the main cause of relatively wider peaks observed in native MS studies seems to be incomplete desolvation of the analyte (51). Many different neutral and/or ionic adducts (sodium, potassium, ammonium, acetate, water, etc.), differing only slightly in mass, can be attached on the same protein complex. Rather than measuring a bare protein ion, one measures the unresolved envelope of the protein with a variety of buffer adducts with closely spaced masses, leading to extensive peak broadening (52).

Buffer adduct formation becomes increasingly likely with larger protein complexes, resulting in broader peaks for higher masses. The current upper mass limit for native MS, still enabling charge-resolved ion signals, is estimated at approximately 20 MDa (51). Relatively little can be gained with an enhancement of the instrumental resolution of the mass analyzer, given that the underlying isotope distributions of all the different adduct peaks will also overlap extensively (and to achieve isotopic resolution on such large analytes at high m/z is currently simply unfeasible). For this reason, the resolving power (and accessible mass range) in native MS studies could be enhanced greatly if buffer adduct formation could be reduced further, moving toward complete desolvation.

Although the ToF has traditionally been the mass analyzer of choice, Fourier transform ion cyclotron resonance (FT-ICR) and Orbitrap mass analyzers are also used to study large protein assemblies. Several native MS studies using FT-ICR mass analyzers have been reported, including the study of protein-protein and protein-oligosaccharide interactions, some of the first systematic studies of gas-phase dissociation kinetics of protein ions and the use of alternative activation schemes for high-mass ions, such as electron capture dissociation (53–57). It was recently shown that the Orbitrap mass analyzer could also be modified to become suitable for native MS (58). Rather than measuring the flight time, the Orbitrap measures the axial frequency of oscillation of trapped ions along a central electrode (59, 60). A schematic of the Orbitrap platform used in those studies is presented in **Figure 2*c***. Unlike the ToF instruments, the front end of the native MS Orbitrap is not operated at elevated pressures. The only differences compared to its use for analyzing small molecules are tuning of the DC potentials of the ion lenses and trapping of the ions in the high-energy collisional dissociation (HCD) cell (which is operated at elevated pressure using a heavier collision gas), before injection into the Orbitrap. It was shown that this Orbitrap platform has high sensitivity and superior resolving power in native MS applications (58, 61), which, for the reasons outlined above, is most probably due to the difference in the front end of the instrument (which apparently promotes loss of buffer adducts), whereas the higher nominal resolution of the analyzer has a relatively minor contribution to its higher performance.

In the past 20 years, native MS has established itself as a valuable tool in studying protein complex composition, structure, and assembly (36). Mass analysis with a precision that is routinely below 0.01% has been conducted on various protein complexes. Recent reports include membrane protein complexes (solubilized in detergent micelles) (62–64), which are particularly challenging for many techniques in structural biology, as well as 18-MDa virus capsids (51), which are thought to be the biggest molecules accessible to the technique with the current state of instrumentation. The resolving power of the technique is often sufficient to monitor posttranslational modifications (such as glycosylation and phosphorylation) and small ligand binding on intact protein complexes up to the megadalton range (18, 58, 61). Data acquisition takes on the order of minutes between samples, allowing kinetic measurements of relatively slow reactions

in the minutes-to-hours timescale (65). Studies on amyloid formation and chaperone and virus assembly illustrate how multiple co-occurring states can be separated, detected, and characterized simultaneously with native MS (66–68). The quadrupole mass filter on the hybrid QToF setup allows one to study the gas-phase stability and subunit arrangement of individual components from these complex mixtures by tandem MS using collision-induced dissociation (CID).

CID is a so-called slow-heating activation process, and it typically results in gradual unfolding of single subunits that are ejected from the complex with a particularly high number of charges (protons are mobile on the protein and rearrange to occupy the newly available surface on the unfolded subunit) (69). Depending on precursor ion charge and the quaternary structure of the protein complex, alternative dissociation pathways may also be observed that include the dissociation of lower-charged compact subunits, dissociation of higher-order multimers from a complex or fragmentation of the peptide backbone (15). As a consequence of the slow heating of ions in CID and the associated unfolding of subunits, the amount of structural information that can be gained from CID is somewhat limited. Assuming peripheral subunits tend to dissociate first, the order of dissociation observed in CID can provide some clues as to the arrangement and connectivity of subunits. However, this is only useful when studying heteromultimeric assemblies, and the concept of a peripheral subunit is meaningless in the context of symmetric oligomers and ring-like structures. Alternative dissociation pathways where higher-order multimers dissociate are much more informative on subunit arrangement and connectivity. These alternative dissociation pathways can be achieved by using an alternative activation method called surface-induced dissociation (SID). In SID, ions collide with an inert surface to achieve dissociation of protein complexes. Because activation in SID is a single-step, high-energy event, subunit contacts are broken without the extensive unfolding that is observed in CID (70). Hence, it is more likely with SID to generate subcomplexes in the gas phase that are informative for resolving the quaternary structure of the assembly (71, 72).

Within the framework of the native MS workflow, additional structural information about the protein complex under study can be obtained by combining mass spectrometry with ion mobility spectrometry (IMMS) (73). Ion mobility is sometimes also used as a stand-alone technique to analyze the size of protein complexes, as is the case with differential mobility analysis (see Section 7.3). In IMMS, a hybrid instrument performs ion mobility separation and MS analysis of the protein ions in sequence (74–76). From the ion mobility of an ion, its collisional cross section can be calculated, a property that is largely determined by the rotationally averaged projection area of the analyte. The collisional cross section of an ion thus relates to its shape, and IMMS measurements have been used to determine the subunit arrangement of protein complexes and to monitor conformational changes. IMMS has also been pivotal in assessing the gas-phase structure of protein ions and has gone a long way to show that global aspects of solution-phase structure are preserved after desolvation (14).

The most popular drift-time separation techniques for native IMMS are drift tube ion mobility (DTIMS) and traveling wave ion mobility (TWIMS). In DTIMS, ions are separated on the basis of mobility by applying a static field on a drift tube that is densely filled with a neutral buffer gas. In TWIMS, ions are separated in a gas-filled stacked ring ion guide under the influence of a traveling wave potential. Whereas the collisional cross section can be directly deduced from DTIMS measurements, TWIMS requires careful calibration with known standards from DTIMS measurements (74, 77).

The collisional cross section has been used in two important ways to assess protein complex structure in the gas phase. In one approach, it is determined how the collisional cross section scales with the molecular weight of the protein assembly. This trend allows one to make the distinction between globular, ring-like, or extended sheet-like structures and has been used in the study of

amyloid and virus assembly (66–68). In a more systematic approach, experimental cross sections are compared to theoretically modeled projections of atomic or coarse-grained models of protein structures obtained by X-ray crystallography or electron microscopy (48, 78). By systematically scanning all possible arrangements of protein subunits in an unknown structure, the experimental cross section can be used as a constraint to exclude a large space of possible subunit arrangements.

6. HIGHLIGHTED RECENT APPLICATIONS OF NATIVE MASS SPECTROMETRY

6.1. F- and V-Type ATPases

A recent breakthrough in native MS has been the analysis of typically insoluble intact integral membrane protein complexes (62, 79). Membrane proteins are notoriously difficult to handle because of their poor solubility, which can be overcome by the use of detergent micelles. However, most ionic detergents are incompatible with ESI, but several nonionic detergents have recently been described to facilitate the analysis of membrane protein complexes with native MS (64). Most notably, recent studies on a variety of ATPases demonstrate how stoichiometry, subunit arrangement, and even lipid and nucleotide binding can be uncovered with native MS (see **Figure 3**) (63, 80). Related to this, attempts to analyze membrane proteins have also been successful using MALDI or LILBID MS, rather than nanoESI (32, 33).

6.2. Bacterial Immune Response–Related Protein Complexes

Recently, it was found that bacteria have defense systems against viral infections, generally known as clustered regularly interspaced short palindromic repeat (CRISPR) systems. In this defense mechanism, a protein-RNA complex plays a pivotal role, termed cascade in *Escherichia coli*, which targets RNA molecules encoded in the genome of the bacteria toward viral DNA, where, upon binding, it hampers viral replication, thereby suppressing infection. The stoichiometry and structures of a variety of such CRISPR-related complexes, which typically contain half a dozen different proteins and a crRNA sequence, have recently been elucidated with the aid of native MS by detailing the stoichiometry, demonstrating subunit connectivity with solution-phase dissociation, and determining subunit arrangement and shape from IMMS (see **Figure 4**) (81–83). The resulting models of the CRISPR-related complexes were shown to be in excellent agreement with cryoEM reconstructions of the same complexes and a critical first step in assigning those EM densities. These studies highlight how native MS serves as a valuable complement in the toolbox of structural biology. The use of solution-phase dissociation and IMMS to determine the topology of a protein complex has been reported in several studies and is becoming an integral part in the native MS workflow (48, 81, 84, 85).

6.3. Antibody Dimerization

Binding constants of protein-protein and protein-ligand interactions can be determined from titration experiments, using native MS as a readout to quantify binding (86–88). As mentioned above, care needs to be taken to ensure that no ESI- or MS-related artifacts are introduced in such analyses. A recent example of the use of native MS to determine binding affinities comes from a study where the determinants of antibody half-body dimerization were uncovered (see **Figure 5**) (89). The binding affinities for dimerization were determined in engineered half-molecules of the IgG4 antibody by native MS. Titration experiments with native MS were used to determine

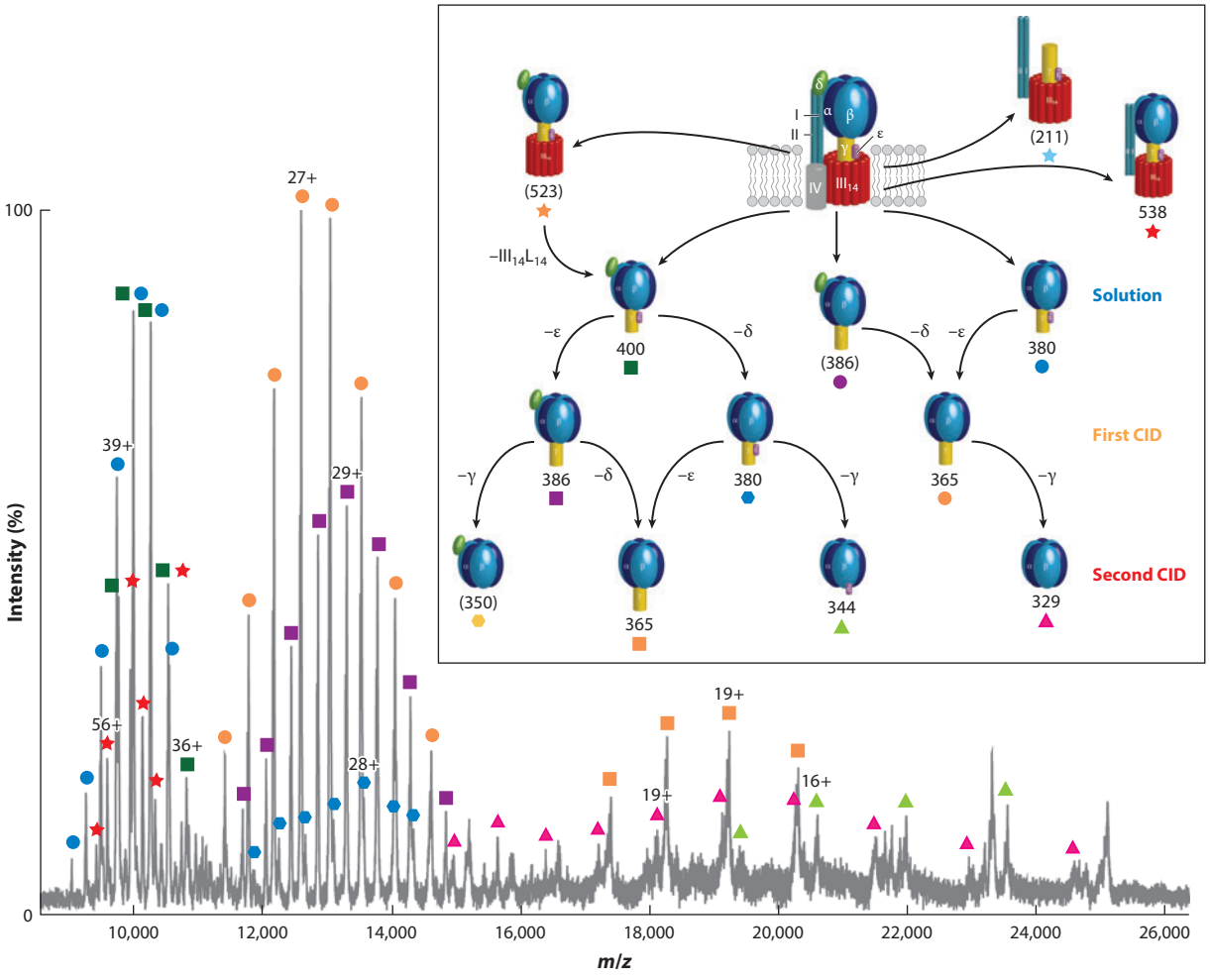


Figure 3

Highlighted application of native MS: subunit arrangement and connectivity from dissociation approaches. Native MS of detergent-solubilized F-ATPase membrane protein complexes. Subunit connectivity was deduced from CID pathways. Taken from Reference 80, with permission. Abbreviations: CID, collision-induced dissociation; MS, mass spectrometry.

the effect of a range of mutations on the dimerization constant of the half-body to reveal which regions are particularly important. Moreover, time-resolved measurements of antibody mixtures of different masses allowed the kinetics of subunit exchange to be determined. The mass-tagged mixing of subunits to determine subunit exchange between complexes has been reported on several occasions (90–92).

6.4. Small-Molecule Binding to Large Protein Assemblies: GroEL with Adenosine Triphosphate and Adenosine Diphosphate

Many supramolecular assemblies rely on small-molecule effectors or substrates for their biological activity. Binding of nucleotides such as ATP is very common but not easily studied in large protein

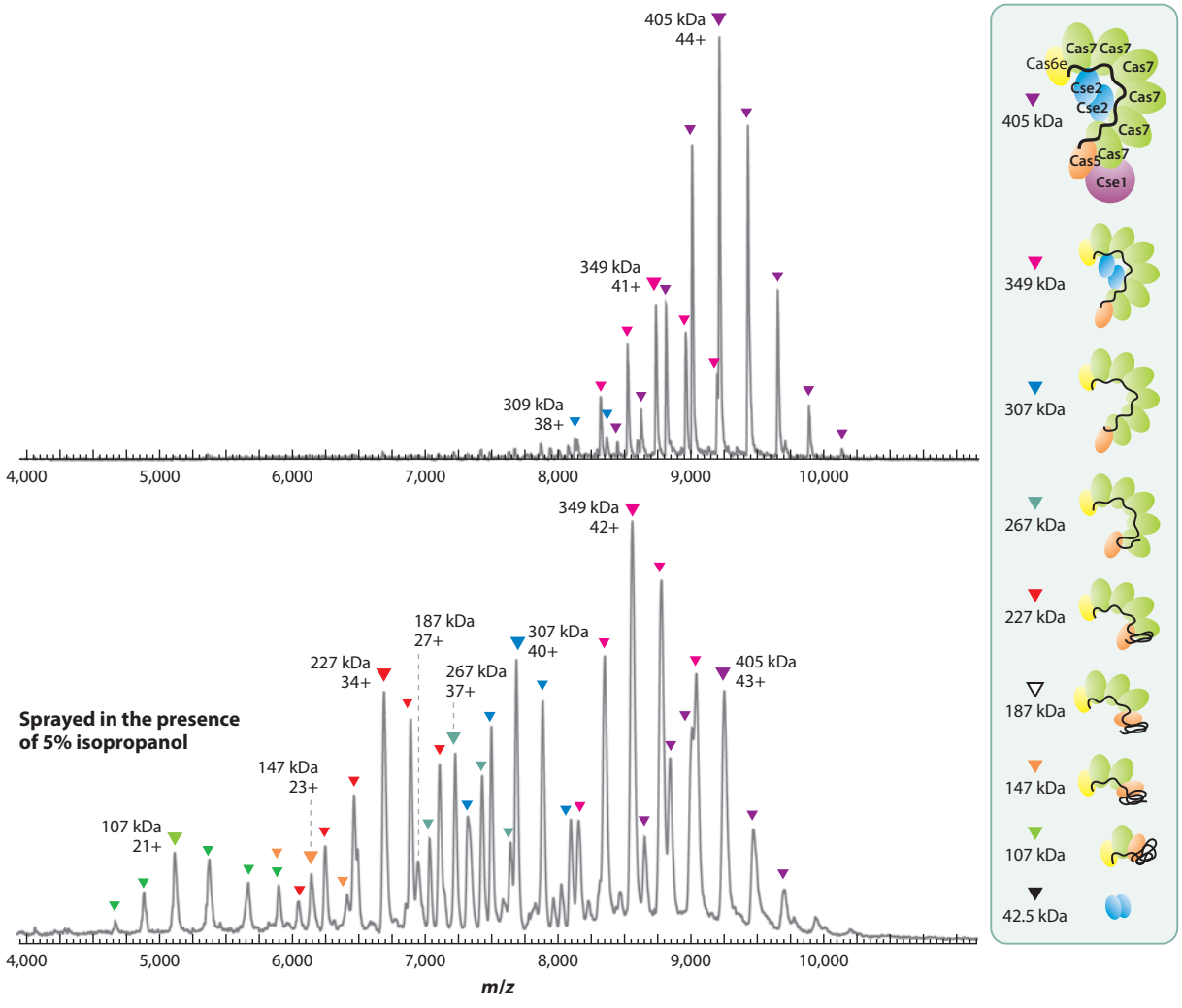


Figure 4

Highlighted application of native MS: subunit arrangement and connectivity from dissociation approaches. Native MS with solution-phase dissociation to determine subunit connectivity of the CRISPR-related complex cascade. Taken from Reference 81, with permission. Abbreviations: CRISPR, clustered regularly interspaced short palindromic repeat; MS, mass spectrometry.

complexes. Information on the ensemble state of a multiprotein complex for ATP occupancy is particularly challenging for many techniques with relatively low resolution. It was recently shown that binding of individual ATP/ADP molecules could be resolved on the 800-kDa GroEL chaperone using the modified native MS Orbitrap platform, which would facilitate detailed studies on small-molecule binding on supramolecular assemblies (see **Figure 5**) (58). Binding of ADP or ATP could be easily distinguished, even though this represents a mass difference of only 80 Da on a total mass of 800 kDa (i.e., 0.01%). With the use of an EDDA solution, rather than the more common ammonium acetate, it was recently shown that ATP binding could also be resolved on a more conventional QToF instrument (18). Here, it was demonstrated that native MS can be used to characterize the ensemble state of ATP binding on GroEL, and this information was used to

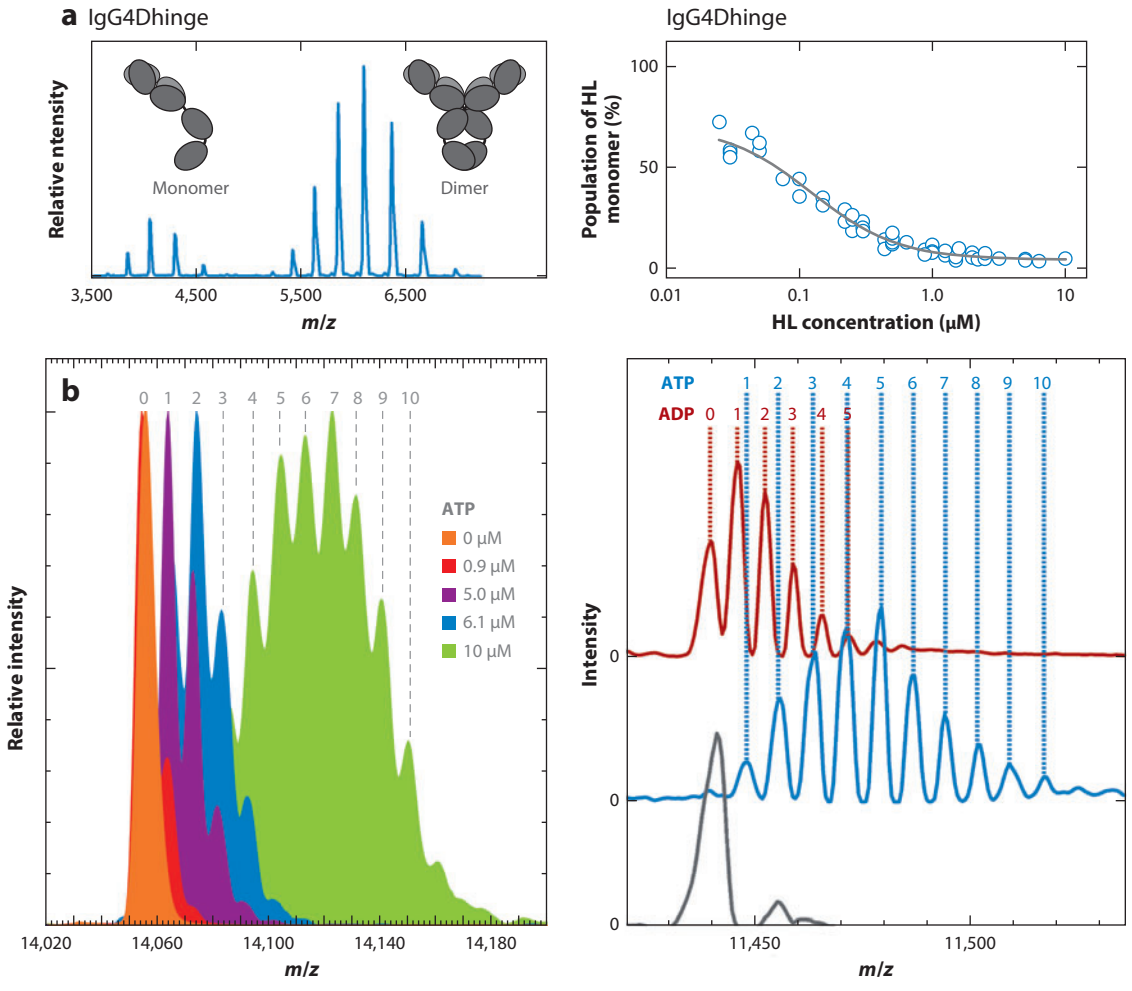


Figure 5

Highlighted applications of native MS: protein-protein and protein-ligand binding. (*a*) Antibody dimerization with native MS. "HL" refers to the "hinge-less" antibody monomer. Taken from Reference 89, with permission. (*b*) (*left*) ATP binding to the chaperone GroEL in EDDA buffer, analyzed on a QToF instrument. Taken from Reference 18, with permission. (*right*) ADP/ATP binding on GroEL in ammonium acetate buffer, analyzed on an Orbitrap instrument. Taken from Reference 58, with permission. Abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; EDDA, ethylenediamine diacetate; HL, hinge-less; MS, mass spectrometry; QToF, quadrupole Time-of-Flight.

determine the allosteric mechanism of ATP binding. These data clearly demonstrate that native MS may provide an ideal tool to monitor effector and/or drug binding to proteins and protein assemblies.

7. ALTERNATIVE APPROACHES IN THE DETERMINATION OF THE SIZE AND MOLECULAR MASS OF PROTEIN ASSEMBLIES

As described above, ToF MS detection is still predominant in native MS applications, but alternative means to sense and record the mass of a particle are emerging, being explored, and present

interesting alternatives for the analysis of protein complexes in the mass range between several kilodaltons to megadaltons.

7.1. Charge and Image Current Detection for Single-Particle Mass Spectrometry

Protein complexes acquire tens to hundreds of charges in ESI. This high number of charges makes it possible to detect single ions by measuring the image current they induce on a variety of detection plates, such as those used in charge-detection MS coupled to ToF, FT-ICR, and Orbitrap MS.

In FT-ICR and Orbitrap mass analyzers, imaging currents are typically induced and detected when an ion packet, containing typically thousands of ions, is in coherent orbiting motion. After being amplified, these currents can be digitized, after which these time-domain signals are Fourier-transformed to yield a mass spectrum. To induce a recordable image current, ~100 charges are needed. Smith and coworkers (93) pioneered the application of FT-ICR to measure the masses of single ions of macromolecular protein assemblies and DNA. They demonstrated the detection of single ions of bovine serum albumin dimers carrying only 60 charges (94). Moreover, using FT-ICR and individual ion detection, large pieces of ~100-MDa DNA and circular double-stranded plasmids (~1.9 MDa) extracted from *E. coli* could be analyzed (95). The plasmid ions trapped in the ICR were reacted with acetic acid molecules to induce shifts in the charge state. Measurements of the multiple peaks arising from the charge-state shifting provided masses of the individual ions with an average accuracy of 99.8%. More recently, Makarov and coworkers (58, 96) have shown that single-ion detection can also be achieved for highly charged proteins and protein assemblies using Orbitrap mass analyzers.

Several groups have explored and constructed in-house charge-detection devices for the analysis of large ions in ToF mass spectrometers. Pioneering work by Fuerstenau & Benner (97) provided an instrument capable of analyzing highly charged macromolecular particles ionized by ESI that contained a very sensitive amplifier, which detects the charge on an ion as it passes through a tube detector. The tube (length of 36.1 mm and a bore of 6.35 mm) acts both as a ToF mass analyzer and as a detector. It must be aligned with the ion beam axis, allowing ions to pass through the tube one at a time. This detected charge is then amplified and measured. This approach has the advantage of being simple and cheap to produce. A velocity measurement of the ion together with its known electrostatic energy provides the ion's mass-to-charge ratio. They demonstrated initially that, with this instrument, the molecular weight of single ions of DNA with masses of ~1 MDa and charge numbers in excess of 400 could be measured. Later on, it was also used to analyze intact viruses (introduced by ESI), namely the rice yellow mottle virus that consists of a single-stranded RNA surrounded by a homogeneous protein capsid with a mass of 6.5 MDa and the rod-shaped tobacco mosaic virus with a mass of ~40 MDa (98).

More recently, a few groups revisited the concept of charge-detection MS for the analysis of macromolecular ions using a (linear) array of charge detectors enabling multiple image charge measurements per ion (99–101). These detectors consist of multiple collinear tubes differentially isolated and connected to amplifiers to reduce the noise and improve the detection limit. For instance, the image charge-detection mass spectrometer of Smith and coworkers (93) encompassed an array of 22 charge-detection tubes, arranged coaxially and divided into two sets of 11 detectors. They claimed that the correlation approach they used to analyze the output from the image charge detectors provides advantages over using a Fourier transform in terms of signal-to-noise ratio. Using correlation analysis, the achievable noise level with the 22 detectors was approximately 10e for a 500 m/s ion. They used the device to measure polyethylene glycol and found that the measured

charge and molecular weight was in agreement with the expected polymer size of 300 kDa. Later on, it was shown that cytochrome c and ADH monomers can be detected and mass analyzed in their device.

The charge-detection approaches outlined above seem to be applicable to protein complexes larger than those accessible to the wider reported native MS approach. These techniques cover a very wide mass range, but they still suffer from a comparatively low precision. The uncertainty in the obtained masses makes for a less powerful constraint in determining complex stoichiometry and composition. Whereas single-ion detection does have a ring of sensitivity to it, the reality is that transfer of the analytes into the MS device is often the bottleneck, such that solutions in the high micromolar range are required for analysis. The generation of ions by ESI, combined with single-ion detection, does make it possible to uncover multiple co-occurring populations from heterogeneous mixtures, and therefore give these techniques a distinct advantage over conventional wet-lab techniques for the determination of protein complex size and mass. In addition, whereas resolution of subsequent charge states is an absolute necessity for precise mass determination in native MS, single-particle, charge-detection MS will yield a mass for every ion, no matter how heterogeneous the mixture of analytes is, which can often be problematic for "conventional" native MS, especially with larger, poorly desolvated ions. The ability of charge-detection MS to deal with particularly heterogeneous mixtures, especially of large protein assemblies, is illustrated in recent work on virus capsids by Jarrold and coworkers (102, 103).

7.2. Nanomechanical Mass Sensors

As another means to analyze macromolecules and nanoparticles, resonant micrometer-scaled cantilevers can be used as mass sensors (104). Cantilever-based mass sensors have been shown to ensure the sensitivity needed to measure the mass of not only single large protein assemblies, nanoparticles, and cells, but also of small molecules such as naphthalene and even Xe atoms (105). In nano(electro)mechanical–mass spectrometry (NEMS), analytes such as DNA (106), protein assemblies, or nanoparticles are introduced into the device by ESI, followed by desolvation and guidance by ion optics toward the detector (105, 107, 108). The detector makes the major difference, as it is an ultrahigh-frequency nanoelectromechanical resonator. The vibrational frequency of a NEMS resonator is a delicate function of its total mass. Small variations in mass, as induced by adsorption of analytes onto the resonator, can measurably alter its resonant frequency. Even individual protein molecules induce an abrupt jump in the resonant frequency when they adsorb onto the sensor. These frequency shifts are proportional to the mass and position of adsorption on the resonator and can be recorded in real time (109).

Individual events can be detected by monitoring successive frequency shifts when a protein assembly of interest is electrosprayed into the instrument. Presumably only a small fraction of the electrosprayed molecules make it to the small resonator surface, and thus the overall detection sensitivity is likely not yet that great. The NEMS detector clearly seems to have a nearly unlimited mass range, capable of analyzing particles in the tens-of-MDa range. A current limitation seems to be the mass resolution and mass accuracy of such devices.

A nice recent application of NEMS has been in the analysis of human IgM antibodies (see **Figure 6**). IgM forms macromolecular complexes in serum, whereby biologically active isoforms can be tetrameric, pentameric, hexameric, or dipentameric assemblies of ~190 kDa subunits. For the prevalent pentamer isoform, an additional small protein (the J chain) helps link the assembly and contributes ~15 kDa to the total ~960 kDa mass of the complex. The NEMS spectrum, accumulated from 74 single-particle spectra, reveals particles of numerous different masses but clearly shows the pentameric IgM complex as the most abundant signal at 1.03 ± 0.05 MDa.

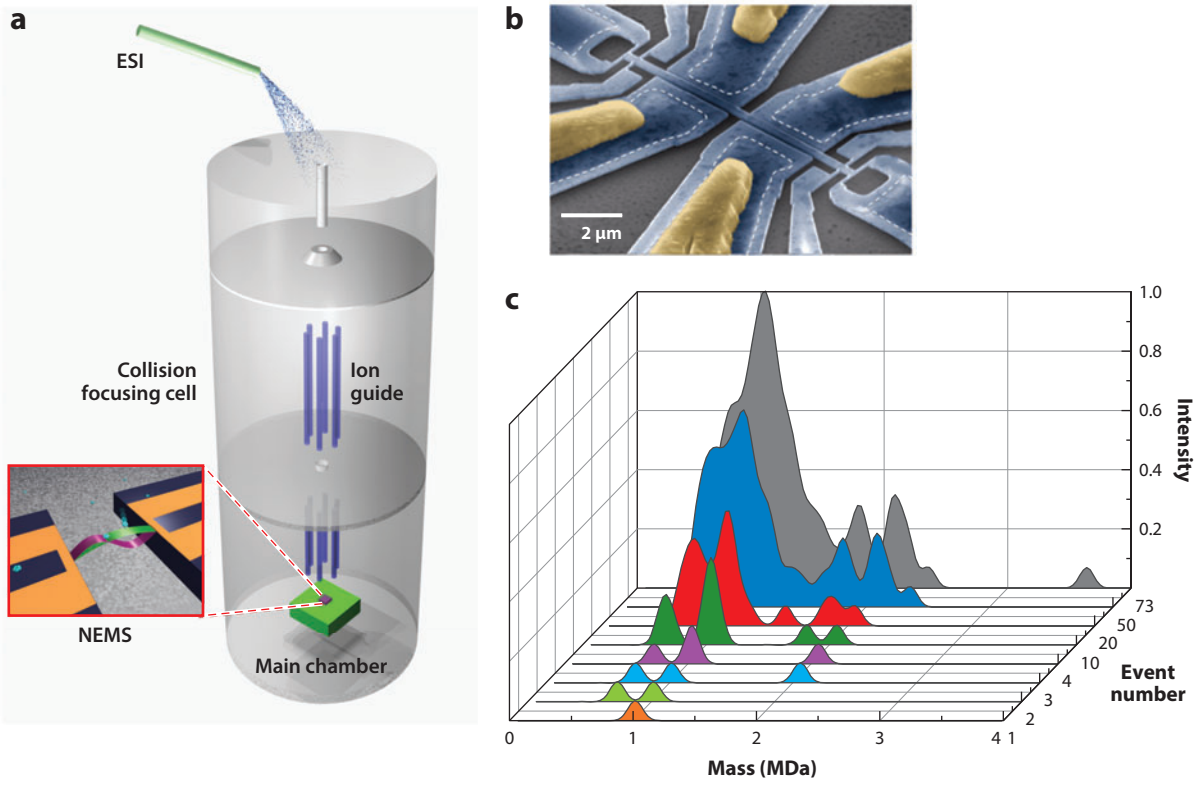


Figure 6

Single-particle MS with NEMS. (*a*) Schematic overview of a NEMS mass spectrometer. (*b*) Scanning electron micrograph of the NEMS device. The white dashed line shows the boundaries of the region beneath the suspended device that anchors it to the substrate. Yellow regions represent Al/Si gate contacts. Narrow gauges near the ends of the beam become strained with the motion of the beam, thereby transducing mechanical motion into electric resistance. (*c*) NEMS mass spectrum of IgM oligomers showing the cumulative spectrum for an increasing number of measured events. Taken from Reference 107, with permission. Abbreviations: ESI, electrospray ionization; MS, mass spectrometry; NEMS, nano(electro)mechanical–mass spectrometry.

Moreover, a dimerized pentameric complex at 2.09 ± 0.05 MDa is also present. These values are very close to the anticipated values 0.96 MDa and 1.92 MDa, which is remarkable as no calibration other than using the nominal mask dimensions of the mass sensor was applied. It is impressive that such data can already be acquired from analyzing only 74 particles, although it would also be interesting to see whether the convoluted spectrum still reveals the additional presence of multiple oligomers (trimer, tetramer, etc.) when the number of analyzed particles, and thus the statistics, could be increased.

7.3. Gas-Phase Electrophoretic Mobility Molecular Analyzers

Representing another interesting alternative to ToF analyzers for the analysis of macromolecular assemblies, ESI has been combined with gas-phase electrophoretic mobility molecular analyzers (GEMMA) (110, 111). Therein, the high charge of particles as obtained via ESI is first reduced by means of bipolar ionized air (typically generated by an α-particle source) to yield predominantly singly charged ions. These charge-reduced species are subsequently separated and sized by their electrophoretic mobility in an ion mobility drift cell. In GEMMA, the particles are typically

detected using a condensation particle counter. The resulting data can be converted into electrophoretic mobility diameters by applying the Millikan equation. These diameters can be converted to a mass spectrum as well, due to the generally good correlation between mobility size and molecular weight, although as in IMMS, exceptions to this rule may apply for nonglobular structures, such as shell-like viral capsid intermediates (66, 112). The GEMMA analyzer has an extended size range and has been successfully used for particles of sizes ranging from 3 nm to 100 nm, which covers a mass range of a few kDa (small proteins) to 100 MDa (whole viruses or even cell organelles and DNA) (113).

Applications of the GEMMA analyzer have ranged from analyzing antibody aggregation, macromolecular protein complexes (114), synthetic polymers (115), intact viruses (116), and lipoparticles (117). GEMMA analysis was successfully applied, for instance, on the 4.6-MDa cowpea chlorotic mottle virus (CCMV) (116) and the tobacco mosaic virus (117). Although the mass resolution of the GEMMA instrument is still too low to enable accurate mass measurement, GEMMA does provide parallel information about the electrophoretic mobility diameter of the analyzed particle. Such analysis indicated that the gas-phase CCMV particle had largely retained its quaternary structure as its measured diameter resembled that of the particle as measured by electron microscopy.

Loo et al. (114) reported a direct comparison of native MS using a QToF-type mass analyzer with GEMMA for studying the macromolecular organization and structure of the 28-subunit 20S proteasome from *Methanosarcina thermophila* and the mammalian (rabbit) proteasome. ESI-MS measurements with a QToF analyzer of the intact 690-kDa proteasome were consistent with the expected $\alpha_{14}\beta_{14}$ stoichiometry. Collisionally activated dissociation of the 20S gas-phase complex was applied on the QToF, resulting in the loss of only α-subunits, consistent with the known $\alpha_7\beta_7\beta_7\alpha_7$ topology. Moreover, the analysis of the binding of a reversible inhibitor to the 20S proteasome showed the expected stoichiometry of one inhibitor for each β-subunit. Using the GEMMA approach, an electrophoretic diameter of 15 nm could be measured for the $\alpha_7\beta_7\beta_7\alpha_7$ complex, in concordance with the diameter estimated from crystallographic measured electron densities. The authors concluded from their work that elements of the gas-phase structure of large protein complexes are preserved upon desolvation and that native MS and IMMS analysis can be used in a complementary manner to reveal structural details of the solution protein complex.

Although GEMMA does not provide the mass accuracy potentially achievable by native MS using ToF analyzers, it is not that much limited by microheterogeneity of the samples. An illustrative study by Allmaier et al. (117) presented differential analysis of intact very-low-density (approximately 35 nm), low-density (approximately 22 nm), and high-density lipoparticles (approximately 10 nm), which represent multifaceted heterocomplexes consisting of cholesterol, lipids, and proteins in different ratios. The measured EM diameter and narrowness of the peaks are indicative of the size and molecular complexity of the analyzed particles. HDL is the smallest/densest lipoparticle due to its high protein content (containing 50% proteins, 25% phospholipids, and 15% cholesterinesters). The broad distribution and bigger size of the VLDL particles correlate to its task of transporting endogenous lipids, and it is estimated to contain 50% triacylglycerols, 20% phospholipids, 10% cholesterinesters, and 10% proteins. These particles could be easily differentiated by GEMMA analysis but would likely present difficulties in native MS as their microheterogeneity would hamper charge-state resolution and thus accurate mass assignments.

8. CONCLUDING REMARKS AND FUTURE OUTLOOK

We have reviewed several electrospray-based analytical methodologies used for the size and mass analysis of naturally occurring as well as synthetically prepared nanoparticles. As the importance of

these particles is evident, in traditional biology as well as in nanotechnology and synthetic biology, the methods described here are essential for better particle characterization and understanding. Improvements are still needed in the areas of sensitivity, selectivity, specificity, and speed. At present, it seems that extending the accessible mass range to over several tens of MDa comes at the expense of mass accuracy, and thus specificity. Additionally, although single-particle analysis may provide certain advantages, current approaches still lack high sensitivity, thus requiring long analysis times. Hybrid technologies may emerge that provide valuable new approaches, either combining some of the analytical methods described above or combining those methods directly with, for instance, electron microscopy or (gas-phase) X-ray diffraction (118, 119). In conclusion, present-day ESI-based approaches have been very beneficial for the size and mass analysis of large protein assemblies and other nanoparticles, but there is still plenty of room and need for further future technology developments.

DISCLOSURE STATEMENT

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