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Native Mass Spectrometry: Recent Progress and Remaining Challenges

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Abstract

Native mass spectrometry (nMS) has emerged as an important tool in studying the structure and function of macromolecules and their complexes in the gas phase. In this review, we cover recent advances in nMS and related techniques including sample preparation, instrumentation, activation methods, and data analysis software. These advances have enabled nMS-based techniques to address a variety of challenging questions in structural biology. The second half of this review highlights recent applications of these technologies and surveys the classes of complexes that can be studied with nMS. Complementarity of nMS to existing structural biology techniques and current challenges in nMS are also addressed.

Keywords

native mass spectrometry; ion mobility; protein complexes; structural biology

INTRODUCTION

In this brief review, we first describe significant technological and methodological developments that have improved data quality and interpretation or enabled new measurements in native mass spectrometry (nMS) (Figure 1). The establishment of nMS as a powerful structural biology tool for biomedical research is then illustrated with several examples in the second half of this review. Limits on text length and reference numbers prevent us from comprehensively covering all contributions to this growing field; the reader is referred to the cited reviews for more detailed coverage.

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NATIVE MASS SPECTROMETRY TECHNOLOGIES

Sample Preparation and Separations

A critical step in an nMS experiment is the preparation or transfer of samples into an MS-compatible solution, which typically involves exchange into a solution of a volatile salt such as ammonium acetate. However, low concentrations of additional components can be retained if required for stability or activity (102). Traditionally, sample preparation is done offline, via buffer exchange spin columns, diafiltration, or dialysis. In recent years, however, more online sample preparation and separation methods have been developed, allowing for increasing throughput and sample complexity. A recent report demonstrated that rapid online buffer exchange (OBE) can be performed directly prior to nMS analysis using short size-exclusion chromatography columns, enabling the separation of proteins and protein complexes from nonvolatile buffer components (158). OBE enables higher throughput than offline buffer exchange and allows the sample to be kept in its preferred buffer until immediately before nMS analysis. More recently, OBE has been coupled with immobilized metal affinity chromatography OBE (IMAC-OBE) to screen overexpressed His-tagged proteins from cell lysates (16). Alternatively, for abundant overexpressed proteins, if lysis is performed in an MS-compatible buffer, then samples can be centrifuged and the supernatant directly used for MS analysis, reducing the sample purification steps required (161, 162).

Recently, a novel method for protein desalting was developed utilizing nanoscale nanoelectrospray ionization (nESI) emitters,which reduce initial droplet size and therefore the number of salt ions in each droplet, leaving fewer adducts on proteins after solvent evaporation (74). Nanoscale emitters permit analysis of protein complexes (153,154) and even membrane proteins (152) over a wide variety of buffer,salt,and detergent conditions and reduce sample preparation requirements.

The coupling of longer size exclusion chromatography (SEC) columns with nMS has also been demonstrated for the separation of protein mixtures directly prior to MS analysis, reducing signal suppression caused by differential ionization efficiencies in direct infusion experiments (32, 39). Online SEC-nMS has been used to study protein mixtures, thermally stressed biopharmaceuticals, and protein–ligand interactions (including nucleic acids) (32). SEC-nMS has the potential to not only increase sample throughput, but also widen the range of sample types that are amenable to nMS. Ion exchange has also been coupled with nMS, using a salt or pH gradient, and has been used to study antibody charge variants and a range of designed heterodimers of similar size but differing pIs (4, 23). Hydrophobic interaction chromatography (HIC) can have high separation power but has been challenging to couple with nMS, as it typically requires buffers with high salt concentrations. Even so, the coupling of HIC with nMS for antibody drug conjugates has recently been demonstrated via the incorporation of an SEC step after HIC separation to allow for salt removal (38). More recently, HIC was directly coupled with MS using MS-compatible buffers (165). As online separation approaches advance, enabling higher sample throughput and improving nMS’s amenability to more complex samples, we anticipate widespread adoption of nMS.

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Ion Sources for Native Mass Spectrometry

Retaining native protein structure during the transfer of analytes from solution to the gas phase is a key stage in then MS workflow and is typically accomplished by nESI (47, 166). Unfortunately, ammonium acetate and residual nonvolatile solution components can adduct with proteins, causing peak broadening and reducing apparent mass resolution. Several strategies have been developed to ameliorate this issue. Collisional activation is effective and widely used for removing adducts after the ionization stage but risks gas-phase restructuring (33, 48, 51, 169).

Aside from influencing gas-phase adduction, variations in source design and solution conditions can manipulate ion charge states, which can alter fragmentation pathways and/or gas-phase conformation (151). Charge-reducing reagents such as triethylammonium acetate (TEAA), ethylene diamine diacetate (EDDA), and trimethylamine *N*-oxide (TMAO) (83, 126) are commonly used to reduce ion charge state, resulting in more native-like fragmentation. In the past several years, many alternative reagents have been described, from imidazole derivatives (159) to alkali metal acetate salts (127) and polyamines (106). For membrane proteins in particular, charge-reducing detergents such as tetraethylene glycol monooctyl ether (C8E4) (90), *n*-dodecyl-*N*,*N*-dimethylamine-*N*-oxide (LDAO) (126), and oligoglycerol detergents (OGDs) (160) can be used, as well as reagents including TMAO (126), and polyamines (106).

Structural features of protein complexes can also be probed using newly developed variable temperature electrospray ionization (vT-ESI) sources, and readers are encouraged to consult Russell and coworkers (89) in this volume.

While nESI is the most commonly used method of ionization for nMS studies, desorption ESI (DESI) has also been shown to be a promising technique in such studies. Recently, the Robinson group demonstrated the utility of DESI in studying lipid and drug binding to a membrane protein [G protein–coupled receptor (GPCR)]. Analyzing membrane samples from a surface allows for the possibility of high-throughput screening and measurement of selectivity of agonists, and enables the formation of a more planar and spatially heterogeneous lipid distribution that is more representative of the cellular surface compared to solubilized membrane proteins (3). In addition, the Cooper lab has demonstrated the power of nano-DESI for nMS imaging of proteins and their complexes from tissue samples. This provides the exciting opportunity to study proteins from their native environment. Furthermore, Hale & Cooper (65) showed that it was possible to perform location-targeted top-down sequencing experiments with this setup.

New Paradigms in Mass Analysis

nMS has benefited from the widespread adoption of high-resolution, high-accuracy MS platforms. The challenges of transmitting, detecting, and resolving charge states and proteoforms of large, heterogeneous macromolecular assemblies have also prompted the development of novel measurement strategies, including collision cross-section (CCS) and charge detection mass spectrometry (CDMS) measurements in Orbitraps (83, 114, 128),

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electrostatic linear ion trap (42, 86) analyzers, and mass analysis of large complexes by mass photometry (146, 147).

CDMS in linear ion traps and Orbitraps has enabled ultrahigh mass analysis because it permits the independent measurement of ion mass and charge simultaneously (86).To this end,the Jarrold group (69) and Williams group (41, 42) have independently developed two distinct CDMS systems based around the electrostatic linear ion trap geometry with reflectron mirrors on either side of detection tubes. The charge accuracy of these devices is high compared to that of Orbitraps; the Jarrold group has demonstrated accuracies as low as 0.20e (87, 125) and detection limits of approximately 7e.

Because the Orbitrap uses image charge detection,CDMS is also possible on this platform (82, 168). The Orbitrap CDMS technique involves the collection of hundreds to thousands of low-intensity scans (time-domain transients corresponding to approximately single-ion events) and specialized data processing (81) to produce a mass spectrum. By allowing determination of charge states and reducing ion–ion interactions in the Orbitrap cell, CDMS has enabled ultrasensitive analysis of heterogeneous protein assemblies such as ribosomes and adeno-associated viruses (82, 168) and improved mass spectral resolution (83, 115).

Ion Mobility

Ion mobility (IM) enables separation of ions in the gas phase based on their rotationally averaged CCS and can provide a wealth of information on oligomeric state and conformation (8, 55). IM is often coupled with prior collisional activation [collision-induced unfolding (CIU) or surface-induced dissociation (SID)] to investigate the conformational space of proteins and their fragments.The main IM techniques employed for nMS are drift tube IM (DT-IM),traveling wave IM (TWIM), and trapped IM spectrometry (TIMS).

In DT-IM, analyte ions are propelled through a pressurized drift tube under a uniform weak electric field. DT-IM enables direct measurement of an ion’s CCS from first principles and therefore does not require calibrants (112). Typically, DT-IM is coupled to time-of-flight (ToF) mass analyzers, as the IM separation is slow (milliseconds) compared to ToF spectrum acquisition (microseconds); this coupling enables the collection of mass measurements across the full arrival time distribution (128, 148). Conversely, higher-resolution trapping MS instruments (i.e., Orbitraps) experience an inherent duty cycle mismatch, as IM separation and mass measurement scans occur on approximately the same timescale (76, 113). Recently, the Russell group developed a new IM-Orbitrap platform that overcomes this duty cycle mismatch. The group designed a reverse-entry ion source (REIS) and a periodic focusing DT-IM analyzer that retains the ability to measure CCS on first principles while also enabling high-resolution mass measurements on the Orbitrap (126, 127). This instrument was able to successfully measure CCS values of protein complexes from 8.6 to 810 kDa, demonstrating the power of this new platform to combine first-principles CCS measurements with high-mass-accuracy Orbitrap analyzers (114).

In TWIM experiments, an oscillating electric field is used to produce a traveling voltage wave that pushes ions through a drift gas. TWIM enables enhanced separation and the use of longer path lengths without significant ion loss compared to DT-IM but cannot directly

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measure CCS (60). Instead, calibrants consisting of ions of known CCS and charge states with similar properties (i.e., shape, mass, and charge) are used to determine the CCS of the analytes of interest (54, 149). Recently, Waters Corporation introduced the SELECT SERIES Cyclic IMS instrument containing a 98-cm path length traveling wave cyclic IM (cIM) device that enables single or multipass separations to increase resolving power. This new instrument has been used to measure conformations and unfolding pathways of monomeric cytochrome C and multimeric Concanavalin A proteins with considerably higher resolution than linear IM instruments (R up to approximately 750 for the reverse peptides SDGRG and GRGDS after 100 passes versus R approximately 45 on a Synapt G2) (40, 61, 62).

TIMS represents one of the newest IM methods and was recently commercialized by Bruker Daltonics. In TIMS, ions are exposed to a parallel gas flow that pushes them toward the detector, but their motion is opposed by an electric field. The field strength is slowly decreased, allowing ions to eject in order of decreasing mobility. Generally, CCS values are obtained based on a calibration curve (20, 130). TIMS has been used to study native protein complexes, protein– nucleic acid complexes, and protein–ligand complexes (17, 77, 78, 100, 123).

Activation Methods

The activation and subsequent dissociation of native-like ions provides important insight into their interactions, organization, inter subunit connectivity, and ligand binding sites. Several activation methods yield fragmentation pathways that correlate with secondary, tertiary, and quaternary structural features (66, 107).

In collision-induced dissociation (CID), ions are activated through a series of low-energy collisions with gas molecules. CID results in slow accumulation of ion internal energy that generally causes dissociation through the unfolding or elongation and subsequent ejection of a highly charged monomer (asymmetric charge partitioning) (46, 80). CID can also cause structural rearrangement without dissociation and should be used with appropriate caution when attempting to derive structural features of complexes (137). CID as part of a CIU experiment, however, has been used extensively to study protein unfolding and changes in protein stability (33).

Electron- and photon-based activation methods, including electron transfer dissociation (ETD), electron capture dissociation (ECD), ultraviolet photodissociation (UVPD), and infrared multiphoton dissociation (IRMPD),have been shown to preserve noncovalent interactions and/or produce backbone fragments that retain bound ligands. Protein backbone cleavages induced by these methods occur in regions of greater structural flexibility and surface exposure, providing insights into protein conformation, sequence, and ligand binding sites (178).

SID, i.e., collisional activation with a surface, has emerged as a promising activation method, as it has been shown to produce compact, charge-symmetric fragments that are reflective of the quaternary structure while also generally preserving bound ligands for a range of complex types (170). SID devices have been installed on a range of instrument

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platforms, and one is now commercially available on the Waters SELECT SERIES Cyclic IMS platform (144, 159). SID has been used to study the stability, structure, ligand binding, and assembly pathways of a range of protein complexes, such as membrane proteins, designed heterocomplexes, and large complexes such as the 20S proteasome (150).

Gas-Phase Chemistry

Once noncovalent complexes are in the gas phase, a wide variety of controlled chemical manipulations can be applied to probe them through ion–ion and ion–molecule reactions (49). Recent developments have focused on reduction of charge state by proton transfer charge reduction (PTCR) (75), cation to anion proton transfer reactions (CAPTR), electron transfer or capture, analysis of protein conformation by gas-phase hydrogen/deuterium exchange (HDX) (120), and other manipulations such as covalent cross-linking and multiply charged ion attachment (49).

PTCR is a tool for reducing spectral congestion by decreasing the charge states of ions at an analyte-dependent rate using ion–ion proton transfer to negatively charged reagent ions such as fluoranthene or perfluoroperhydrophenanthrene (71, 75), although it has been used only sparingly with nMS (6). A similar technique, CAPTR, involves a reaction between cation analytes (proteins) and reagent anions [e.g., perfluoro-1,3-dimethylcyclohexane (PDCH)] to reduce the analyte charge state (95). The Bush lab has published several studies using CAPTR to resolve mixtures of native proteins and complexes (e.g., 95). The Bush lab also showed that ubiquitin ions of various charge states generated from CAPTR of the 13+ precursor exhibited a range of CCS,despite originating from the same precursor structure (Figure 2), suggesting the occurrence of protein folding or restructuring in the gas phase. For the larger multidomain serum albumin (66 kDa), the final CAPTR CCS depended on starting solution conditions (57), supporting the kinetic trapping hypothesis that has allowed nMS to flourish.

Electron transfer and electron capture are equally viable methods for manipulating charge states of native protein complexes. Lermyte and coworkers (98, 99) demonstrated extensive electron transfer without dissociation (ETnoD) charge reduction for several proteins, to as low as singly or doubly charged species, on Synapt G2 and G2-S platforms using 1,4-dicyanobenzene and *p*-nitrotoluene as reagent anions. The Barran lab investigated conformations of cytochrome c and myoglobin, observing depletion of compact conformers after charge reduction and, as in CAPTR, changes in protein folding in the gas phase upon charge reduction (79). The Koltashov lab has used electron transfer charge reduction to determine the binding ratio of heparin of varying chain lengths with antithrombin-III (175) and has combined solution-phase supercharging and gas-phase charge reduction to investigate heterogeneous hemoglobin and haptoglobin samples (172).

Software Tools for Native Mass Spectrometry

Data analysis is often a bottleneck in nMS studies, but fortunately multiple tools have emerged in recent years to aid data interpretation. Software can be divided into two broad categories: tools to aid deconvolution (converting from *m/z* to mass domain) and tools for IM data interpretation. Data deconvolution can be performed using both commercial tools

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[e.g., Intact Mass from Protein Metrics (10) or BioPharma Finder from Thermo Scientific] and noncommercial options such as UniDec (111, 129) and iFAMS (27). Intact Mass (10) and MetaUnidec (129) nMS software have enabled batch deconvolution and reporting of data files, increasing data analysis throughput. Batch deconvolution coupled with automated sample running has the potential to transform nMS into a truly high-throughput method.

As discussed above, rotationally averaged CCS can be determined by IM. Combining experimental CCS with computationally determined theoretical CCS can provide additional information, allowing the comparison of models and proposed structures to experimental values (67, 114). Several approaches have been previously reported for CCS determination (108, 117, 140). The simplest approach, projection approximation (PA), models ions using a collection of overlapping hard spheres, with radii equal to the hard sphere collision distances (108). PA often leads to underestimation of the CCS, but it is a less computationally demanding method and is often used with an empirically derived correction factor (7). The PA model has been implemented in many forms, including in a tool called Ion Mobility Projection Approximation Calculation Tool (IMPACT), which can determine CCS from X-ray crystallography, nuclear magnetic resonance (NMR) spectroscopy, small-angle X-ray scattering (SAXS), and electron microscopy (EM) data (31, 110). An alternative method is the projection super approximation (PSA), which determines CCS in a similar fashion to PA but also accounts for collective shape and size effects, which can increase accuracy (11). The trajectory method is computationally more expensive but considers the ion as a collection of atoms and accounts for long-range interactions, collisions between the ion and buffer gas, and multiple collisions (117). A version of the trajectory method has recently been implemented into a package known as Collidoscope (44), which offers faster analysis than previous versions. Specialized tools have also been developed to analyze IM-based studies on the stability, unfolding, and interactions of proteins and complexes (2, 43, 118). Recent progress on computational tools for top-down MS and nMS can be found at http://nativems.osu.edu/training.

DRIVING BIOMEDICAL RESEARCH THROUGH NATIVE MASS SPECTROMETRY

As the rapid advances in nMS technology enable more in-depth analysis of a broader range of macromolecular complexes in the gas phase, the technique is gaining in popularity for driving biomedical research through collaborative structural biology. This section highlights selected achievements in which nMS provided critical structural information to complement other structural biology approaches.

Protein–Protein Complexes

Most proteins interact with other biomolecules to form assemblies essential for their biological function. nMS has emerged as a leading tool to characterize various properties of complexes, including their individual subunits, stoichiometries, relative binding affinities, and architecture (177). Ahnert and colleagues (1) used nMS and large-scale analysis of existing protein structures to elucidate some guiding principles of protein assembly and topology that can be accessed in the gas phase.

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Recently, Vimer and colleagues (160) integrated several nMS techniques to study structural features among 20S proteasome orthologs from five species (Figure 3). Each proteasome adopts a cylindrical structure composed of four hetero-heptameric rings (approximately 700 kDa), but these structures vary in complexity. Subunit connectivity was determined using SID and CID, while rough architecture was determined from CCS values calculated from IM. The kinetic stabilities of each complex were measured using CIU, and top-down MS^3 was used for sequence analysis. The combined results of these experiments demonstrated that these complexes vary in their size, kinetic stability, and subunit variants. The nMS results were corroborated by the solving of the rat 20S proteasome structure by cryo-EM, demonstrating how nMS approaches can reliably guide structural studies, even for complexes that lack high-resolution structures (160).

Many protein–protein interactions are mediated by specific inter- and intramolecular interactions. Double-mutant cycle analysis (DMC) is a strategy to measure the energetic coupling between specific amino acids at a binding interface or within proteins (122, 145). In DMC, two residues are mutated individually and in combination, and the effects on binding or folding are measured. If the residues interact directly or indirectly, then the effect of the double mutant will differ from the sum of the two individual mutations. DMC has been used to identify the binding location of capsaicin in the ion channel TRPV1 (171) and protein–peptide interactions (84) and has been combined with nMS to study protein–protein interactions in *Escherichia coli* lysates (30) and interprotein contacts in the gas phase (145).

nMS has also been used to probe endogenous protein–protein interactions in tissues and cells (9, 58, 121, 133). For example, Skinner et al. (141) used nMS and multistage tandem MS to identify and characterize 125 intact endogenous complexes and 217 distinct proteoforms from mouse heart and human cancer cell lines, providing insight into how protein complexes exist in the cell, including preservation of endogenous interactions, modifications, and ligands. The Sun group has developed and utilized native capillary zone electrophoresis (CZE) to separate heterogeneous protein complexes from ribosomal isolates and cell lysates (139). Further work with native CZE has focused on characterization of antibodies, as well as structural and conformational analysis of large protein complexes (21).

Protein–Ligand Complexes

Protein function is often regulated by conformational changes imparted by ligand interaction. For protein systems with multiple ligands, the binding cooperativity is also critical. Therefore, deciphering allosteric and cooperative properties upon ligand binding provides mechanistic insight into protein complex function. nMS has emerged as a leading tool to probe the stoichiometry, binding constants,and allosteric properties of ligand binding, as well as their effects on overall complex stability (63, 138).

For example, Holmquist and colleagues (72) used nMS to measure the cooperativity of tryptophan (Trp)–*trp* RNA-binding attenuation protein (TRAP), a ring-shaped homo-oligomeric complex composed of 11 subunits and 11 Trp binding sites located at the subunit interface. A Trp titration experiment and thermodynamic modeling revealed that Trp binds to TRAP according to a nearest-neighbor cooperative model whereby binding of Trp to one subunit modestly enhances Trp binding to immediately adjacent subunits (Figure 4). Some

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other notable examples of probing ligand cooperativity and allostery using nMS include studies of lipid binding to membrane proteins (28, 101, 124) and nucleotide and substrate binding to chaperones (37, 92).

Ligand binding sites can also be mapped using nMS coupled to single- or multistep activation techniques. In general, CID causes ligand loss and thus is not often used for these purposes. However, CIU can provide information on the overall conformational landscape and stability of a protein upon ligand binding (33, 45). Electron- and photon-based activation methods (e.g., ETD, ECD, UVPD) and SID generally preserve bound ligand, and the covalent fragmentation of the protein backbone in ETD, ECD, and UVPD can enable localization of ligand binding sites based on which fragments retain the ligand (178).

Nucleoprotein Complexes

Nucleoprotein complexes present some additional challenges in nMS analysis: (*a*) Nonvolatile cations such as $Mg^{2}+$ are sometimes required for assembly; (*b*) ionization suppression from metal ions and/or free RNA or DNA can result in weak signal intensity; (*c*) adduction of nonvolatile cations may reduce apparent mass resolution and accuracy; and (*d*) charge–charge interactions (common in nucleoproteins) are difficult to dissociate by CID, although they can be dissociated by SID in some cases (56, 109). Despite these challenges, nMS has been used to rigorously study many nucleoprotein complexes, including their stoichiometries, that have evaded analysis by complementary techniques due to their size, heterogeneity, and/or stability (22, 94, 134). nMS has also recently been used to measure the interaction between the SARS-CoV-2 nucleocapsid protein and a range of ligands including RNA, antibodies, and cyclophilin A (103).

When combined with IM and molecular modeling, nMS has also measured the stability, assembly pathways, and/or structure of nucleoprotein complexes, including DNA Pol III subcomplexes (109),nucleosomes (132), topoisomerase (77), aminoacyl-tRNA synthetase complexes (22), HIV-1 interaction with Gag protein (134), viral matrix proteins (94), and RNase P (93, 106). For example, nMS has been used to study the assembly pathways of Redβ oligomerization on DNA and has provided mechanistic insights into DNA repair processes in bacteriophage λ (18).

Top-down nMS has also been used to map binding locations of nucleic acids on nucleoprotein complexes and ligand binding on nucleic acids (135, 136, 164, 174). For example, Schneeberger and colleagues (136) used nMS and CID to locate the binding site of rev response element (RRE) RNA on rev protein complexes, an important step in HIV-1 virus assembly. The results demonstrate that rev protein initially binds to the upper stem of RRE IIB RNA but is then relocated to a binding site on RRE that enables rev protein dimerization, highlighting the utility of nMS techniques in probing the assembly pathways, stoichiometry, and binding interfaces of nucleoprotein complexes.

Membrane Proteins

Due to their low expression yields, heterogeneity, and requirement for solubilization in a membrane mimetic, membrane proteins have proven challenging to structurally characterize. nMS's low sample requirements and ability to handle heterogeneous samples have brought

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it to the forefront in membrane protein studies. An initial challenge in such studies was how to solubilize the proteins under nMS-friendly conditions (5). In recent years, multiple membrane mimetics have been utilized, including detergent micelles (91), nanodiscs (85), bicelles (73), amiphols (97), and styrene maleic acid lipid particles (SMALPs) (68, 70), and analysis has even been conducted directly from membranes and in destabilized lipid vesicles (25, 26). nMS reports on individual states, as opposed to a bulk ensemble measurement, and thus has revealed the specificity of lipid interactions, stability imparted upon lipid binding, and even the thermodynamics of binding (2, 12, 29, 90). In addition, nMS has been used to study nucleotide and drug binding to GPCRs, a particularly challenging class of proteins due to their low yield and instability post–membrane extraction (173). IM-MS has also been used to study the ATP-binding cassette transporter P-glycoprotein, demonstrating that the protein exists in an equilibrium between different conformational states that can be readily interconverted upon ligand and lipid binding (109).

nMS has recently been exploited to identify ligands and endogenous lipids bound to membrane proteins, which often appear as unassigned or poorly resolved density in solved structures (59). One such approach has identified endogenous lipids by combining nMS, controlled delipidation, and solution-phase lipid profiling techniques (64). Recently, a multistage MS approach (MS^n) was also presented in which membrane proteins are introduced into the MS within detergent micelles. In the first stage of activation, the protein complex is released from the micelle (MS^2); the assembly is then isolated and dissociated to release proteins or ligands (MS^3) for further fragmentation (MS^4) for proteoform sequencing or ligand identification. Using this approach, Gault & coworkers (59) were able to observe lipid binding and identify the endogenous lipid species to the outer mitochondrial membrane translocator protein (TSPO), which could then be fit into the previously solvedX-ray structure. Multiple lipids could often be modelled into poorly resolved maps, and thus, defining the lipid headgroup, side chain asymmetry, and chain length distribution can improve phospholipid modeling (59).

Antibodies and Glycoproteins

nMS, often coupled with liquid-phase separation techniques, has been applied to the study of antibody–drug conjugates, antibody–antigen complexes, and bispecific antibodies (19). While the intact mass alone can be informative (e.g., providing information on the extent of glycosylation or insight into antibody–antigen complexes), coupling with complementary methods such as activation can often provide more information. For example, the Ruotolo group has shown that intentionally unfolding antibodies with gas-phase collisions (known as CIU) and monitoring the unfolding with IM can distinguish among antibody isoforms containing different numbers and patterns of disulfide bonds and differing extents of glycosylation (155, 156). Glycosylation is the most complex protein modification and is not only essential for many cellular functions, but also often present on biotherapeutic proteins, influencing their efficacy and safety. The characterization of glycosylation is therefore of great interest, and a recent review has summarized the role of nMS in studies of glycosylation (151). The heterogeneity and flexibility of oligosaccharides often pose a problem for traditional, higher-resolution structural biology techniques, frequently resulting in proteins being deglycosylated before analysis, which has motivated the development

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of alternative methods. High-resolution nMS is proving useful in glycoprotein analysis, enabling the identification of previously unreported glycosylation sites on human C9 and C8 proteins (52, 53). High-resolution nMS can be coupled with complementary techniques such as IM, to obtain conformational information, or 193-nm UVPD, to map binding epitopes (100, 116).

Computationally Designed Proteins

Protein re-engineering and de novo design have great potential in the generation of novel materials for chemical and medical applications. Characterizing designs using traditional structural biology techniques can be time consuming and sample intensive. nMS has shown promise as a rapid,high-throughput method of screening different designs to confirm complex formation (158) and of studying pH-driven conformational changes (14), co-operativity of designed protein-logic gates (24), and transmembrane β-barrels (163). When coupled with SID, nMS has also been used to confirm subunit arrangement (23, 131). The study of designed protein complexes has also been aided by online native separations coupled with nMS (23, 158). In one such study, to test the interaction specificity of 16 heterodimer designs, the dimers were mixed, denatured, reannealed by dialysis, and then characterized using ion exchange chromatography coupled online with nMS. Significantly, the mixing experiments highlighted the specificity of the designs, with all 16 designed pairs recovered and only a low number of off-target dimers observed (23), as shown in Figure 5. The advent of CDMS has also aided in the characterization of designed multimeric complexes, particularly hexamers designed to form asymmetric virus capsids (176).

Measuring Large Molecules

While the mass range and transmission efficiency of conventional Orbitrap, FT-ICR, and ToF mass spectrometers are continuing to increase, unconventional means of mass analysis still lie at the forefront for measuring MDa-size particles (86). As described above, CDMS is pushing the limit of what can be measured, including intact virus particles (13, 35, 36, 105), exosomes (15), lipoproteins (104), amyloid fibers (34), and a 552-protein nuclear pore complex (NPC) from *Saccharomyces cerevisiae* (88) (Figure 6). The NPC gates RNA and proteins between cytoplasm and nucleoplasm (Figure 6***b***) and is amenable to measurement by CDMS (Figure 6***a***). The measured mass of approximately 80 MDa implies that the large complex and its many substituents remain intact and measurable by CDMS in the gas phase. A variety of complementary tools (e.g., cross-linking, cryo-electron tomography, etc.) were used in this study to determine the masses and orientations of the individual subunits in the complex. In another recent study, the SARSCoV-2 spike protein was characterized, better defining the heterogeneous glycosylation profile and average glycan mass (119).

Orbitrap mass spectrometry has also demonstrated the ability to analyze MDa-size particles, including viruses (50, 51, 82, 143, 157, 167, 168) and bacteria (142, 143). In one study, the Heck lab used an Exactive Plus Orbitrap to study the stoichiometry of viral and bacterial nanoparticles up to approximately 4.5 MDa, which was possible due to the reduction of rf frequencies throughout the instrument (143). Both conventional ensemble measurements and CDMS have been applied in these studies (82). For example, Kafader et al. (82) compared spectra of 27 nm (3.2 MDa) MS2 virus-like particles using standard ensemble MS

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measurements on an Orbitrap with CDMS measurements. While the charge states remain unresolved by standard MS, the latter approach has allowed measurement of *m/z* and charge and thus determination of mass. CDMS clearly has advantages over conventional MS in these cases, where high sample heterogeneity or limited instrument resolution hinders mass determination, and is likely to expand its presence in nMS in the near future.

CONCLUSIONS AND FUTURE DIRECTIONS

nMS is one important tool in a suite of complementary structural biology tools that are used in parallel to solve structural questions. nMS is complementary to cryo-EM, for example, in (*a*) identification of multiple proteoforms that make high-resolution structural characterization difficult; (*b*) identification of the ligand corresponding to missing density in an X-ray or cryo-EM structure; (*c*) definition of stoichiometry, subunit connectivity, and CCS for a complex for which no or only low-resolution structures can be observed using cryo-EM; and (*d*) identification of subunit mixing in cases where partners of a complex are too similar to be distinguished via their cryoEM structure. Innovations in nMS technology and techniques have led to improved separations; sample introduction; ionization; activation; and mass, *m/z*, and shape measurement, enabling increasingly robust structural and dynamic analysis of macromolecular complexes. Complementary advances in computational frameworks will increasingly allow the integration of nMS data into structural modeling, for example, in cryo-EM 3D reconstructions. All of these nMS advancements have enabled applications to increasingly complex sample systems and illustrate the emerging role of nMS as an important member of an integrated suite of structural biology tools.

Despite these exciting advances in nMS, some challenges remain to enabling faster and more rigorous analysis on a broader swath of sample types. For example, existing platforms are often unable to resolve heterogeneous protein or nucleoprotein complex mixtures containing small mass differences (e.g., addition of small ligands or post-translational modifications), although charge changing experiments can sometimes solve that problem. Improvements to single molecule or charge detection MS, including improvements to throughput, would also solve this problem. Another challenge is the need to resolve and desolvate complexes that are sprayed in high concentrations of additives such as metal ions, small molecules (e.g., ATP), specific and nonspecific lipids, or nMS-incompatible buffer components that may be required for maintaining the integrity of the complex. Improving gas-phase desalting and adduct removal techniques, or removal of membrane mimics, while also being gentle enough to prevent restructuring of the complex would also aid in improved identification and resolution. Further improvements in the resolution of IM would enable better separation and characterization of different conformers of a protein or nucleoprotein complex or subcomplex and aid in the identification of species that overlap in *m/z* space. Coupling IM techniques with different analyzer types that are useful in nMS (e.g.,Orbitrap), and developing platforms where IM can be placed in different positions within the instrument without worries about pressure, will increase the versatility and utility of IM techniques in nMS. However, increasing IM resolution without sacrificing ion transmission or increasing the instrument footprint is an important challenge. Another remaining challenge is increasing the throughput and ease of nMS analysis, for example,

through the streamlined integration of software, development of online separation techniques to enable screening, and integration of multiple MS/MS and IM approaches on a single instrument platform. We are especially excited about the future possibility of integrating nMS and other structural biology tools into a single platform using the same sample for MS- and non-MS-based measurements.

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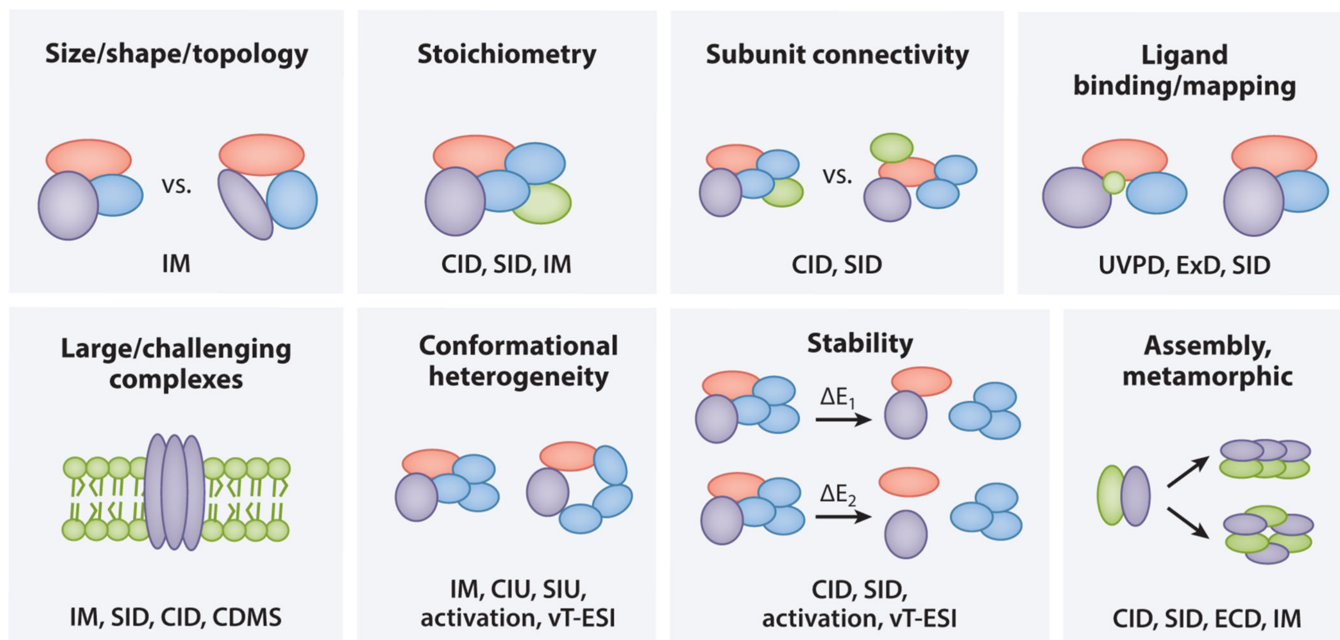

Figure 1.

Diverse applications of nMS. Each box includes an application of nMS depicted with a schematic, and the nMS-based techniques used to address that application are listed in the lower portion of the box. Abbreviations: CDMS, charge detection mass spectrometry; CID, collision-induced dissociation; CIU, collision-induced unfolding; ECD, electron capture dissociation; ExD, electron transfer dissociation (or electron capture dissociation); IM, ion mobility; nMS, native mass spectrometry; SID, surface-induced dissociation; SIU, surface-induced unfolding; UVPD, ultraviolet photodissociation; vT-ESI, variable temperature electrospray ionization.

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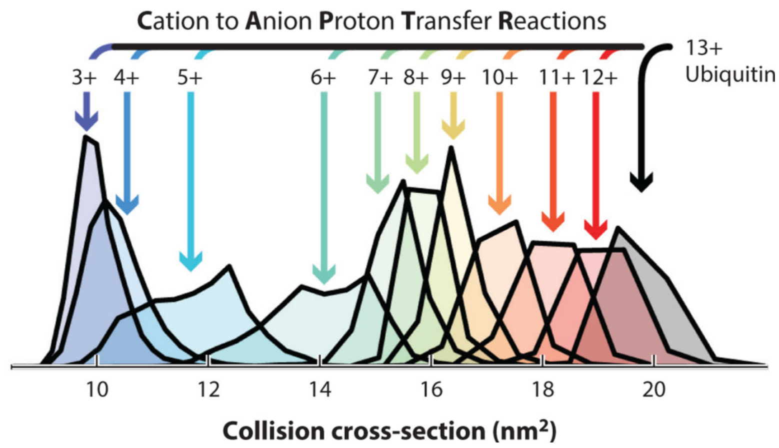

Figure 2.

Collision cross-sections of ubiquitin ions generated from the cation to anion proton transfer reaction of the 13+ charge state. Figure adapted with permission from Reference 96; copyright 2016 American Chemical Society.

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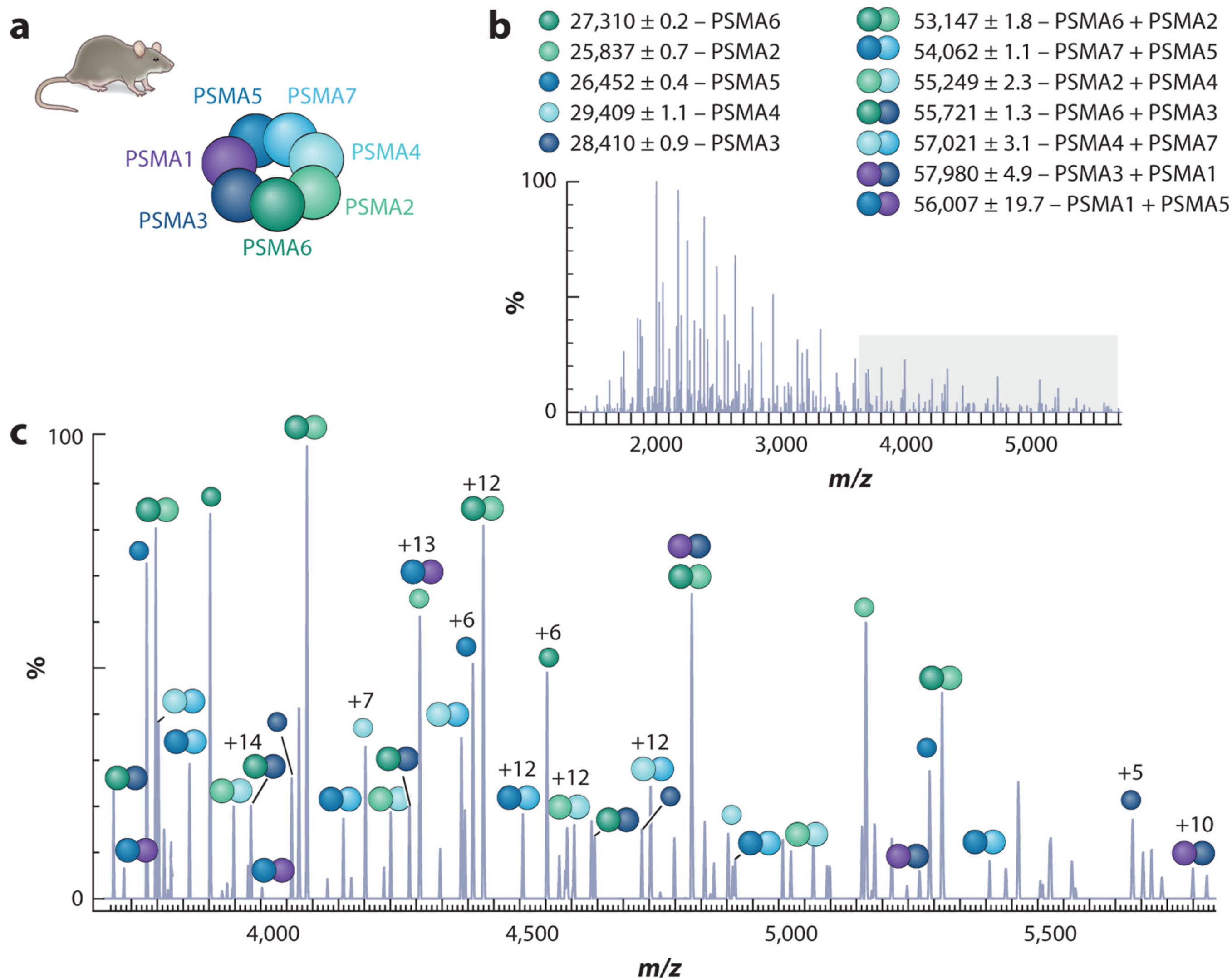

Figure 3.

MS^3 analysis of rat 20S proteasome α-ring containing seven different α subunits (PSMA1–7). Samples were subjected to HCD, and fragments were identified based on mass. (*a*) Deduced structural organization of the α-ring based on identified subcomplexes labeled in panels *b* and *c*. Panel *c* represents an enlarged view of the gray-shaded area in panel *b*. Figure adapted with permission from Reference 160; copyright 2020 American Chemical Society. Abbreviations: HCD, higher-energy collision dissociation; MS, mass spectrometry.

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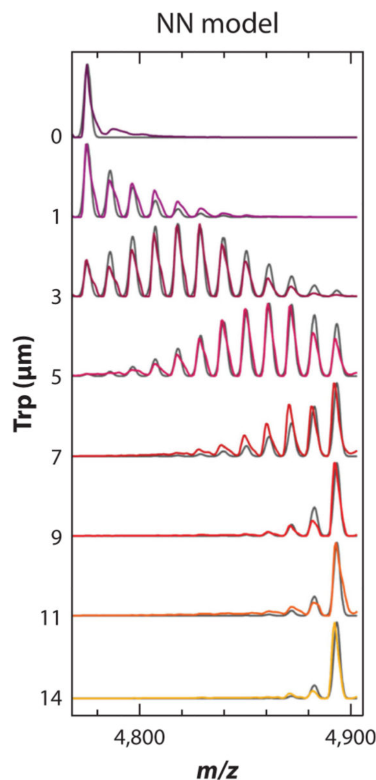

Figure 4.
Population distributions of tryptophan (Trp)–*trp* RNA-binding attenuation protein (TRAP) complexes reveal homotropic cooperativity in Trp binding to TRAP. The mass spectrum for the 19+ charge state of 1 μM TRAP incubated with the indicated concentration of Trp is displayed (*color*), overlaid with simulated populations computed from fits to the nearest-neighbor (NN) cooperative model (*gray*). Figure adapted with permission from Reference 72; copyright 2020 American Chemical Society.

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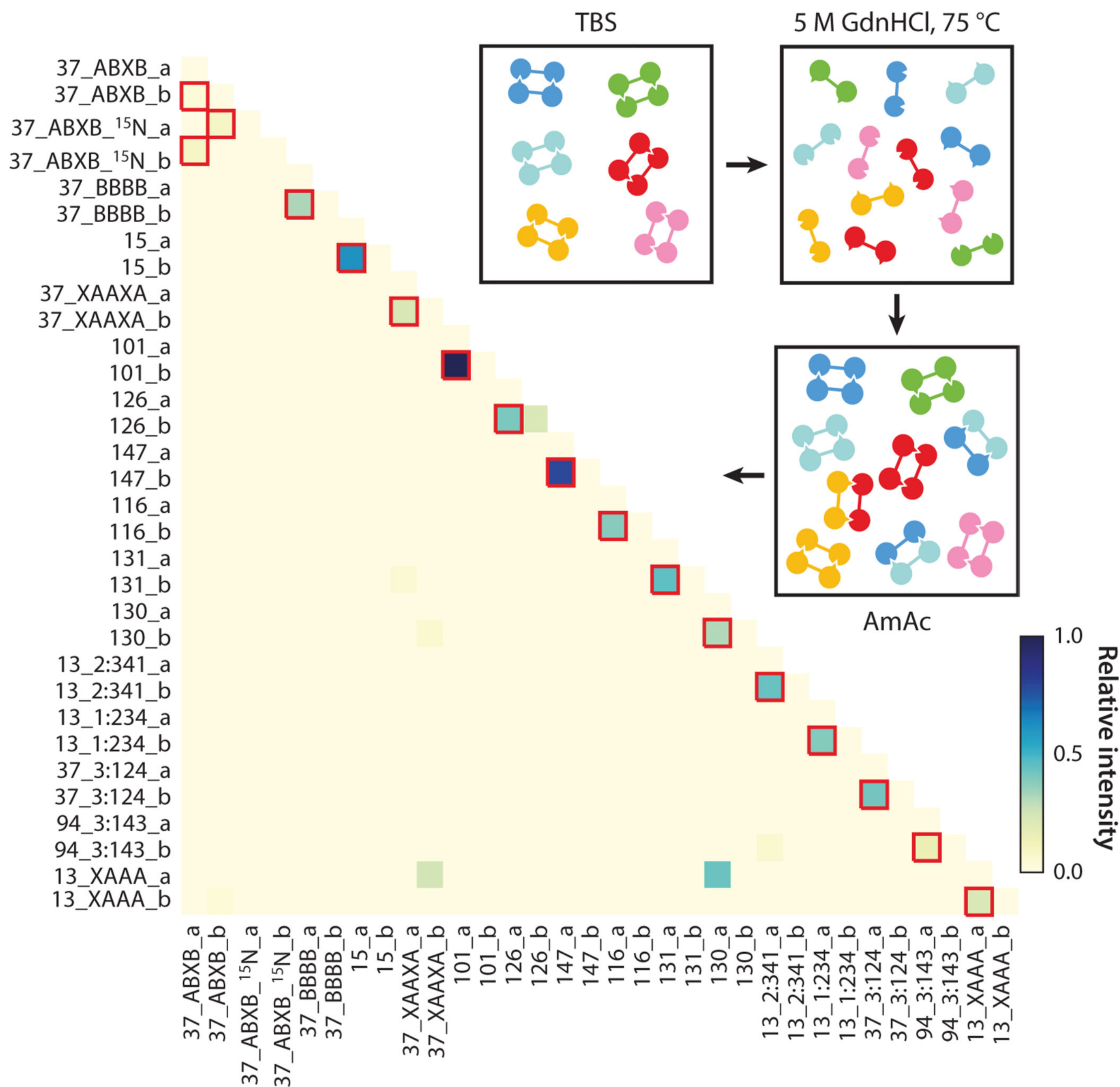

Figure 5.

All-against-all orthogonality of 16 pairs of heterodimers assessed by online ion exchange chromatography coupled with native mass spectrometry. Red boxes indicate designed cognate pairs. Exchange of unlabeled and partially ^{15}N-labeled DHD37_ABXB results in a distribution of overlapping species. Figure adapted with permission from Reference 23; copyright 2018 Springer Nature Limited. Abbreviations: AmAc, ammonium acetate; TBS, Tris-buffered saline.

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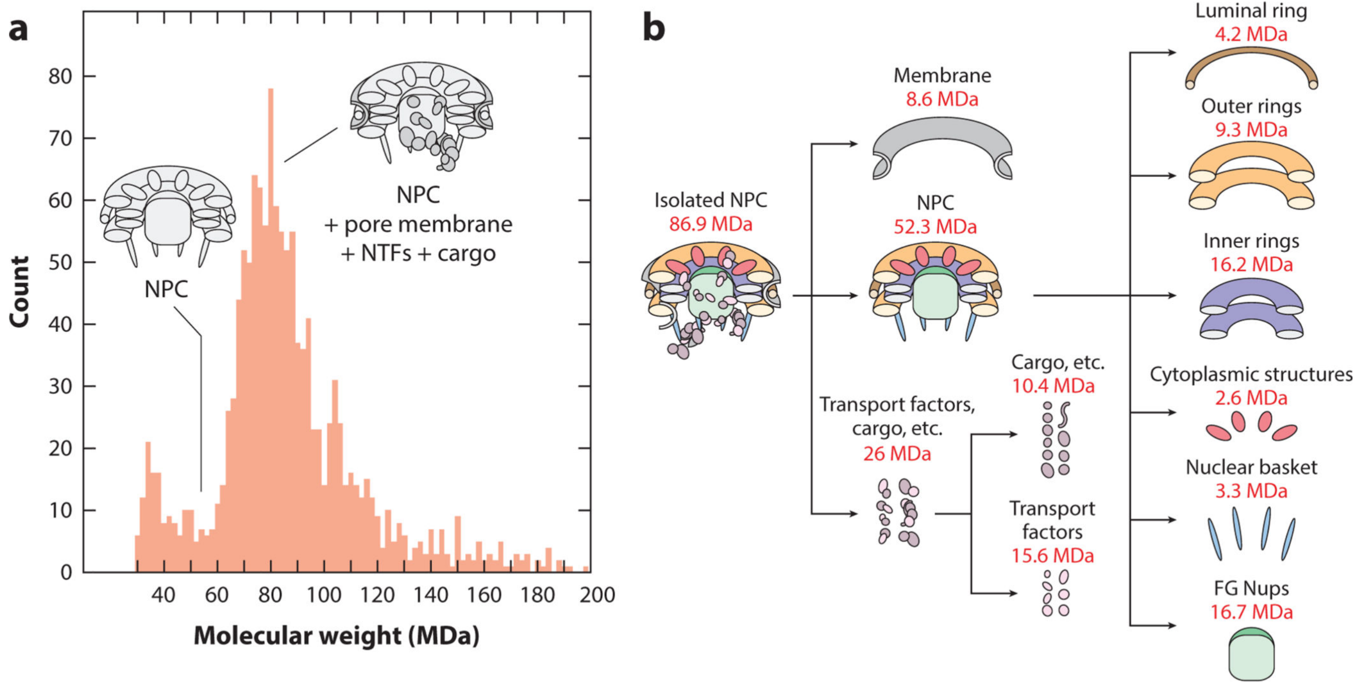

Figure 6.

(*a*) Charge detection mass spectrum of a 552-protein NPC and (*b*) breakdown of its composition. Figure adapted with permission from Reference 88; copyright 2018 Macmillan Publishers Limited, part of Springer Nature. Abbreviations: FG Nups, phenylalanine-glycine nucleoporins; NPC, nuclear pore complex; NTF, nuclear transport factor.