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Abstract
Mass spectrometry (MS) is an effective approach for determining the mass of biomolecules with high accuracy, sensitivity and speed. Over the past 25 years, MS performed under non-denaturing conditions ("native MS") has been successfully exploited to investigate non-covalently associated biomolecules. Here we illustrate native MS applications aimed at studying protein-ligand interactions and structures of biomolecular assemblies, including both soluble and membrane protein complexes. Moreover, we review how the partial dissociation of holo-complexes can be used to determine the stoichiometry of subunits and their topology. We also describe "native top-down MS", an approach based on Fourier Transform MS (FT MS), whereby non-covalent interactions are preserved while covalent bonds are selectively fragmented. Overall, native MS plays an increasingly important role in integrative structural biology, helping researchers to elucidate the three dimensional architecture of intricate macromolecular complexes.

Keywords
Native mass spectrometry (MS), native top-down MS, protein-ligand interactions, macromolecular complexes, stoichiometry, 2D interaction map, integrative structural biology

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Abstract

Mass spectrometry (MS) is an effective approach for determining the mass of biomolecules with high accuracy, sensitivity and speed. Over the past 25 years, MS performed under non-denaturing conditions – native MS – has been successfully exploited to investigate non-covalently associated biomolecules. Here we illustrate the use of native MS to study protein-ligand interactions and structures of biomolecular assemblies, including both soluble and membrane protein complexes. Moreover, we review how the partial dissociation of holo-complexes can be used to determine the stoichiometry of subunits and their topology. We also describe native top-down MS, whereby non-covalent interactions are firstly preserved and then covalent bonds are selectively broken. This allows the detailed probing of intersubunit and subunit-ligand interactions within a biomolecular assembly. Overall, native MS plays an increasingly important role in integrative structural biology, helping researchers to elucidate the three dimensional architecture of intricate macromolecular complexes.

Keywords

Top-down mass spectrometry (MS), protein-ligand interactions, macromolecular complexes, stoichiometry, 2D interaction map, integrative structural biology

1. Introduction

Knowledge of the three-dimensional (3D) structure of a macromolecular complex is often critical for achieving a detailed understanding of its biological role and of the molecular mechanisms that underlie its functional activity. Traditionally, methods such as X-ray crystallography, Nuclear Magnetic Resonance (NMR) spectroscopy and cryo-electron microscopy (cryo-EM) have been used to decipher the atomic organization of biomolecules. Recently, mass spectrometry (MS) has emerged as an important tool in structural proteomics for investigating 3D structures [1-5]. Several MS-based methods have been introduced for monitoring the structural and conformational changes of biomolecules and for characterizing their non-covalent interactions both in well-defined biochemical conditions and in complex biological environments such as cell lysates and intact cells. These techniques include MS coupled to: chemical cross-linking (XL) [6], hydrogen/deuterium exchange (HDX) [7-9], hydroxyl radical footprinting (HRF) [10, 11], limited [12] or pulse proteolysis [13], cellular thermal shift assay (CETSA) [14], Drug Affinity Responsive Target Stability (DARTS) [15, 16] and Stability of Proteins from Rates of Oxidation (SPROX) [16]. XL-MS allows the identification of spatially proximal residues and can be used to validate an experimentally determined 3D structure or to deduce the structure of a complex for which the individual subunit structures are known [17]. MS combined with HDX, HRF or limited proteolysis can be used to identify residues whose surface accessibility becomes altered upon complex formation, thereby providing information on intermolecular interfaces and on the conformational dynamics of complexes. MS coupled to pulse proteolysis, CETSA, DARTS and SPROX can identify the protein targets of small-molecule ligands.

Below we review the role of native MS in investigating the structure and dynamics of biomolecular assemblies, including soluble and membrane protein complexes. In particular, we describe the different approaches used to assess the stoichiometry and topology of non-covalent complexes. These include the partial dissociation of assemblies under controlled conditions in the gas phase and in solution. Moreover, we present recent progress in native top-down MS, whereby both covalent bonds and non-covalent interactions (i.e., primary and quaternary structures) can be investigated using the same mass spectrometer. Finally, we outline prospects for the future development of native MS and its application to the study of macromolecular complexes.

2. Native MS and its role in investigating macromolecular complexes

Over the last 25 years native MS (also known as structural MS [18]) has emerged as a key tool for investigating macromolecular and protein-ligand complexes because it allows non-covalent interactions (hydrogen bonds and van der Waals, electrostatic, hydrophobic and π-interactions) to be preserved during experimental analysis [19-21]. This distinguishes native MS from other types of biological MS (such as MS-based proteomics) which generally require denaturing conditions that disrupt such interactions. Using native MS one can determine the mass and stoichiometry of intact macromolecular complexes, identify direct interactions between their components and, in the case of multi-subunit assemblies, identify stable subcomplexes and assign the relative position (core vs. periphery) of subunits [22-27] (**Figure 1**). One can deduce the hierarchy of an assembly pathway by mixing subunits in a stepwise manner [18] or by using different sample buffer conditions to induce a change in oligomeric state [28, 29]. Equilibrium dissociation (K_d) constants for protein-protein and protein-ligand interactions can also be determined by native MS [30-32]. The K_d values obtained by native MS were consistent with those obtained by isothermal titration calorimetry (ITC) [30-33]. Native MS combined with “double-mutant cycle” analysis successfully determined pairwise interaction energies [33]. Native MS coupled with ion mobility (IM; MS-related abbreviations are summarised in **Table 1**) allows one to investigate the shape of macromolecular assemblies [34-38], and has been combined with bioinformatics to study the evolutionary history of protein complexes [17, 39].

3. Strengths and limitations of native MS

Native MS presents several advantages compared to other structural approaches. First, it can be used to study a wide diversity of biological samples that differ in mass, polydispersity, symmetry and dynamic flexibility [2]. Second, native MS does not require samples to be labelled or crosslinked. Third, different oligomeric states can be investigated simultaneously. Specific information is obtained for each individual species present, without data being averaged over different species. Therefore, the dynamics of quaternary structure can be studied in real time [40, 41]. Finally, native MS represents a quite sensitive approach. Successful analyses have been reported in which only a few microliters of sample at relatively low (μM) concentration were required.

Nonetheless, native MS experiments can be biased because (as all MS analyses) they are performed under vacuum conditions and so macromolecular complexes are detected in the gas phase [42, 43]. Therefore, the relative abundances of detected assemblies may differ from those in solution because distinct complexes may present different ionisation, transmission and detection probabilities [30]. Moreover, hydrophobic bonds are weaker in the gas phase than in solution, whereas electrostatic interactions become stronger. Therefore, certain assemblies need to be stabilized by crosslinking to enable their detection [44]. Nevertheless, computational and experimental data indicate that the transition from solution to the gas phase does not drastically alter biomolecules [45, 46]. For instance, enzymes such as lysozyme and trypsin retain their catalytic activity after their ionisation, mass selection, and soft-landing onto surfaces [47]. Also, when solution conditions such as pH and concentration were modified, the gas-phase spectra correspondingly changed [30]. Changes in MS spectra that mirror pH changes have been recently confirmed using designed homo-trimers (3-mers) and hetero-dimers (2-mers) engineered to undergo a pH-responsive conformational change upon protonation of buried histidine residues [48]. These complexes were stable above pH 6.5, but underwent cooperative, large-scale conformational changes when the pH was lowered, as shown by native MS and size-exclusion chromatography [48]. Recent evidence regarding the stability of folded biomolecules in vacuum has been obtained by low-energy electron holography [49]. Using electrospray ionisation (see next section) and soft-landing, two monomeric (1-mer) proteins (cytochrome C and BSA) and a hetero-4-mer (haemoglobin) were deposited on freestanding graphene. Then, sub-nanometer resolution images of individual biomolecules were obtained. These low-energy electron micrographs showed that the samples were in a folded state after being subjected to ultrahigh vacuum [49].

To conclude, native MS presents many advantages for investigating biomolecular assemblies in terms of accuracy, selectivity, sensitivity and speed of analyses. However, the limitations of native MS should be kept in mind when the results of such experiments are evaluated.

4. Preserving non-covalent interactions in the gas phase

Native MS requires a gentle ionisation of macromolecular complexes that preserves non-covalent interactions. Although in some cases this has been achieved using matrix-assisted laser desorption/ionization (MALDI) [50-54], by far the more prevalent method is electrospray ionisation (ESI) [55-57], which more easily preserves non-covalent interactions. ESI-MS investigations can be influenced by the nature of the intermolecular interactions, by the composition, ionic strength and pH of the sample buffer, and by the voltages and pressures within the mass spectrometer. Consequently, it is important to consider these parameters during data acquisition and analysis. Unlike other types of ESI-MS analyses that require the use of acidic conditions or organic solvents, native MS experiments are usually performed using volatile buffers such as ammonium acetate [58], ethylenediammonium diacetate [59] or alkylammonium acetate [60]. Typically the sample buffer is exchanged immediately prior to native MS analysis [23], although in some cases the sample purification protocol may also require modification.

Generally, non-covalent assemblies are investigated using nano-electrospray ionisation (nano-ESI), which requires a sample emitter with an orifice diameter (1–10 μm) smaller than that used for conventional ESI (approximately 100 μm) [61, 62]. The small opening enables

the use of a low flow rate (20-200 nL/min) to analyse a 2-5 µL sample volume at micromolar concentrations [63]. Recently, 0.5 µm emitter tips have been introduced to study proteins and their complexes using buffers containing non-volatile salts (such as Na^+ or K^+) at 135-150 mM concentration that more closely resemble the intracellular environment [64-66]. Therefore, experiments can be performed utilizing widely used biochemical buffers, such as phosphate and Tris. It has been hypothesized that the ESI droplets generated by submicrometer emitters contain a lower salt-to-biomolecule ratio than those generated by traditional nano-ESI needles [64]. Another advantage of submicrometer ESI tips is that they do not require buffer exchange into volatile solutions. These emitter tips have also been used for the study of membrane proteins [67]. Overall, controlled experimental conditions using appropriate buffers and ionisation conditions allow the maintenance of weak non-covalent interactions during native MS experiments.

5. Native MS experiments are performed using Q-TOF and Orbitrap instruments

Native MS requires mass spectrometers able to transmit and detect large fragile assemblies. Early studies showed that relatively small complexes could be analysed by conventional nano-ESI-spectrometers [68-71] (see **Figure 2** for timeline). However, larger complexes (>60 kDa) generate ions with mass-to-charge (*m/z*) ratios above 4000 that exceed the detection limit of such instruments. This led in the 2000s to customise nano-ESI-quadrupole-time-of-flight (Q-TOF) mass spectrometers to detect non-covalent complexes [43, 72]. The use of modified Q-TOFs made it possible to investigate remarkably massive particles, such as an 18 MDa viral capsid [73], and to analyse membrane protein complexes [74-80] (see section 11 below). Fourier transform (FT) based mass spectrometers have also successfully been used to study non-covalent interactions. These include Orbitrap instruments [81-84], which in 2012 were used to analyse intact protein complexes 150-800 kDa in mass [85-88], and were subsequently utilized to study virus-like nanoparticles with a mass up to 4.5 MDa [89]. To perform such analyses, the Orbitrap *m/z* range was extended up to 40000 to transmit “heavy ions” [85, 90]. Using only a 1 nM sample concentration, this modified instrument reached an excellent resolution at high *m/z*, deciphering different glycosylated forms of IgG antibodies [86]. Such high resolution enabled the characterisation of the type (ADP or ATP) and number of nucleotides bound to a large assembly such as GroEL (800 kDa) [85]. The performance of Orbitrap instruments utilised for native MS is continuously being improved by different groups [42, 91-93]. For example, in 2017 a Q Exactive Plus™ mass spectrometer was further modified to enhance its sensitivity and increase transmission up to 70 000 *m/z* [92, 93].

6. Dissociation of macromolecular complexes in the gas phase and subunit fragmentation

Tandem MS (also called MS^2 or MS/MS) is based on two MS steps, whereby selected ions are broken down in the gas phase and the product ions are analysed [94]. It is possible to distinguish between (a) the breaking of non-covalent interactions to dissociate complexes and (b) the fragmentation of covalent interactions to study the primary sequences of subunits and their post-translation modifications (PTMs). Here we outline the most common dissociation/fragmentation methods, including collision-induced dissociation [known as Higher Energy Collisional Dissociation (HCD) in Orbitrap instruments [42, 91]], surface-induced dissociation, photodissociation and electron-based dissociation techniques (e.g., electron-capture and electron-transfer dissociation) [95-97].

(i) Collision-induced dissociation (CID). Normally, dissociation/fragmentation experiments require that a single ion population, characterised by a specific mass (*m*) and charge state (*z*) and referred to as a “precursor ion”, is selected according to its *m/z* ratio. In the case of CID, when non-covalent assemblies are investigated, a precursor ion is selected according to its *m/z* ratio in a first mass analyser (e.g., a quadrupole in the case of Q-TOF instruments) and then accelerated toward a cell filled with a collision gas such as argon or xenon. Precursor ions are subjected to numerous, low-energy collisions that convert part of

their kinetic energy into vibrational energy, causing the internal energy of the precursor ions to increase. Since many small steps of energy conversion take place, CID is considered a "slow heating" process [98]. Non-covalent interactions are broken by collisions and the dissociation products are further analysed (e.g., in a TOF region). With some exceptions [99-101], 1-meric proteins are ejected from biomolecular assemblies one after the other and this phenomenon allows the assessment of stoichiometry with high accuracy. Ion charges are distributed across the dissociated particles in a highly asymmetric manner. For instance, a dodecamer (12-mer) generates highly charged 1-mers and lowly charged "stripped" 11-mers that can further dissociate into 1-mers and 10-mers [99].

The dissociation behaviour of assemblies during CID has been studied, but many questions remain open [29, 59, 102-104]. Behaviour can be influenced by sample features such as charge density, salt bridges, flexibility of subunits, isoelectric point (pI) and strength of intersubunit interfaces [99-101, 105]. For example, the pI of subunits has been shown to affect the dissociation of protein complexes [105]. Leney studied two 6-mers (i.e., phycoerythrin and allophycocyanin), composed of alternating alpha and beta subunits [105]. The subunits of the phycoerythrin presented identical pIs and in the CID experiments both types of subunits were ejected. In contrast, the alpha and beta subunits of allophycocyanin showed distinct pIs and only alpha 1-mers were detected upon CID. CID is very useful for investigating biomolecular complexes because it provides an insight into the location (core vs. periphery) of subunits within the complex [106], as peripheral subunits are expelled at lower energy compared to the core subunits [18]. CID can also provide important structural information such as subunit interactions and their relative spatial arrangements [99, 100].

(ii) Surface-induced dissociation (SID). In the case of SID, an ion population is accelerated toward a surface [97, 98, 107]. The non-covalent interactions are broken by collision against the surface and the masses of the dissociation products are further assessed using, for example, a TOF analyzer [108] or an Orbitrap [109]. Compared to the slow-heating effects of CID, SID is regarded as a fast, single-step, and energetic activation process [98]. SID makes non-covalent complexes dissociate into folded subunits that carry ion charges proportional to their mass without major structural rearrangements. This means that ion charges are distributed across the dissociated particles in a symmetric manner and that oligomers (as opposed to only 1-mers in the case of CID) are ejected from assemblies. For instance, a 4-mer composed of a 2-mer of 2-mers could be dissociated into individual 2-mers at low SID energy, and these in turn could be further dissociated into 1-mers at higher SID energy [108].

(iii) Photodissociation (PD). PD is based on the use of photons irradiated on target ions [95, 97, 110]. Different sources of photons have been used. For instance, ultraviolet (UV) lasers can deposit a large amount of energy into ions, allowing dissociation and fragmentation pathways different from those generated by other MS^2 methods. Remarkably, it has been shown that ion charges can be distributed across the dissociated particles in both symmetric and asymmetric manners by modulating the laser power [103]. Moreover, UVPD induced covalent bond cleavage of the 1-meric subunits, most frequently within highly flexible loop regions.

(iv) Electron-based techniques. In techniques such as electron-capture dissociation (ECD) [111-114] and electron-transfer dissociation (ETD) [96, 115], the fragmentation of biomolecules is based on the addition of electrons to the target molecules. In the case of ECD low-energy photoelectrons are released and captured by even-electron $(M+nH)^{n+}$ biomolecular ions (peptides, proteins or macromolecular assemblies) [116, 117]. Thus, radical charge-reduction products $(M+nH)^{(n-1)+}\cdot$ are generated and selective cleavage of the N-Cα bond, a highly bond specific process, takes place. ETD is a fragmentation method based on the transfer of electrons originating from radical anions [96, 118, 119]. Similarly to ECD, N–Cα bonds are broken in ETD experiments. Both ECD and ETD allow non-covalent bonds and PTMs to be preserved [120, 121], whereas covalent interactions such as peptide bonds are fragmented. The mechanism of ECD and ETD has been extensively studied

theoretically and experimentally, but remains a subject of debate [116]. ECD and ETD are mainly used in FT instruments, but can also be incorporated in ion traps [113], [114, 122] IM mass spectrometers (such as Synapt® instruments [123]) and Q-TOF mass spectrometers. For instance, ETD was utilised to sequence fragments of a non–covalent protein complex (ADH) using a Q-TOF instrument [124].

To conclude, a diversity of dissociation/fragmentation methods are available that enable one to dissociate non-covalent assemblies or fragment covalent bonds with distinct outcomes. These techniques are of key importance for confirming stoichiometry, studying protein-ligand interactions and determining protein sequences and PTMs (see sections 7, 9 and 12 below).

7. Native top-down MS to study non-covalent interactions

In the late 1990s the use of ECD led to the introduction of top-down MS, an approach to efficiently characterise intact proteins [125]. Unlike bottom-up proteomics that requires samples to be digested enzymatically [126, 127], samples subjected to top-down MS are analysed as intact molecules and fragmented inside appropriate mass spectrometers [128]. Top-down MS allows the investigation of different proteoforms [129, 130], protein conformations and PTMs [131-135]. Recent developments allowed native top-down MS experiments to be performed to study intact assemblies and to localize ligand binding sites on proteins [19, 127, 136-139].

These experiments require three different analysis steps [42, 91, 127, 136, 140] (Figure 1). In the first (MS) step, the mass of an intact macromolecular complex is assessed. In the second (MS^2) step, protein assemblies are often dissociated by CID and 1-mers are ejected. This step allows the stoichiometry of the complex to be confirmed [127]. In the final (MS^3) step, the ejected 1-mers are fragmented by backbone cleavage using various methods such as ECD or UVPD. This last step is useful to investigate the primary sequence of the ejected subunits of interest. According to the type of dissociation/fragmentation utilised, non-covalent interactions can be preserved and covalent ones broken [19, 141-143].

The complexity of samples that can be analysed by this approach has increased over the years. In early studies, small non-covalent complexes [144] and a protein-ligand complex [145] were fragmented using ECD. Since 2010, the approach has been applied to large non-covalent assemblies [63, 146-148]. Specifically, a 4-meric yeast alcohol dehydrogenase (ADH, 147 kDa) was analysed using an ECD and ESI-FT-ICR instrument [146]. By breaking covalent bonds within monomers, Gross and coworkers fragmented ADH subunits without disrupting non-covalent interactions. This was the first example in which information on both the primary and quaternary structures was obtained using the same instrument.

A recent example illustrates the use of native top-down MS and of nanodiscs to study a copper-dependent membrane metalloenzyme, particulate methane monooxygenase (pMMO), composed of three subunits (PmoA, PmoB, and PmoC), each present in three copies [149]. Using traditional biochemical and biophysical approaches, the assessment of the stoichiometry of metal centers and their localization represents a major challenge for large, multisubunit metalloprotein complexes, particularly when these cannot be heterologously expressed. In the case of pMMO, spectroscopic and crystallographic studies provided inconclusive information regarding the metal stoichiometry [149]. Using the three step MS-based approach (MS, MS^2, MS^3, see above), it was possible to assess the copper stoichiometry of the pMMO subunits and to investigate their PTMs. In an MS experiment performed to assess the total mass of the pMMO holocomplex, the observed mass of the main species was 559.7 Da lower than the theoretical one. Subsequently, the possible presence of PTMs and metal cofactors was investigated by MS^2 and MS^3 experiments. In MS^2, subunits were ejected from the intact pMMO and their masses were determined. The PmoA subunit had an experimental mass 89.1 Da smaller than that expected, consistent with removal of the first methionine and acetylation of the new N-terminus. This hypothesis was confirmed by MS^3. Similarly, these experiments revealed a methylated lysine within PmoB and the truncation of six N-terminal residues from PmoC. Moreover, they confirmed

the presence of a Cu(II) ion in PmoB and revealed an additional Cu(II) ion bound to PmoC that was shown in activity assays to be important for enzyme activity.

To conclude, native top-down MS experiments combine the ability to study non-covalent assemblies with the ability to fragment individual subunits using using a single mass spectrometer.

8. Dissociation of macromolecular complexes in solution

In addition to gas-phase dissociation, macromolecular complexes can also be dissociated in solution. Destabilising agents such as dimethyl sulfoxide (DMSO) or methanol or an increase in buffer ionic strength are used to selectively break intermolecular interactions within a complex before introducing the sample into the mass spectrometer [24]. In-solution dissociation of hetero-complexes allows one to identify direct interactions between subunits by generating subcomplexes [105, 150-152]. The analysis of these subcomplexes can lead to a two-dimensional interaction map that subsequently can be combined with other structural data to try to infer 3D organization. In-solution dissociation has also been exploited to elucidate the assembly and disassembly pathways of certain complexes, revealing that these pathways recapitulate the evolutionary routes by which different quaternary structures arise [39, 153, 154]. In summary, the ability to dissociate assemblies in solution is highly useful for elucidating intersubunit interactions, assembly/disassembly pathways and molecular evolutionary history.

9. Soluble protein-ligand interactions studied by native MS

The use of native MS to analyse non-covalent interactions between proteins and small ligands has been extensively reviewed [19, 34, 37, 136, 155-157]. Here we outline a few examples mainly in the context of structural studies [59, 158-162]. For instance, native MS was combined with X-ray crystallography to study two nickel import proteins of *Staphylococcus aureus* [163]. One of these was successfully crystallised bound to a Ni-(L-His)$_2$ ligand. The other protein could not be crystallised, but native MS showed that it bound Ni(II) ions via a distinct His-dependent chelator [163]. In another example, native MS combined with IM was used to quantify the stabilizing effects of ligands on soluble proteins, and of lipid binding on membrane proteins [164].

In many cases, the identification of ligand-binding sites within macromolecular complexes made use of appropriate dissociation/fragmentation techniques such as ECD, SID and UVPD (see above) [165-170]. In one of the first examples of non-covalent binding studied by ECD, published in 2002, homo-2-mer formation by different peptides and the binding of two glycopeptide antibiotics, vancomycin and eremomycin, to their bacterial tripeptide target were investigated [144]. These experiments allowed the authors to localize the binding sites and determine primary sequence information.

In a recent example, ECD was utilised to investigate the interaction between tau, a protein implicated in neurodegenerative disorders such as Alzheimer's disease, and a synthetic "molecular-tweezer" compound (CLR01) that inhibits the aggregation of amyloidogenic polypeptides through hydrophobic and electrostatic interactions [143]. Using ECD, Nshanian *et al.* identified phosphorylation sites on tau and localized the binding site of CLR01 to a 38-residue microtubule-binding region within the protein, providing novel insights into the mechanism of CLR01-based inhibition. Another example from the Loo laboratory combined ECD with CID to localize the binding sites for cobalt and manganese in an intrinsically disordered protein, α-synuclein [142].

The usefulness of SID was demonstrated in a recent study involving two homo-5-meric complexes, C-reactive protein (CRP) and cholera toxin B (CTB) [141]. These proteins have different ligand binding site arrangements: the site is located within each subunit in CRP, but between adjacent subunits in CTB. The authors were unable to obtain information on ligand location using CID, which caused ligand-bound 5-mers to dissociate into 4-mers and ligand-free 1-mers in a manner suggesting that ligands were lost or migrated away from their binding site. In contrast, SID of 5-mers led to the dissociation into 1-mers, 2-mers, 3-mers and 4-mers. The predominant species detected for each CRP *n*-mer contained *n* ligands,

whereas CTB *n*-mers contained *n*-1, *n* and *n*+1 ligands, consistent with the known ligand binding site locations of these proteins [141].

Another fast activation technique, UVPD, has also been proved useful for investigating ligand binding sites [171, 172]. For instance, UVPD-MS was used to investigate the catalytic cycle of adenylate kinase (AK) [172]. This enzyme, which catalyzes the interconversion of adenine nucleotides via phosphoryl transfer from ATP to AMP to generate ADP, undergoes a large conformational change from an open inactive state to a closed active state. Native MS and top-down UVPD-MS were used to investigate different complexes of AK with four ligands (AMP, ADP, ATP, and a small-molecule inhibitor) to monitor different steps of the catalysis. Holo fragment ions produced in UVPD experiments revealed specific regions of AK that exhibited substantial differences in fragmentation throughout the catalytic cycle. In particular, changes in backbone cleavages involving three α-helices and adenosine binding regions of AK indicated that these elements were in different structural microstates as AK progressed through its enzymatic cycle. Moreover, enhanced fragmentation of the loop binding the phosphates of ATP suggested that the metal ion cofactor Mg^{2+} increases the flexibility of this loop, consistent with a role for Mg^{2+} in disrupting interactions between AK side chains and ligand phosphate groups. Changes in backbone cleavage efficiency also corroborated the hypothesis that a specific conserved residue (Arg138) stabilizes the donor phosphoryl group during catalysis.

Overall, the above examples illustrate that native MS combined with dissociation/fragmentation experiments represents a versatile approach for studying protein-ligand interactions and characterising binding sites.

10. Employing native MS to probe the structure of soluble protein complexes

Numerous studies exemplify the great ability of native MS to elucidate structural features of soluble protein assemblies (reviewed in [18, 90, 106, 173-179]). The examples below illustrate how native MS can be integrated with other structural approaches, provide valuable knowledge when high-resolution structural data are unavailable, and elucidate assembly pathways of macromolecular complexes.

(i) Native MS complements other structural methods

MS based-structural proteomics plays an increasingly important role in characterising the architecture of non-covalent assemblies, complementing traditional approaches [178, 180, 181]. 3D models of macromolecular complexes have been deduced by combining MS experiments with data from crystallography, NMR, small-angle X-ray scattering and EM [28, 182-188]. Computational methods have been developed for merging native MS information with other MS-based data (such as IM-MS and chemical crosslinking) to generate pseudo-atomic models of biomolecular assemblies [189-192].

For instance, native MS was used in combination with EM to characterise protein cages formed by lumazine synthase, a bacterial enzyme that self-assembles into a 60-subunit dodecahedral shell (~1 MDa) [193]. Sasaki and colleagues engineered two variants of this enzyme that resulted in much larger assemblies of either ~3 MDa or ~6 MDa. Accurate mass measurements by native MS revealed that the smaller assembly comprised 180 subunits and that the larger assembly formed a distribution of particles comprising 240-420 subunits.

Native MS was combined with X-ray crystallography in structure/function studies of a kinase, Receptor Interacting protein kinase 2 (RIP2). This study investigated the wildtype (WT) form of the RIP2 kinase domain as well as two kinase-dead point mutants [194]. While both point mutants showed suppressed autophosphorylation activity, native MS revealed that they differed significantly in oligomeric state. The WT and one mutant (D146K) formed stable 2-mers, whereas the other mutant (K47R) was in a 1-mer/2-mer equilibrium. Interestingly, crystallography revealed that the D146K and K47R mutants adopted the active and inactive conformations, respectively. The combined findings provided valuable insights by revealing a link between dimerization and kinase activation [194].

Native MS has also been used to shed light on the dynamics of macromolecular assemblies. For instance, transthyretin (TTR), an amyloidogenic protein, has been extensively studied by this technique [40, 41, 195-197]. Native MS allowed scientists to

assess the effect of point mutations and to monitor the exchange of unlabelled and labelled TTR subunits over time. In a recent report, MS-based subunit exchange experiments combined with neutron crystallography data and modelling studies led to propose a novel mechanism of TTR fibrillation [40] (**Figure 3**).

Large (several MDa) viral assemblies have also been successfully analysed by native MS [90, 198-203] as well as by other MS-based methods such as charge detection MS [204-206] and nanomechanical resonator MS (NEMS) [207] (see below). For instance, the bacteriophage HK97 was investigated by native MS and HDX MS, label-free quantitative proteomics and single-particle cryo-EM to gain insights into the function of the HK97 protease during maturation of the bacteriophage capsid [199]. The masses of protease-free and protease-containing procapsids and of the mature capsid (17.9, 21.4 and 12.9 MDa, respectively) were determined by native MS, allowing for the number of protease molecules in the procapsid and the efficiency of procapsid cleavage to be evaluated.

(ii) ***Native MS provides useful structural information in the absence of high-resolution 3D structures***

Native MS provides important structural information even when no high-resolution 3D data are available [151, 208-212]. For instance, native MS was combined with size exclusion chromatography and multi-angle light scattering (SEC-MALS), isothermal titration calorimetry (ITC), epifluorescence microscopy and *in vivo* functional studies to investigate a bacterial complex called MukBEF, a member of the Structural Maintenance of Chromosomes (SMC) complexes involved in chromosome organization and segregation [213]. The MukBEF complex is formed by an ATPase, MukB, which associates with two other proteins, MukE and MukF. When the three proteins were incubated with ADP, three major complexes were detected: a 2-mer ($MukB_2$), 6-mer ($MukE_4$:$MukF_2$) and 8-mer ($MukB_2$:$MukE_4$:$MukF_2$). In the presence of a non-hydrolysable ATP analogue, an additional 10-mer ($MukB_4$:$MukE_4$:$MukF_2$) was detected. Combined with additional biophysical data, these results shed important light on the architecture of the MukBEF complex and the changes induced upon ATP binding and hydrolysis [213].

Another interesting example showed that the energy of SID experiments, which informs on the relative strength of intermolecular interfaces (see above), can be used as an experimental restraint to improve the ability of computational protein–protein docking studies to predict the quaternary structure of protein complexes. Such studies are useful when structural data are unavailable for the complex but NMR or crystal structures are individually available for the constituent subunits [214].

Excellent recent reviews discuss the application of IM-MS and molecular modelling to study unknown structures [192, 215]. In one example, the Ruotolo laboratory investigated a labile 18-subunit assembly, a urease pre-activation complex from *Klebsiella aerogenes* [216]. By combining previously reported SAXS and chemical crosslinking data with IM-MS data and molecular modeling, the authors identified a discrete population of putative structures that was consistent with all the available data for this 610-kDa assembly [216]. A second example involves studies of the plant photoreceptor UVR8, a homo-2-meric protein that dissociates into 1-mers upon UV-B light activation [217]. The authors used IM-MS and molecular dynamics simulations to study the UVR8 β-propeller core domain, whose crystal structure is known, and the full-length protein that includes intrinsically disordered N- and C-terminal regions. The authors found that the full-length 2-mer exists in two conformational families that differ in the conformation of the terminal regions. Upon UV illumination the C-terminal tail promotes the destabilization of the core domain of the UVR8 1-mer.

(iii) ***Native MS allows the investigation of assembly pathways***

An advantage of native MS is the ability to detect several different oligomeric and compositional states simultaneously and to monitor how the distribution of these states changes in real time. This makes native MS ideally suited for studying the assembly and disassembly pathway of macromolecular complexes [73, 218, 219]. Native MS was combined with cryo-EM to investigate the assembly behaviour of a circadian oscillator from

cyanobacteria composed of three proteins, KaiC, KaiB, and KaiA [187]. Incubation of the three purified recombinant proteins with ATP reconstitutes the oscillator, which can maintain a stable rhythm autonomously for weeks *in vitro*. Native MS experiments were used to monitor how the stoichiometry and phosphorylation state of Kai protein complexes changed during the circadian cycle. These experiments revealed the formation of more than 10 different complexes (including $KaiC_6$, $KaiC_6A_2$, $KaiC_6A_4$, $KaiC_6B_1$, $KaiC_6B_6$ and $KaiC_6B_6A_{2n, n=1-6}$) that ranged in mass from ~350 to ~820 kDa over the course of 24 h (**Figure 4**). The data allowed the authors to identify conditions in which the KaiA-binding site of the $KaiC_6B_6$ complex was fully occupied, allowing them to determine the cryo-EM structure of a $KaiC_6B_6A_{12}$ complex. The combined native MS and cryo-EM data allowed them to propose a detailed model for the cyclic phosphorylation-dependent assembly of the protein oscillator.

11. Investigation of membrane protein complexes and their binding to ligands

In the last fifteen years, interactions involving membrane proteins have been studied by MS [1, 220-223], including protein-drug interactions [74, 224], protein-lipid complexes [75, 225-232] and multiprotein assemblies [76, 77, 149, 179]. Heteromeric membrane protein complexes containing both soluble and transmembrane subunits were successfully analysed by native MS [78, 79, 233-235] (**Table 2**). Here we point out a few of the most recent studies.

Native MS was instrumental in deciphering the role of lipids in tuning different conformational states of membrane proteins and the transfer of small molecules or drugs through membranes [224, 236-241]. For instance, native MS was used to investigate the bacterial ATP-Binding Cassette (ABC) transporter P-glycoprotein (P-gp) and its binding to lipids, nucleotides, and drugs in real time [242]. By determining lipid binding rates and apparent K_d values, it was shown that P-gp preferentially binds anionic (versus zwitterionic) phospholipids and short-chain (versus long-chain) cardiolipins, and that the interaction with cardiolipin is enhanced by the binding of cyclosporin A, a P-gp inhibitor. Other native MS studies of bacterial ABC transporters revealed the significance of specific annular lipids in the ATPase activity of TmrAB [227], the propensity of the lipid-A flippase MsbA to bind negatively charged phosphatidyl glycerol lipids [229], and the influence of substrate and nucleotide binding on complex formation by the molybdate importer ModBC-A and by the vitamin B12 importer BtuCD-F [230].

Native MS was also recently used to study a member of the mycobacterial membrane protein Large (MmpL) transporters from *Mycobacterium tuberculosis* [243]. These transporters shuttle fatty acids and lipid components to the mycobacterial cell wall, which is abundant in mycolic acids (MA), and are critical for mycobacterial physiology and pathogenesis. Native MS analysis revealed that the transporter MmpL3 was able to specifically bind phosphatidylethanolamine, a previously unknown MmpL3 ligand, as well as trehalose monomycolates (TMMs), which are precursors of MA-containing trehalose dimycolates (TDMs), but was unable to bind TDMs [243].

Native MS was also helpful to study allosteric effects concerning lipid interactions with the ammonia channel AmtB from *E. coli* [241]. Specifically, the authors investigated mixtures of a fluorescent-labelled cardiolipin (TFCDL) with one of six other lipids and determined K_d values for AmtB binding up to a total of five lipid molecules. This allowed the authors to estimate how some lipid-protein interactions influenced the binding of other lipid types to AmtB, revealing for example that TFCDL exhibits positive and negative allostery on the binding of phosphatidylethanolamine and phosphatidic acid lipids, respectively [241].

Several MS-based studies employed detergents (such as β-octylglucoside and dodecylmaltoside) to maintain membrane protein complexes in their native oligomeric state. The development of detergent-free alternatives indicated that native forms of proteins and macromolecular assemblies can also be preserved if lipid bicelles, amphipols or nanodiscs are used [1, 244-247]. Native MS and nanodiscs were used to study the interactions between glycolipids and two toxins (i.e., the B subunit homo-5-mers of cholera toxin and heat labile toxin) and their relative affinities ranked by screening the proteins against different glycolipid-incorporating nanodiscs [248]. Small, soluble protein-lipid complexes (~3

nm), named picodiscs, were exploited for time-resolved enzymatic studies, antibody-antigen interaction assays and bacterial toxin-glycolipid interaction analyses [249].

In 2018 a detergent-free approach was developed for obtaining mass spectra of biomolecular complexes from native membranes without chemical disruption [250]. Lipid vesicles isolated from prokaryotic and eukaryotic membranes were disrupted and multiple protein complexes ejected “directly” into the instrument gas phase [250]. Quantitative proteomics, lipidomics and native MS allowed the authors to identify numerous complexes, including a chaperone-porin complex in the outer *E. coli* membrane, different subassemblies of multidrug efflux pumps in the inner membrane, and a complex between F_1F_0 ATP synthase and the protein translocation channel SecYEG.

12. Analysis of macromolecular complexes by hybrid MS-based methods

Hybrid MS-based methods address biological problems through the use of more than one MS approach and instrument. For example, van de Waterbeemd *et al.* combined bottom-up proteomics, top-down MS and native MS to study ribosomal particles from bacteria, plants and humans [93, 251]. Using proteomics, they identified the ribosomal proteins and their PTMs. Using top-down MS, they assessed the presence of different proteoforms (carrying multiple PTMs and truncations) [129, 135]. Using MS^3, they determined the composition and stoichiometry of intact ribonucleoprotein complexes, including the human 40S ribosomal subunit bound to viral internal ribosome entry site (IRES) RNA elements.

Several studies have reported native MS analyses of membrane proteins and membrane macromolecular coupled with lipidomics. The latter approach enables LC-MS/MS-based identification of extracted lipids to characterize the function of the bound lipidome [229, 250, 252]. A hybrid MS approach has also been used to study the heterogeneity of monoclonal antibodies [253]. Specifically, native MS was combined with targeted glycan profiling (the MS/MS-based investigation of glycans released by PNGase F digestion). Native MS analysis provided a general overview of the glycoform distribution and indicated the coexistence of antibody modifications such as glycations and truncations. Profiling of released glycans elucidated the linkages within carbohydrate residues and enabled the differentiation of structural isomers.

Overall, these examples illustrate the utility of hybrid MS-based methods to gather insights on inter-subunit interactions within macromolecular complexes, on primary sequences of subunits and on features of bound molecules such as metals, lipids and glycans.

13. Concluding remarks and outlook

Twenty five years ago, the impressive results achieved using bottom-up proteomics firmly established MS as a key technology for biological research [254-257]. At that time, MALDI- and ESI-MS exhibited excellent sensitivity, exquisite accuracy of mass measurement and high speed of data acquisition [258, 259]. Currently, bottom-up proteomics allows one to exhaustively describe the complete proteome of a model organism in a few hours [257] as well as to profile multiple cancer proteomes per mass spectrometer per day, paving the way for proteomics applications in personalized cancer medicine [260]. Soon it may even be possible to profile the proteome of individual cells [261].
Although the first MS studies of non-covalent interactions were reported over two decades ago, [68-71], native MS is currently far less widely used than bottom-up proteomics. Nevertheless, native MS-based studies have steadily gained momentum in the field of integrative structural biology thanks to its wide applicability, speed of analysis, sensitivity and selectivity [179, 262-265]. This latter feature is especially valuable, as it enables several species with different masses to be simultaneously analysed and separated. Indeed, the fact that mass selectivity was recently exploited to image individual proteins and a protein complex by low-energy electron holography demonstrate that MS can be exploited to purify heterogeneous assemblies for structural studies [49].

A key strength of native MS that will prove increasingly useful is its ability to inform on the identity, solubility, oligomeric state, and stability of overexpressed biomolecules directly in crude cell lysates and culture media (i.e., without the need for purification) [266-268]. Moreover, the investigation of endogenous macromolecular assemblies without the need for their recombinant over-expression in prokaryotic and in eukaryotic hosts is also emerging. Several examples of endogenous complexes studied by native MS point to an increasingly important role in deciphering the function and regulation of PTMs, cofactors and transient complexes assembled in a cellular milieu [150, 250, 251, 263, 269, 270].

The use of native MS to investigate endogenous complexes will undoubtedly improve with technical advances that allow the enhanced purification yield of complexes present in cells at their natural expression level [271, 272] and with the development of new instruments having increased sensitivity, resolution and ionisation efficiency. Orbitrap mass spectrometers already present an improvement in sensitivity and resolution compared to Q-TOF instruments [85, 86, 88]. Novel ionisation approaches are of key importance for increasing the amount of sample introduced in mass spectrometers [273-278]. Moreover, new detectors advance the applicability of MS to study biological nanoparticles. For instance, nanoelectromechanical systems (NEMS) detect large masses with unprecedented sensitivity, requiring only a few hundred single-molecule adsorption events to detect megadalton molecules [207, 279]. Recently, empty and DNA-filled bacteriophage T5 capsids with masses up to 105 MDa were investigated, using less than 1 picomole of biological material and reaching an instrument resolution above 100 [207].

Novel detectors were also developed for charge detection mass spectrometry (CDMS), whereby the *m/z* and *z* are simultaneously measured for each ion [206, 280-282]. Recently CDMS was combined with cryo-EM, lipid and glycan analysis to study Sindbis viruses (>50 MDa) produced from arthropod and vertebrate hosts [283]. These studies revealed differences in lipid composition between the Sindbis viruses that influenced the assembly, budding and stability of viral particles, explaining differences in infectivity [283]. CDMS was also combined with transmission EM to investigate the heterogeneity and polymorphism of amyloid fibers [284, 285].

A key area that requires improvement is data analysis software. Currently, some MS software packages are available for the characterisation of macromolecular complexes (e.g., [286-289]), but manual evaluation remains critical. Programs as effective as those developed for bottom-up proteomics will enable high-throughput studies of non-covalent assemblies.

Overall, multiple complementary techniques are often necessary to decipher the structures of challenging macromolecular complexes. The examples cited in this review suggest that native MS will play an increasingly important role in such structural studies.

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Figure captions

Figure 1. Native MS and "native top-down MS" experiments provide key knowledge about non-covalent assemblies.

Grey boxes depict a holocomplex and its derivative components, including subcomplexes, stripped complexes, individual subunits and polypeptide fragments. MS measurements of these species provide information regarding dynamics (green box), binding interactions (blue box) and structural organization (purple box) of a macromolecular complex. Coloured arrows indicate the flow of information.

The mass of an intact assembly is determined by native MS (MS mode). High-performance liquid chromatography (HPLC) performed under denaturing conditions followed by MS measurements yields the experimental mass of each individual subunit [23, 290]. Experimentally assessessing the masses of individual subunits is important since the measured masses often differ from the theoretical ones because of unexpected truncations and post-translational modifications. Destabilising conditions [e.g., dimethyl sulfoxide (DMSO) or methanol] allow one to partially dissociate the complex in solution before loading the sample into the mass spectrometer. These subcomplexes (such as 2-mers and 3-mers) enable one to identify direct interactions between proteins [105, 151, 152]. This type of experiment also assesses the stability of interfaces between subunits [153][154]. In MS^2 experiments, non-covalent bonds are broken and assemblies are dissociated in the gas phase [19, 43]. According to the type of dissociation methods, 1-mers or oligomers can be ejected from the assembly (see section 6 of text). In MS^3 experiments of "native top-down MS" [42, 91, 136, 140], subunits are fragmented by backbone cleavage using various methods (e.g., ECD or UVPD) (see section 7). These experiments provide access to information about primary sequence, PTMs and sites of protein-ligand interactions [141-143, 171, 172]

Dynamics. The dynamic behaviour of complexes can be studied by MS. For instance, the presence of different oligomeric states and their changes in equilibrium induced by different solution pH values and concentrations can be monitored by MS [28, 30, 48]. By incubating light and heavy isoforms of a protein (such as those labelled with ^{13}C and ^{15}N), the changes in subunit composition can be studied as a function of time. The subunit exchange revealed

distinct behaviour for wild-type and mutant proteins (e.g., involved in amyloidosis [40, 196]). Native MS can be instrumental in providing information about allostery [59, 241].

Binding. The experimental masses assessed by native MS, HPLC-MS, dissociation in solution and MS^2 allow one to determine the direct interactions between proteins [105, 150-152], stability of interfaces between subunits [39, 153, 154] and stoichiometry of subunits [23, 150]. The MS^3 experiments [42, 91, 136, 140] provide access to information about primary sequence, PTMs and sites of protein-ligand interactions [141-143, 162, 171, 172].

Organization. Overall, the data collected during native MS and "native top-down MS" experiments allows the reconstruction of a two dimensional (2D) map of the interactions between subunits within a macromolecular complex. These data can be combined with structural information (obtained by X-ray crystallography and SAXS) [28, 182-188], to model a 3D architecture of an assembly [189, 291]. Moreover, individual proteins or subcomplexes can be mixed in solution to assess the formation of (sub-) complexes. In this case, a mass shift is detected [292] and allows one to characterise the assembly pathway of a complex and its evolution [39, 153, 154].

Figure 2. Timeline of MS-based investigations of non-covalent assemblies
ESI [259, 293] and nano-ESI [294] paved the way for the advent of native MS. For the first time, the MS investigation of macromolecular complexes was performed in 1991 and electron capture dissociation (ECD) was developed in 1998 [112]. Non-covalent complexes were fragmented by ECD in 2002 [144] and during the same year MS^2 experiments on protein complexes were described [43]. The first ECD fragmentation of a ligand-protein complex was reported in 2006 [145]. After four years, intact protein complexes were fragmented by ECD [146] and using an Orbitrap instrument subunits ejected from intact protein assemblies were broken down in 2013 during MS^3 experiments [42]. In 2018 NEMS measurements of T5 bacteriophage capsids took place [207] and during the same year membrane proteins were successfully ejected from native membranes [250].

Figure 3. Native MS experiments to study the kinetics of dissociation of transthyretin (TTR).
Dissociation of 4-meric TTR is thought to be a key step in the formation of amyloid fibrils. Using native MS, the exchange of TTR subunits was monitored for 11 days. In particular, the effect of two mutations (i.e., S52P and T119M) and that of a drug (tafamidis) on the rate of dissociation of TTR were assessed. A-D) Mass spectra of subunit exchange experiments when deuterated S52P TTR was mixed with hydrogenated (A) WT, (B) S52P, (C) T119M, and (D) S52P bound to tafamidis, respectively. The spectra were recorded at the beginning of the reaction (left panel) and after 11 days (right panel). The *mass-to-charge* ratio is indicated as *m/z*. E) Scheme of the native MS experiments: 4H (hydrogenated) and 4D (deuterated) 4-mers were mixed in equal parts; following dissociation, four hetero- 4-meric species were formed. F) Results of the subunit exchange experiments of D-S52P with H-WT, H-S52P, H-T119M, and H-S52P bound to tafamidis, respectively (from the left panel to the right one). Changes in relative abundance of homo- and hetero- 4-meric species over the course of 11 days are shown, along with an estimate of their association/dissociation rates. These figures were reproduced with permission from [40], ©(2019) Nature publications, under a Creative Commons Attribution 4.0 International License.

Figure 4. The dynamic assembly of KaiCBA can be efficiently monitored during native MS experiments.
A) Native mass spectra of the circadian oscillator recorded at 30°C. Peaks corresponding to KaiC, KaiCA, KaiCB, and KaiCBA are highlighted with different colours. The relative signal

intensity (RSI) is shown on the y axis and the *m/z* on the x axis. B) Labelled mass spectrum of Kai system after 12 hours of incubation at 30°C. The identified Kai complexes are schematically drawn and colour-coded as follows: $KaiC_6$, blue and green; KaiA, yellow; KaiB, pink. In the spectrum the peaks corresponding to the Kai complexes are labelled with different coloured diamonds and circles. Light pink diamonds: $KaiC_6$; pink diamonds: $KaiC_6A_2$; light violet circles: $KaiC_6B_1$ and $KaiC_6B_6$; violet circle: $KaiC_6B_6$; blue $KaiC_6B_6A_4$; light blue: $KaiC_6B_6A_6$. This figure was reproduced with permission from [187]

Table 1. Summary of main abbreviations mentioned in the text.

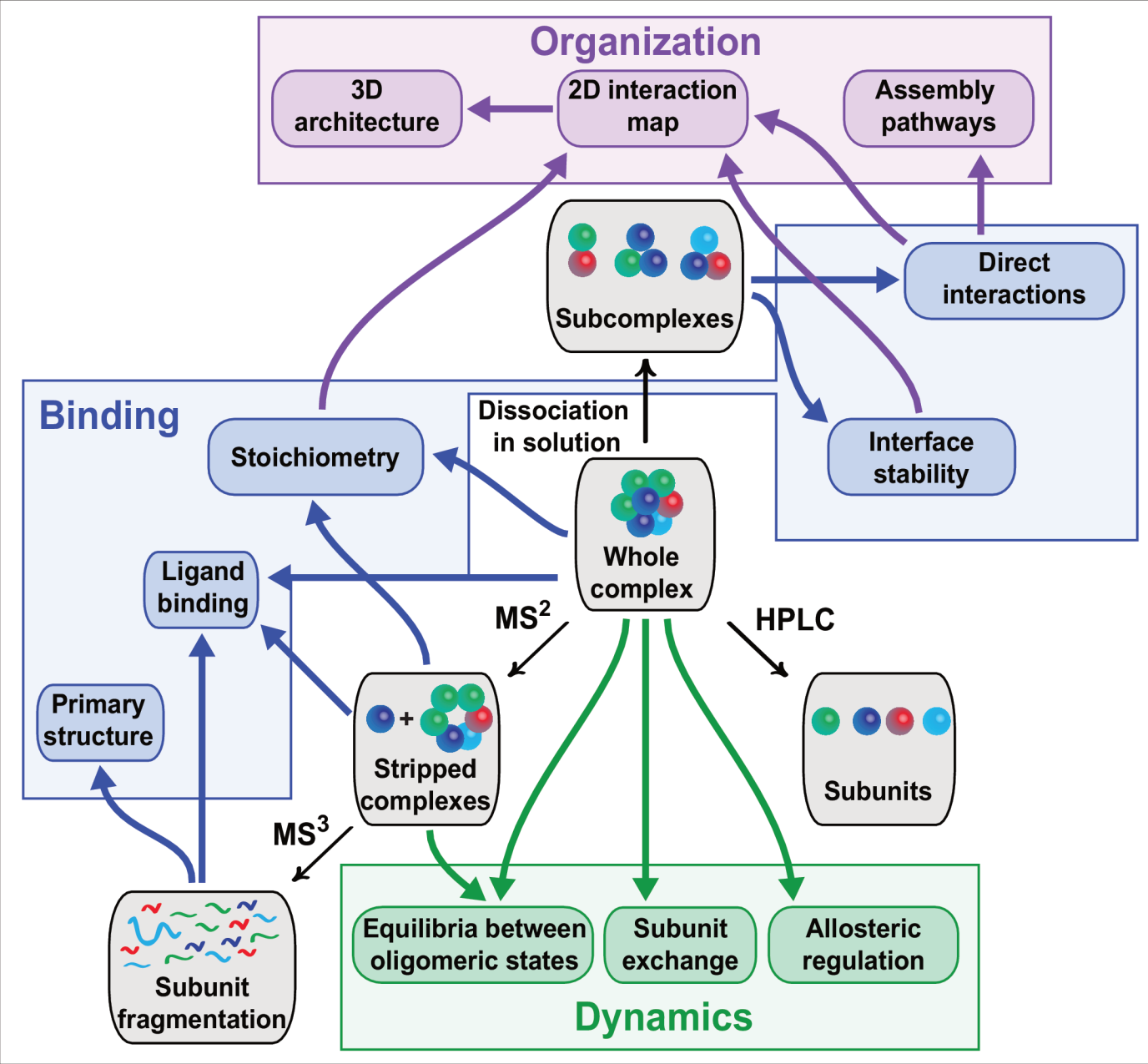
Acronym	Technique	Description
CID (or CAD)	Collision induced dissociation (Collisionally activated dissociation)	Approach to dissociate ions in the gas phase, whereby ions are accelerated by an electrical potential and collide with neutral gas molecules such as argon or xenon.
ECD	Electron capture dissociation	Technique for fragmenting ions in the gas phase. Ions directly interact with low-energy free electrons.
ESI	Electrospray ionisation	Soft ionisation technique whereby ions are produced in the gas phase by applying a voltage to a solution of sample and creating an aerosol.
ETD	Electron transfer dissociation	Approach for fragmenting ions in the gas phase by transferring electrons to them from a radical anion (e.g., anthracene and azobenzene).
FT MS	Fourier Transform based MS	Technique for determining the *m/z* ratio of ions through the frequency signals that the ions generate within the instrument. The mass spectrum is derived by applying a Fourier transform to the frequency data.
FT-ICR	Fourier Transform ion cyclotron resonance	Type of FT-based MS in which mass spectra are determined from the cyclotron resonance frequencies produced by ions when they rotate in a magnetic field.
HCD	Higher energy C-trap (or collisional) dissociation	A CID technique specific to Orbitrap instruments in which the fragmentation of ions takes place outside the Orbitrap analyser.
HDX	Hydrogen/deuterium exchange	Technique used to monitor covalently bonded hydrogen atoms replaced by deuterium atoms. Overall, it can be utilized to investigate conformations of individual proteins and macromolecular complexes.
IM	Ion mobility	Technique that separates molecular ions in the gas phase based on their mobility in a buffer gas under the influence of a weak electric field.
ISCID	In-source collision induced dissociation	Type of CID whereby ions are fragmented in the source region of a mass spectrometer.
IRMPD	Infrared multiphoton dissociation	Approach to fragment ions in the gas phase by the absorption of multiple infrared photons.
MALDI	Matrix-assisted laser desorption/ionisation	Soft ionisation technique whereby molecules are embedded in a solid organic acid (known as matrix) and subsequently desorbed and ionised by a pulse of laser light.
MS/MS or MS^2	Tandem MS	Method of analysis involving two stages of MS selection. The first MS stage separates sample components according to their *m/z*. During the second MS stage, the selected ions are subsequently subjected to fragmentation/dissociation and mass spectra of the products are obtained.
MS^3	-	Using Orbitrap instruments, the 1-mers, ejected during MS^2 experiments, are fragmented by backbone cleavage using various methods such as ECD or UVPD.
-	Orbitrap	A FT-based analyser composed of an inner spindle-like electrode and an outer barrel-like one. Ions are trapped in an orbital motion around the spindle and the frequency signals, which arise from the resulting image current, are utilised for calculating mass spectra.
NEMS	nanoelectromechanical systems	A nanoscale device that resonates at high frequency and functions as a highly sensitive mass sensor. When species are adsorbed on NEMS, the sensors register changes in frequencies that are directly proportional to the mass of the adsorbed species.
Q	Quadrupole	An analyser composed of four parallel metal rods to which a radio frequency (RF) voltage and direct current (DC) voltage are applied. For a given ratio of voltages, only ions with the appropriate *m/z* ratio can travel through the analyser, while others present unstable trajectories and collide with the rods.
SID	Surface-induced dissociation	Technique for dissociating ions in the gas phase, whereby ions are accelerated to collide against a surface such as that self-assembled monolayers of $CF_3(CF_2)_{10}CH_2CH_2S$- on gold serve.
TOF	Time-of-flight	A type of analyser in which ions are accelerated in an electric field and then allowed to drift through a field-free region to a detector. The square of the time taken to reach the detector is directly proportional to the *m/z*

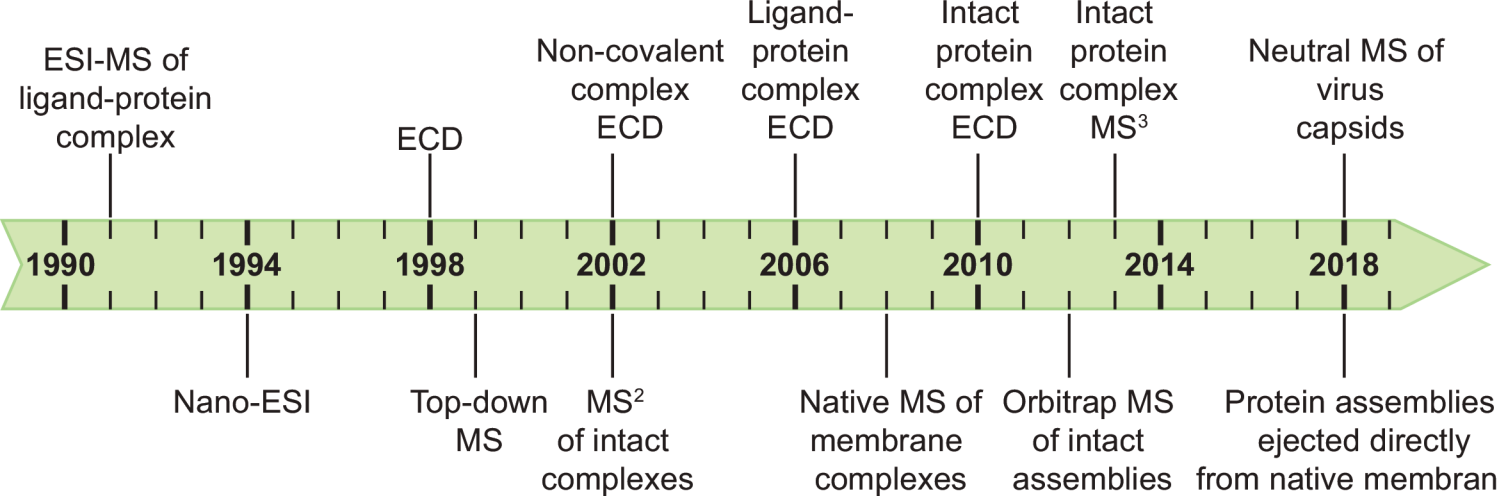
		of the ions.
UVPD	Ultraviolet photodissociation	Absorption of photons by gas-phase ions leads to energization that induces the fragmentation of the ions.

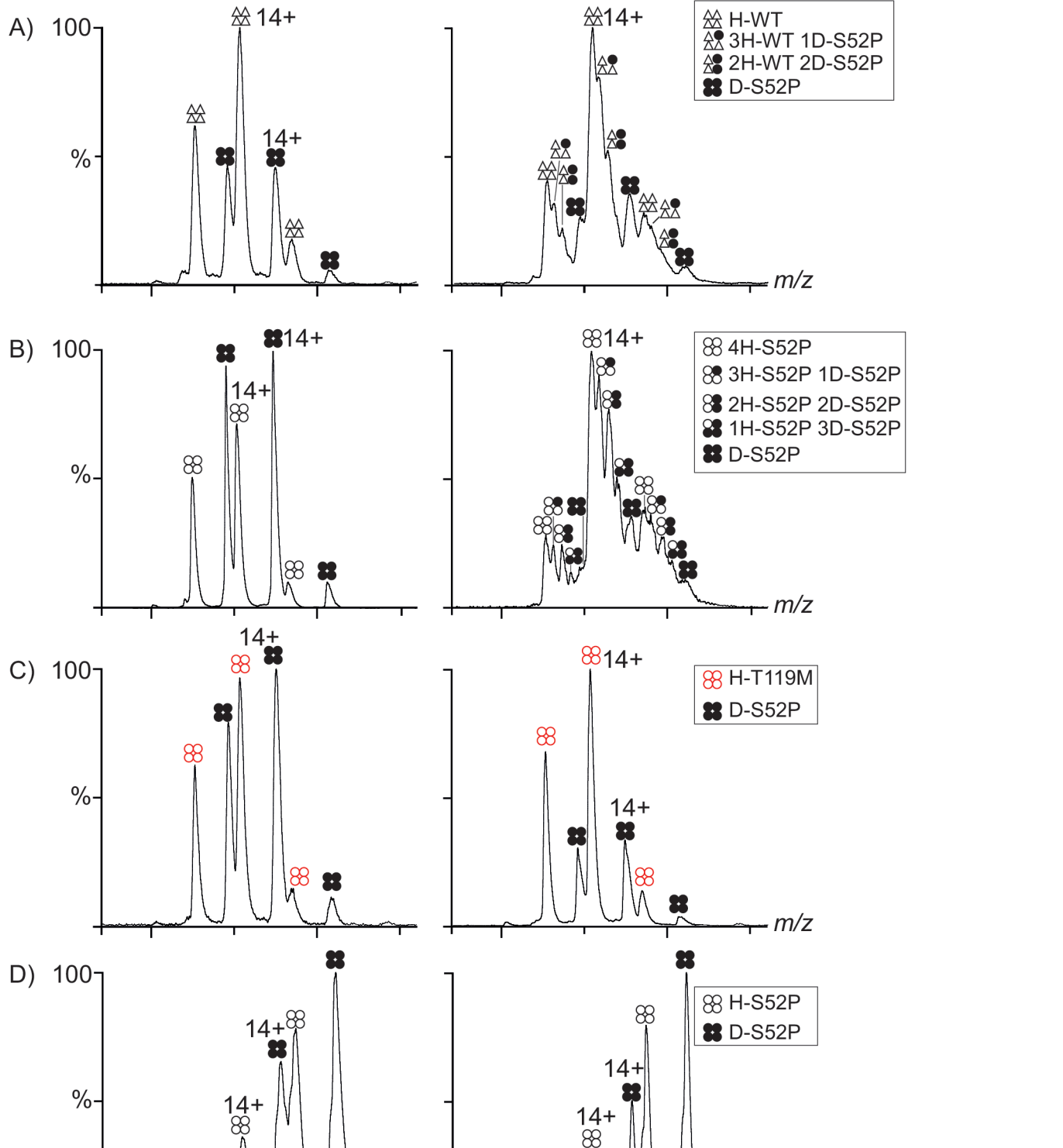
Table 2. Examples of soluble and membrane protein complexes analysed by native MS

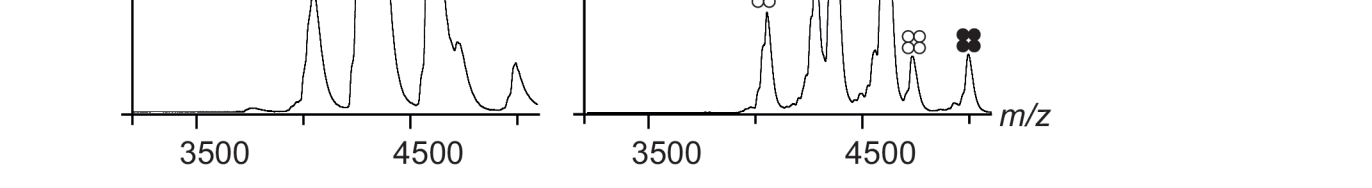
Complex	Mass (kDa)	Outcome of analysis	Prior atomic structure known	References
Soluble Complexes				
Cascade	405	Two dimensional map	N	[210]
Csy (CRISPR system yersinia)	350	" " "	N	[295]
EcoP15I, EcoPI, PstII	63-311	Functional insights	N	[296]
Structural Maintenance of Chromosomes complex (SMC)	424-589	" "	N	[213]
Kinase domain of RIP2 (receptor Interacting protein 2)	34-69	" "	Y	[194]
Lumazine synthase	3000-6000	" "	Y	[193]
Transthyretin	55	" "	Y	[40]
Bacteriophage HK97 capsid	18000	Assembly pathway	N	[73]
TrV virions (from *Triatoma infestans*)	8300	" "	N	[219]
Norwalk virus-like particles	10100	" "	N	[218]
Kai system	624-823	" "	Y	[187].
HK97 Prohead-1 particles	~21400	" "	Y	[199]
DegP oligomers	143-575	Model for the transition between the resting and active states of an enzyme	Y	[297]
Protruding (P) domain of the norovirus capsid protein oligomers	72-1361	Discovery of multiple oligomeric states controlled by buffer conditions (e.g., pH)	Y	[198]
Membrane Complexes				
E. coli Translocon (ColE9-Im9 complex, BtuB, OmpF trimer, and TolB)	296	Functional insights	Y	[234]
MscL	78	" "	Y	[298]
DgkA, pSRII, LacY-GFP	13-78	Reconstitution in detergent, amphipols, bicelles and nanodiscs	Y	[244]

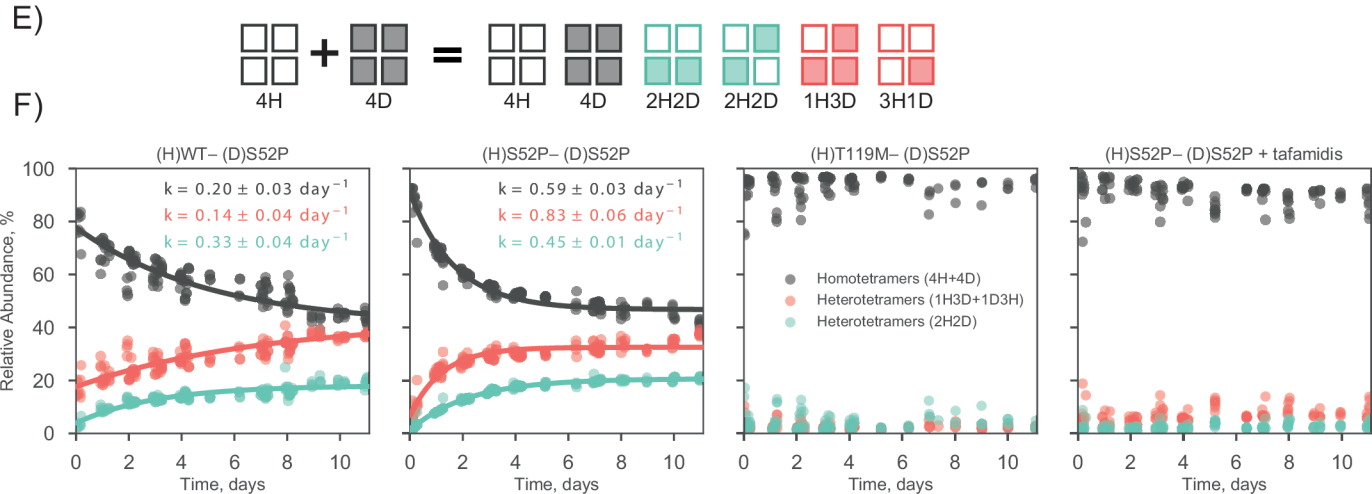
PagP and OmpT, Mhp1 and GalP	20-54	Same as above.	Y	[245]
Many complexes	7-809	Complexes ejected from native membranes into the gas phase	Y	[250]
ATP-Binding Cassette transporter P-glycoprotein (P-gp)	141-147	Ligand binding affinities	Y	[242]
MsbA	134	" " "	Y	[229].
ModBC-A and BtuCD-F	29-159	" " "	Y	[230]
MscL, AqpZ and AmtB	85-126	" " "	Y	[225]
B subunits of cholera and heat labile toxins	58-62	" " "	Y	[248]
Mycobacterial membrane protein Large 3	83-110	" " "	Y	[243]
AmtB	127	Allosteric interactions	Y	[241].

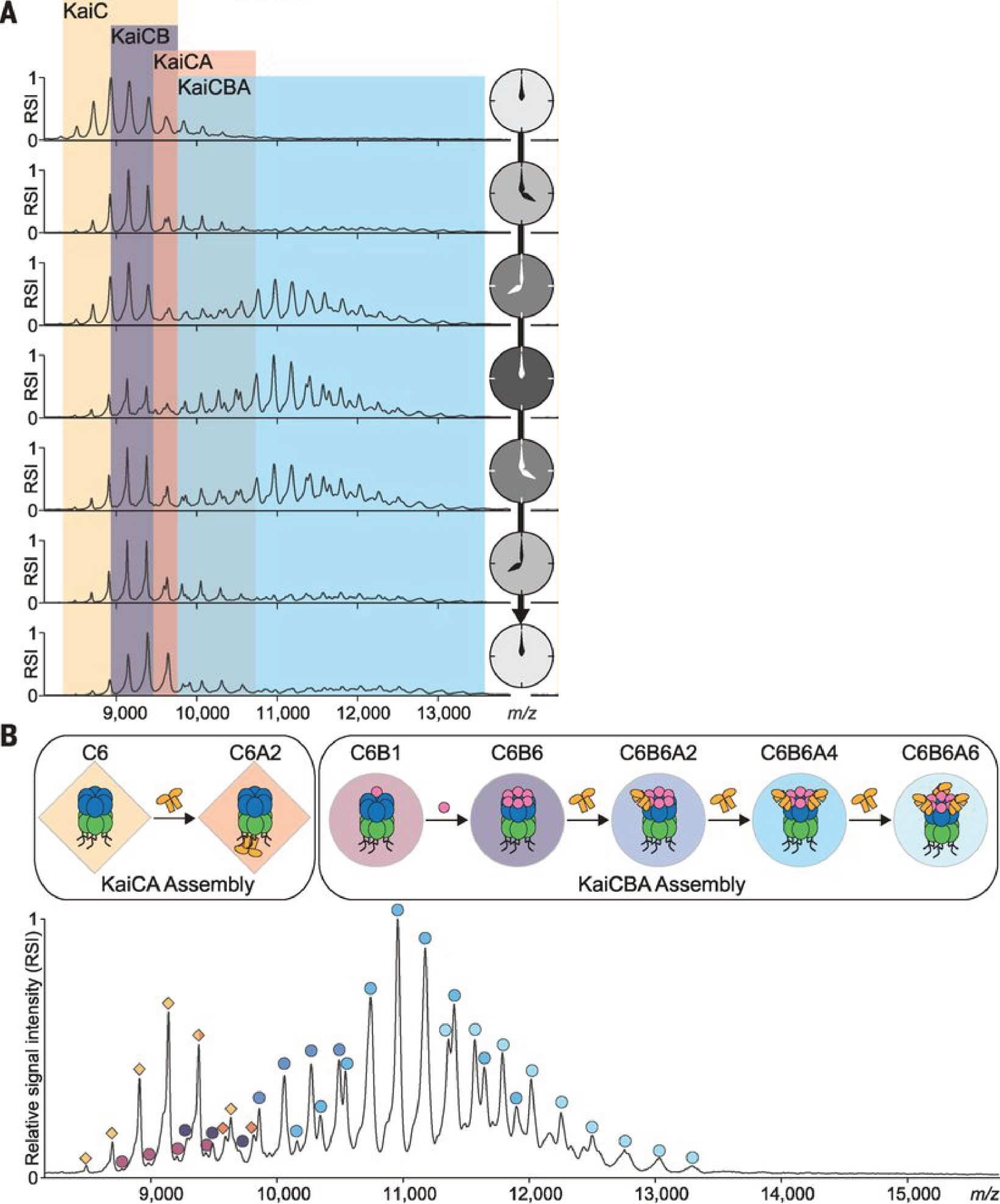

Organization
3D architecture
2D interaction map
Assembly pathways
Subcomplexes
Direct interactions
Binding
Dissociation in solution
Stoichiometry
Interface stability
Whole complex
Ligand binding
MS2
HPLC
Primary structure
Stripped complexes
Subunits
MS3
Subunit fragmentation
Equilibria between oligomeric states
Subunit exchange
Allosteric regulation
Dynamics


ESI-MS of ligand-protein complex
ECD
Non-covalent complex ECD
Ligand-protein complex ECD
Intact protein complex ECD
Intact protein complex MS3
Neutral MS of virus capsids
1990
1994
1998
2002
2006
2010
2014
2018
Nano-ESI
Top-down MS
MS2 of intact complexes
Native MS of membrane complexes
Orbitrap MS of intact assemblies
Protein assemblies ejected directly from native membranes


A)
100
%
14+
14+
14+
H-WT
3H-WT 1D-S52P
2H-WT 2D-S52P
D-S52P
m/z
B)
100
%
14+
14+
14+
4H-S52P
3H-S52P 1D-S52P
2H-S52P 2D-S52P
1H-S52P 3D-S52P
D-S52P
m/z
C)
100
%
14+
14+
14+
H-T119M
D-S52P
m/z
D)
100
%
14+
14+
14+
H-S52P
D-S52P
m/z
3500
4500
3500
4500


E)
4H
4D
4H
4D
2H2D
2H2D
1H3D
3H1D


F)
(H)WT– (D)S52P
k = 0.20 ± 0.03 day⁻¹
k = 0.14 ± 0.04 day⁻¹
k = 0.33 ± 0.04 day⁻¹
(H)S52P– (D)S52P
k = 0.59 ± 0.03 day⁻¹
k = 0.83 ± 0.06 day⁻¹
k = 0.45 ± 0.01 day⁻¹
(H)T119M– (D)S52P
Homotetramers (4H+4D)
Heterotetramers (1H3D+1D3H)
Heterotetramers (2H2D)
(H)S52P– (D)S52P + tafamidis
Relative Abundance, %
Time, days
Time, days
Time, days
Time, days


A
KaiC
KaiCB
KaiCA
KaiCBA
RSI
1
0
9,000
10,000
11,000
12,000
13,000
m/z
B
C6
C6A2
KaiCA Assembly
C6B1
C6B6
C6B6A2
C6B6A4
C6B6A6
KaiCBA Assembly
Relative signal intensity (RSI)
14,000
15,000