Writing assignment

Recent innovations in native mass spectrometry: a valuable tool for the study of protein interactions and kinetics

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Layman's summary

Many proteins do not act individually, but together with other proteins to form complexes and perform reactions. Protein interactions and kinetics can explain how proteins function in these complexes. Many different analytical techniques can be used to study protein interactions, binding, and reactions, including UV/VIS and infrared spectroscopy, and nuclear magnetic resonance spectroscopy. However, this review will focus on the advantages of the use of mass spectrometry (MS), more specifically native MS. With MS the mass-to-charge ratio of a charged molecule is measured, and from that the total mass of that molecule can be calculated. To become a charges species, the molecule most undergo ionization, and with native MS this is achieved in a way that retains non-covalent interactions. Thereby, the mass of the entire complex can be analysed, and the individual components of the complex can be identified.

Some advantages of native MS compared to other methods is that it is quick and requires less sample. However, protein interactions are highly dependent on the environment and some analytes require a biological environment, which contains high salt concentrations. Most other analytical techniques are compatible with many different buffers, and their performances are not influenced by salt concentration or polarity of the solvent. High salt concentrations interfere with MS analysis, leading to low resolution and high background signals. To circumvent this, nano-emitters, online buffer exchange or direct MS can be used. Another external parameter that influences protein interactions and kinetics is temperature. An easy method to test the influence of temperature is by incubating the samples at a specific temperature, and then performing the measurement. However, equilibrium reactions require a stable temperature upon infusing the sample, as small changes in temperature can shift the equilibrium. Two different groups have shown that a stable infusion temperature can be achieved by building temperature control systems.

A big advantage of NMR is that it gives information on the conformational changes of the protein, and that it can pinpoint these dynamic regions to specific residues. Native MS can be extended with ion mobility, a technique that separates ions on their charge and size, thus on their conformation. Additional fragmentation can be used to provide more region-specific information.

In this review different innovations are discussed that have enlarged the native MS toolbox for the analysis of protein interactions and kinetics, displaying that native MS is a valuable alternative for traditional methods. However, native MS can even be further exploited, as it is especially valuable for the analysis of heterogenous samples. For example, native MS can be used to study enzymatic reactions or complex formation of a mix of different proteoforms or post translational modification. Additionally, combined with IM, it might be used to study dynamic interactions of these heterogenous samples.

Abstract

Protein interactions and kinetics can explain a proteins functions and activity. Protein interactions can be studied by a variety of high- and low-resolution methods. Native MS, an MS based technique where proteins native conformation and interactions are conserved, is a valuable tool to identify and quantify protein interactions. Unlike other methods, native MS does not require labelling for identification and qualification of different species in a sample, which makes it suitable for the analysis of proteins that are extracted from patient material. Additionally, native MS is particularly useful when analysing heterogenous samples. This review focusses on recent innovations to extent the native MS toolbox for the study of protein interactions. The showcased innovations, which are both online and offline, allow for extended usage of native MS, for example for the analysis of (thermo)dynamic properties. Also sample preparation and fragmentation innovations are discussed. Although innovation is rapid and there seems to be no limit, we believe that the current native MS toolbox can be exploited for the

functional analysis of heterogenous samples, for example caused by post translational modifications or a mix of competitive binding partners.

Introduction

Protein interactions and kinetics are the basics for understanding a protein's function and pathways(1). Methods to study protein interactions include UV/VIS spectroscopy(2), circular dichroism spectroscopy(3) surface plasmon resonance(4), nuclear magnetic resonance spectroscopy (NMR)(5), and mass spectrometry (MS)(1). A variety of different MS techniques exist to study protein interactions and kinetics, such as hydrogen deuterium exchange (HDX)(6), chemical cross linking(7) and native MS(8).

UV/VIS spectroscopy, circular dichroism and surface plasmon resonance are analytical techniques based on light(9,10). With UV/VIS spectroscopy, the rate of the reaction is followed as the absorbance or fluorescence changes as a chromophoric or fluorophoric reactant or product is converted. If the reactant or product does not have chromophoric or fluorophoric properties, a fluorescent tag might be used(9). Circular dichroism spectroscopy measures the differences in absorption of left and right circularly polarized light and it gives information on the secondary protein structure. This technique is label-free, however structural resolution is low(10). Lasty, surface plasmon resonance measures the reflection of light on a surface in different angles. The analyte is immobilized on this surface, and at a specific angle the light is absorbed instead of reflected; binding of the analyte with a ligand will cause a shift in this angle. This technique is also label free, but it requires immobilization of the analyte, which can change its physicochemical properties(11). All three techniques have a low structural resolution and are thus not capable for the identification of binding regions and you cannot distinguish between different conformers.

NMR does have a high structural resolution. This technique is based on the detection of the spins of nuclei, which are influenced by their chemical environment. In this way, each nucleus has a unique spin and will give a unique signal, which will change upon conformational change or interaction with another molecule or protein(12). This change can be seen as peak splitting or peak shifting, depending on the exchange rate; however, the peaks in the spectrum represent an average of all equal atoms in the mixture and single events are not resolved. Not all nuclei spins can be measured with NMR, as it requires an odd number of protons and neutrons. For example, hydrogen (^{1}H) can be measured, but deuterium (^{2}D) cannot. As proteins are made up from carbon and nitrogen, which naturally occur as ^{12}C and ^{14}N, this requires enrichment of isotopically labelled ^{13}C and ^{15}N(13). Thus, proteins must be recombinantly expressed. Other drawbacks include: high sample consumption (several milligrams) and time-consuming data acquisition and analysis.

In native mass spectrometry (MS) a biological analyte is analysed in a non-denaturing solvent, thereby retaining to some extent its native-like structure(14). The analyte is ionized by electrospray ionization (ESI), which is considered a 'soft' ionization technique(15). Native MS is especially capable of quantifying the heterogenous nature of a sample, for example when analysing different post-translational modifications on a protein with high resolving power(16). Additionally, the combination of soft ionization by ESI and the retainment of the native-like structure make this technique extremely useful for the examination of protein complexes and interactions; as non-covalent interactions are preserved under these conditions(17).

When comparing NMR and native MS, native MS has a lower sample consumption, and that the data acquisition is less time consuming. However, the resolution of MS is influenced by high salt concentrations and standard biochemical buffers cannot be used in MS analysis. Additionally, there is no option for temperature control in the mass spectrometer, which for example is available in an NMR spectrometer or for some UV/VIS spectrophotometers. Lastly, another aspect of protein interactions is the conformational changes of the proteins. Since conformational changes do not change the mass

of the protein, additional techniques are required to study this in MS. This review will discuss these drawbacks and recent innovations by which they can be circumvented.

Protein interactions and reactions

It has been estimated that over 80% of all proteins interact with one another to form complexes. These protein-protein interactions are generally dependent on complex mechanisms; and binding by metals or cofactors, or different post translational modifications, can alter the affinity and kinetic parameters of these interactions(18). Here, the basic principles of binding and enzymatic kinetics will be explained.

Binding

Protein binding can be described as the following equilibrium:

$$P + L \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} PL$$

where P is the protein and L is a ligand, which for example can be another protein, a small molecule or a metal, and PL is the protein-ligand complex. The binding rate is described by k_{on} and the dissociation rate is described by k_{off}; the units for the k_{on} and k_{off} are $M^{-1}s^{-1}$ and s^{-1}, respectively. Binding affinity is often represented as the dissociation constant (K_d), which is defined by:

$$K_d = \frac{k_{off}}{k_{on}} = \frac{[P][L]}{[PL]}$$

where the square brackets represent the molar concentration of all components at equilibrium. A low K_d equals high affinity.

Quite a few methods have been established for the identification of protein-protein interactions and their kinetic parameters. Several of these methods, amongst them fluorescence polarization, NMR, fluorescence and bioluminescence resonance energy transfer and display systems, are well suitable for analysing general binding parameters(10). Data derived by these methods can be plotted into the equations above to derive the K_d. However, when investigating different properties of a specific protein with multiple proteoforms (for example different glycosylation patterns) or proteins that exist in different stoichiometric regimens (Figure 1), the prior mentioned methods are not sufficient.

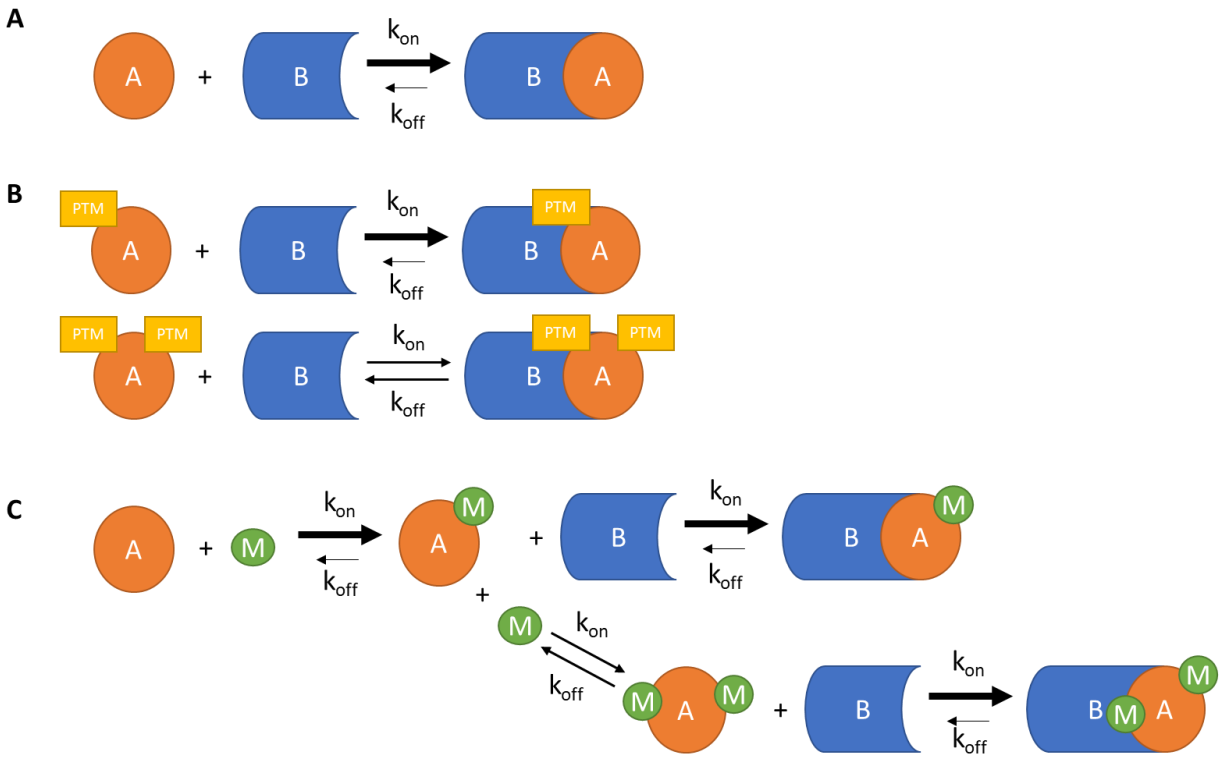

Figure 1: Examples of binding interactions of a protein. *A) The simplest case of binary binding is where protein A and B bind with each other. The k_{on} and k_{off} are represented with the forward and backward arrows, respectively. This binding interaction can be analysed with most conventional techniques. B) represents a reaction where protein A exists in a mixture of different proteoforms, because it can have one or two post translational modifications (PTMs). Here, native MS might be used to analyse individual binding parameters, because the different proteoforms have different masses and are thus resolved separately in the mass spectrum. C) Here, protein A can bind either one or two metal ions (M), and then bind to protein B. Again, each intermediate has a unique mass, and can be analysed with native MS.*

Enzyme kinetics

Apart from binding, many proteins can also initiate and catalyse chemical reactions, the simplest enzymatic mechanism is as follows:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \overset{k_2}{\longrightarrow} E + P$$

where E is the enzyme, S is the substrate, ES is the enzyme-substrate complex, and P is the product. Kinetic parameters are commonly described for the steady state, when the concentration of the enzyme-substrate complex is constant. The reaction rate (v) is defined by the Michaelis-Menten equation:

$$v = \frac{k_{cat}[S]}{K_M + [S]}[E]_0$$

where [S] is the molar substrate concentration, $[E]_0$ is the molar enzyme concentration at the start of the reaction, K_M is the Michaelis-Menten constant, and the k_{cat} is the turn-over rate in s^{-1}. Kinetic parameters k_{cat}/K_M ($M^{-1}s^{-1}$) and k_{cat} describe enzyme efficiency and activity, respectively(19).

This simple mechanism is however not always applicable, as the above mechanism misses the conversion of ES to the enzyme-product complex (EP). Additionally, in complex biological systems branched pathways can occur simultaneously, or additional binding to co-factors are required for a conversion of a substrate.

Proteins in solution are not static, but flexible and capable of adapting different conformations. Protein interactions are therefore also dynamic processes(20). Two major binding models exist: induced-fit and conformational selection. The former model describes that conformational change is the result of ligand binding, while the latter describes that conformational change enables binding of a ligand(21).

Fundamentals of native MS

With electrospray ionisation (ESI) the sample, dissolved in a volatile aqueous buffer, passes through a highly charged needle, resulting in a spray of charged droplets. These droplets evaporate rapidly, resulting in gas-phase ions suitable for MS analysis(15) (Figure 2A). Due to the gentleness of this ionisation technique, non-covalent interactions are retained, which enables the analysis of native-like folded proteins and protein complexes.

The first mass spectrometer for native MS was developed in the early 1990's, it was a time-of-flight (ToF) mass spectrometer with an electrospray ion source(22). And in the 2010's, orbitrap and Fourier transform ion cyclotron resonance (FTICR) mass spectrometers were equipped for native MS(23,24).

After ESI-MS, a mass spectrum is generated where each peak corresponds to a specific mass-to-charge ratio(m/z). Ionisation of proteins and protein complexes usually results in multiple charge states. These charge states can be used to analyse different species that co-exist in solution(22,25,26). By deconvoluting the data, a mass plot can be drafted that disregards these different charge states to give a general overview of the total abundance of each unique mass species (Figure 2B).

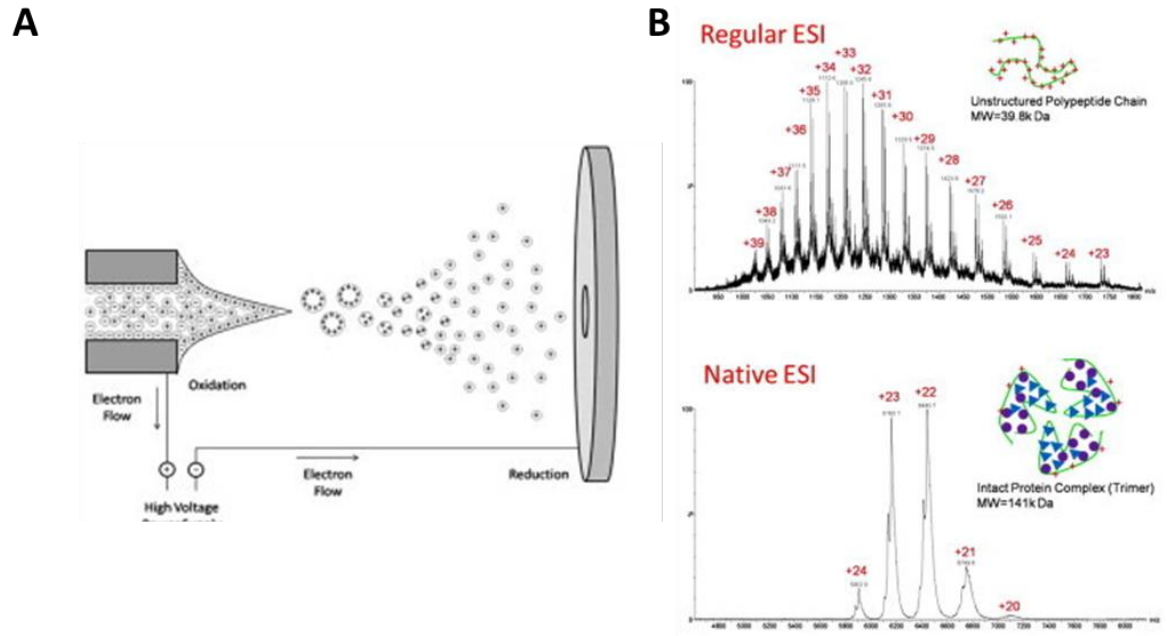

***Figure 2: schematic representation of the principle of native mass spectrometry**. A) ESI: charged droplets are formed by passing the solvent through a small, highly charged emitter. The small droplets evaporate, resulting in charged proteins, which enter the mass spectrometer. B) A typical charge spectrum of a denatured (top) and native (bottom) protein. Charges are lower than with denatured MS and following deconvolution individual proteoforms can be resolved. Image adapted from Zhang et al. 2013*(27).

Recent innovations

Buffer solutions

When analysing biomolecular interactions with non-MS-based methods, for example with NMR or UV/VIS spectroscopy, the analyte is usually dissolved in a biochemical buffer, such as Tris/HCl or phosphate. However, these buffers are not frequently used in native MS, as resulting salt adducts lead to decreased sensitivity and signal-to-noise ratios(28). Additionally, these adducts may interfere with the assignment of the different species in a sample, especially when examining for example protein interactions with multiple competing ligands. Therefore, analytes in native MS are preferably analysed in buffers containing volatile salts, most frequently ammonium acetate, which often requires desalting and buffer exchange steps(29,30). However, not all analytes can withstand buffer exchange conditions and some biomolecular complexes require biological buffers and high metal salt concentrations for structural stability(31), and could therefore not be readily analysed with native MS. Here, several innovations made in the last few years that enable native MS for samples in biochemical buffers will be discussed in detail.

It has been reported that the use of nanoscale ESI emitter tips can decrease salt adduct formation when using non-volatile buffer, and that salt adduct formation decreases as the tip diameter decreases (Figure 3A) (32). It was shown that such an emitter tip with an inner diameter of 250 nm can be used to measure the binding affinities of small ligands to proteins in high concentrations of non-volatile buffers (Figure 3B). Although signal-to-noise ratio is still lower than when using a volatile buffer, it is sufficient to identify and quantify protein and protein-ligand-complexes to derive binding constants. Additionally, nano-scale emitters increase the resolution when using volatile buffer; using this technique, the competitive binding of six ligands, with mass differences up to 0.06 %, could be measured directly in a single experiment(33). However, nano-emitters might also lead to increased adherence of the proteins to the glass(34), or surface-induced protein unfolding(35). Alternatively to nano-emitters, resolution can be improved with the addition of ammonium acetate to biological buffers; the addition of 1.6 M ammonium acetate to different biological buffers led to well resolved peaks of different charge states for a tetrameric protein complex, and even lower concentrations of ammonium acetate were sufficient in the study of a protein-DNA complex(36).

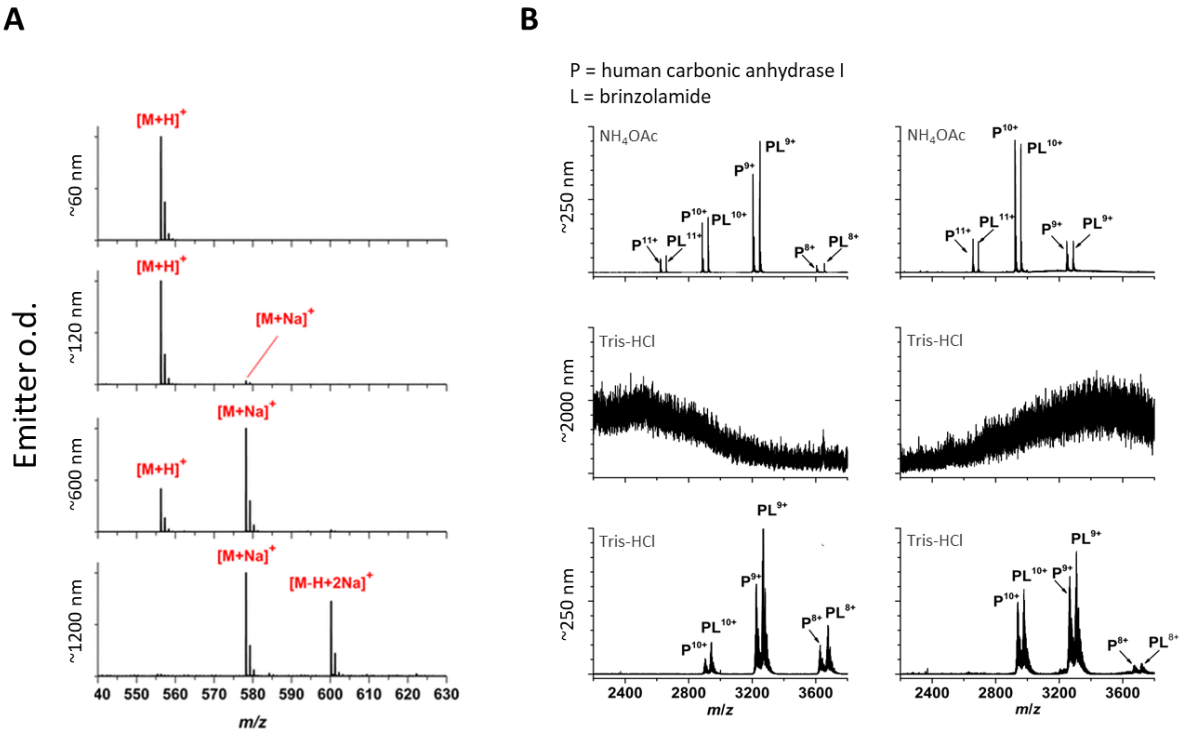

Figure 3: The use of nano-emitters improves signal-to-noise ratio of ESI-MS spectra and decreases salt adduct formation. *A) Mass spectra of 10 μM Leu-enkephalin in a 100 μM NaCl solution. Decreasing the emitter opening also decreases salt adduct formation. B) The binding of 5 μM human carbonic anhydrase I and 3 μM brinzolamide (left), and 5 μM human carbonic anhydrase II and 3 μM indapamide (right) is studied in different buffers and using different nano-emitters. The analytes are studied in (a, b) 70 mM ammonium acetate (pH 7.4) and (c-f) 50 mM NaCl and 20 mM Tris-HCl buffer (pH 7.4). Images adapted from Hu et al. 2017* (32) *and Nguygen et al. 2019* (33)*.*

However, not all biologically relevant proteins are soluble in aqueous systems, such as membrane and lipo-proteins. These analytes require additives to the buffer, to create membrane mimicking conditions, for example the addition of detergent micelles or the use of nano-discs. These and other methods to analyse non-soluble proteins by native MS have been excellently described elsewhere(37,38), and will therefore not be discussed here.

For samples that do not require biochemical buffers and high salt concentrations to retain their interactions, buffer exchange can still lead to loss of sample due to centrifugation at high speed. For these samples, the use of an online buffer exchange system might be beneficial. This concept, where a small size-exclusion chromatography (SEC) column is used to separate non-volatile salts from protein complexes, was introduced in the early 2000's(39); and recently a detailed and optimized protocol was published for the use and preparation of these small columns consisting of PEEK tubing filled with P6 Bio-Gel that allows fully automated and fast (<5 min per sample) analysis of samples up to 800 kDa (Figure 4)(40). Applications of this method include rapid quality control(41), and analysis of labile protein complexes(42).

Additionally, the advantages of online buffer were demonstrated in the analysis of iron-sulphur cluster complexes. Recently, native MS has emerged as a popular method for the study of binding interactions and kinetics in iron-sulphur cluster complexes (43). In 2021, a general method was published for the use of native MS when studying iron-sulphur proteins(44), which has been used to explore several mechanism involving iron-sulphur complexes(45–47). However, these methods were not suitable for the characterization of iron-sulphur clusters located on the protein-protein interface, as these specific complexes are susceptible to dissociation into monomers upon ionization. These problems could be circumvented with the use of online buffer exchange combined with nano-electrospray(48). By these means, new intermediates were found, and a mechanism could be drafted for the substrate exchange of the iron-sulphur clusters on the protein-protein interface.

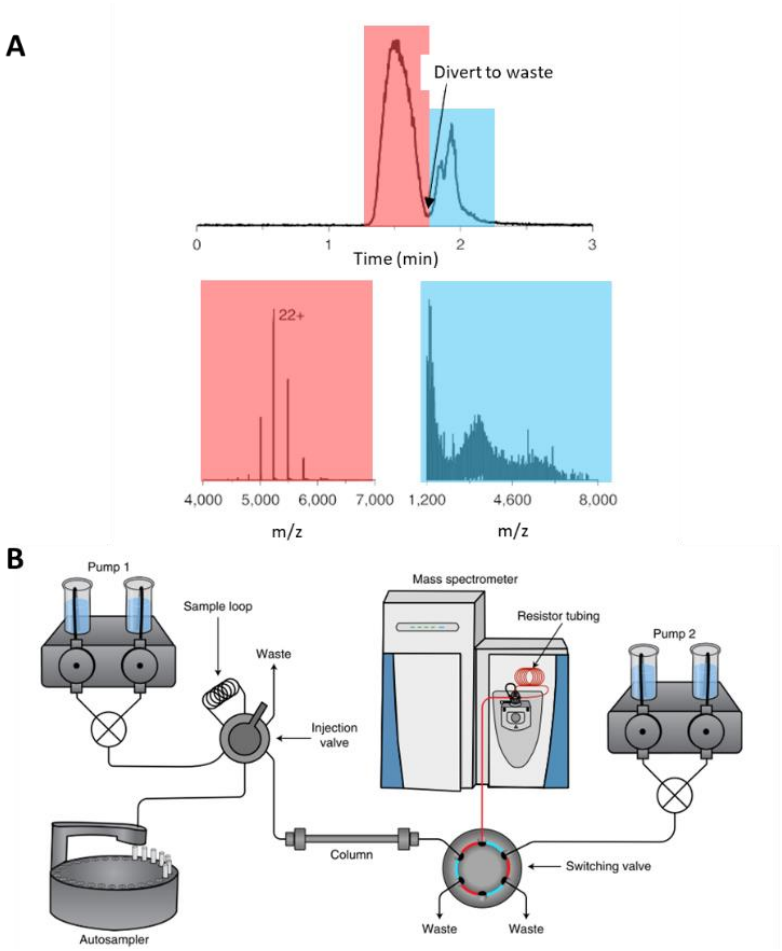

Figure 4: Online buffer exchange (OBE) coupled to native MS. *A) A typical total ion chromatogram and corresponding ESI-MS spectra of OBE-MS. The peak in red is the protein of interest, and in blue the non-volatile PBS components. B) The experimental setup for OBE native MS. The sample is injected and separated by the size-exclusion column, which can either be directed towards the MS, or to the waste, by the switching valve. The red lines show the initial position of the switching valve, when the protein elutes. The blue lines show the position for the diversion of non-volatiles to waste, during which clean buffer from pump 2 is directed to the MS. Image adapted from VanAernum et al. 2020* (49).

Online buffer exchange minimizes sample preparation steps, however, direct-MS, another emerging technique, diminishes sample purification and preparation all together when analysing recombinant proteins. With this method, the overexpressed recombinant proteins are examined from crude medium without any purification steps. For proteins expressed in bacteria, the cells are lysed using sonication in a ammonium acetate buffer, which can directly be injected into the mass spectrometer (Figure 5A) (50,51). When using secretory expression systems (yeast, insect and mammalian), the medium containing the protein of interest is collected and then buffer exchanged 1-3 times to ammonium acetate, after which it is directly injected into the mass spectrometer(51,52). This method has for example been applied in the analysis of antibody-antigen complexes; the antigen was spiked into the medium and subsequently analysed using native MS (Figure 5B) (53). The major advantage of direct-MS is that it enables quick quality control of recombinantly expressed proteins, while preventing loss of sample in purification procedures.

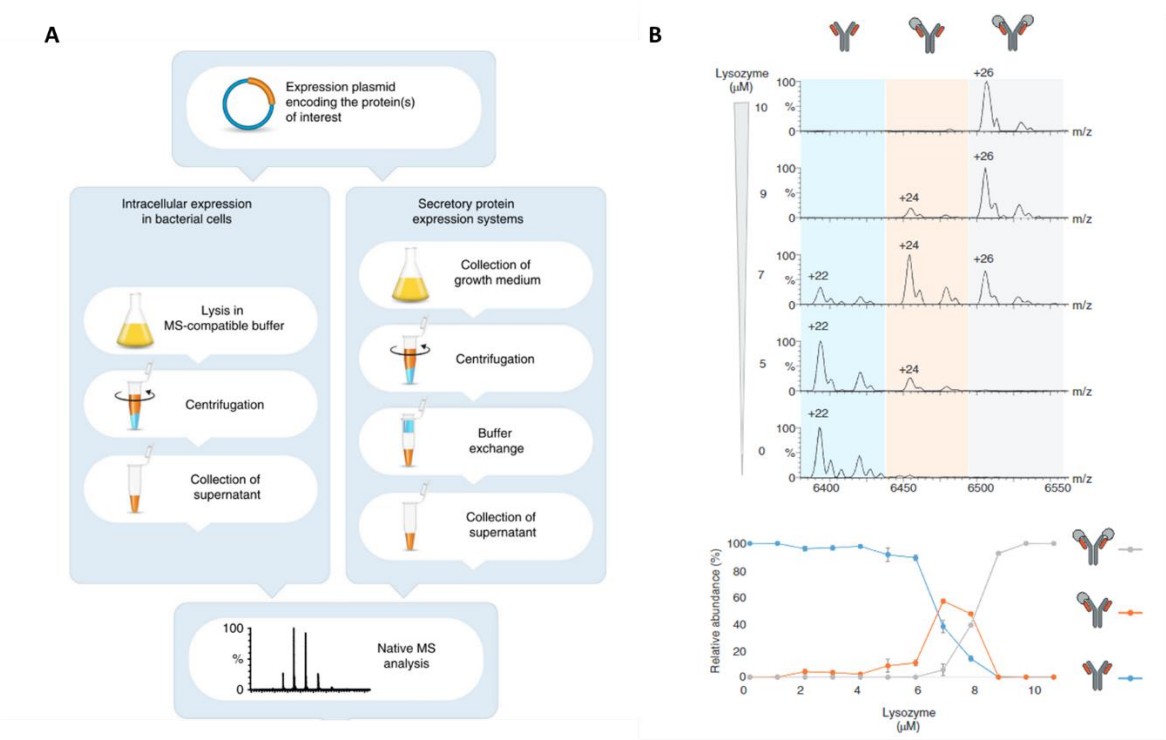

Figure 5: With direct MS, an overexpressed protein can be analysed directly from the cell lysate or medium, or the medium can be spiked with a protein of interest to identify binding partners or study binding parameters. *A) The workflow of direct MS. With intracellular expression systems, the lysis is performed in an MS-compatible buffer, followed by centrifugation to remove insoluble parts and subsequent MS analysis. For secretory expression systems, such as yeast, insect or mammalian cells, the growth medium is collected and buffer exchanged to an MS-compatible solvent, which can then be analysed directly with MS. In B), an anti-lysozyme antibody was expressed, and buffer exchanged into MS compatible solvents, as shown in A. This solution was subsequently spiked with different concentration of lysozyme and analysed with MS, to study the stoichiometric binding of the antibody to the ligand. The top panel shows the ESI-MS chromatogram, the colours correspond to the unbound (blue), single-bound (orange), and double-band (grey) antibody. In the bottom panel their relative abundances are plotted against the ligand concentration. Images adapted from Vimer et al. 2020* (51) *and Vimer et al. 2021*(53)*.*

Temperature

Protein interactions are highly influences by external factors, such as pH and temperature. The pH is buffer dependent, which was already discussed above. The temperature effect can be of interest when examining the thermodynamics, or energetics, of a reaction. This effect can be studied by performing a reaction at different temperatures and following the conversion of products over time. High resolution native MS has for example been used to determine the transition state thermodynamics of intrinsic GTPase activity of K-RAS and its oncogenic mutants. First, the heterogeneity of the isolated K-RAS was investigated, as it was bound to GTP, GDP, 2'-deoxy-GTP, and 2'-deoxy-GDP. Different ratios of these complexes suggest different affinity for these substrates of different mutants. By time-dependent titrations, the hydrolysis rate could be established. Then, the temperature dependence was investigated, by performing the hydrolysis at different temperatures and monitoring the hydrolysis rate(54).

When using this method to analyse thermodynamics of complex formation or reactions, the sample is not infused at the incubation temperature. This might cause a shift of the equilibrium. When examining a reaction which is dominantly forward, this will not create biased results. However, when examining equilibrium states of certain complexes, this will be a problem. Thangaraj *et al.* developed a variable-

temperature inlet for native MS to circumvent this. They build a system to keep the reaction mixture at constant temperature and keep a continuous flow, and used it to study subunit exchange (Figure 6A) (55).

Cong *et al.* designed a different setup for online temperature control, as they used a heatsink and airflow to control the temperature of the sample within the capillary and the air surrounding the source chamber(56). With this setup, the entire nano-ESI tip heated, and the sample temperature reaches an equilibrium within ~40 seconds (Figure 6B). This enabled thermodynamic analysis of binding parameters for three different soluble protein-ligand complexes. Here, native MS provided an advantage over traditional methods for the study of thermodynamics (isothermal titration calorimetry and surface plasmon resonance) as individual binding events could be resolved.

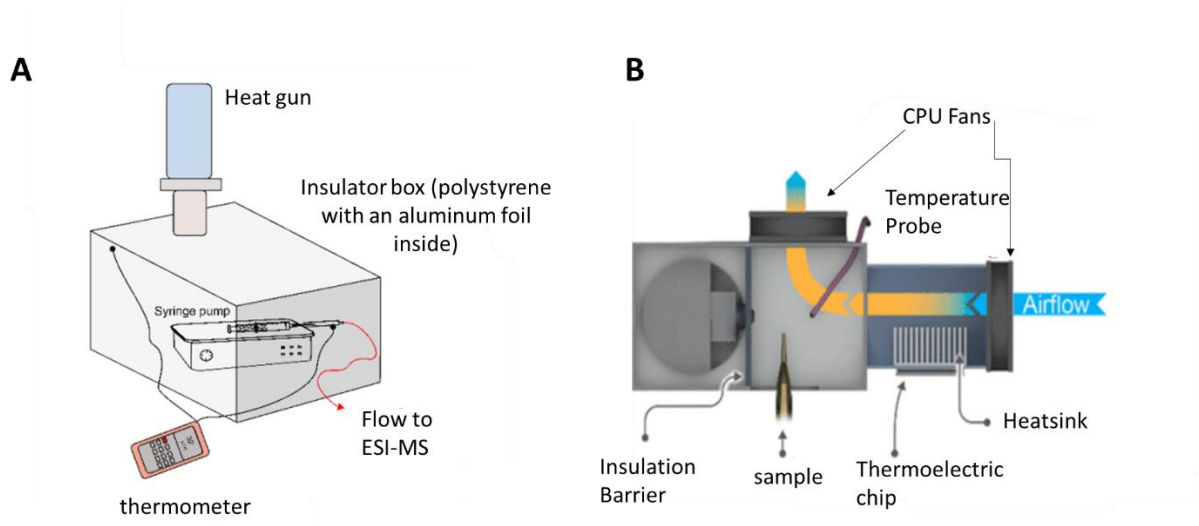

Figure 6: schematic set-up for online temperature control. *A) Set-up used by Thangaraj et al. Temperature control is achieved using a heat gun to warm-up an insulated box containing the sample, which is constantly infused into the MS by the syringe pump. Image adapted from* (55)*. B) Set-up used by Cong et al. Here, the temperature of the sample and the air surrounding the source are simultaneously managed by the hot airflow. Image adapted from* (56)*.*

Dynamic protein interactions

The interactions of proteins with other molecules is a highly dynamic process, as different conformers and energy states of the same protein exist(57). These dynamics can be studied using several high-resolution techniques, such as NMR spectroscopy, where different conformations cause peak broadening or splitting, or HDX-MS. A big advantage of NMR, over HDX-MS, is the additional information on the timescale of the dynamic transitions(20). However, NMR experiments requires high amounts of protein and can take a long time, on top of that, additional experiments are necessary for the assignment of peaks. Moreover, heterogenous samples make peak assignment nearly impossible.

Native MS alone is not capable of revealing small conformational changes. But combined with the use of ion mobility (IM) spectrometry, where ions are separated as they move in the gas phase through an electric field, it can be used to study protein dynamics. The mobility of the ion can be converted into the collision cross section (CCS), which gives direct information about the conformation of the ion(58), thus different conformers will have different CCSs and will enter the mass spectrometer at different retention times (Figure 7). This technique can be exploited to study the dynamics of protein-ligand binding, and to distinguish between conformational selection and chemical fit. For example, Stojko et al. have shown that small conformational changes (Δ CCS < 1%) as a result of ligand binding can be studies using IM-MS. They examined the binding of 3 different ligands to a peptide deformylase 1B, both in an individual as in a competitive manner; and their data showed that different protein:ligand

complexes exhibited different CSS values, e.g. different conformations, which shows that binding occurs through an induced-fit process (59).

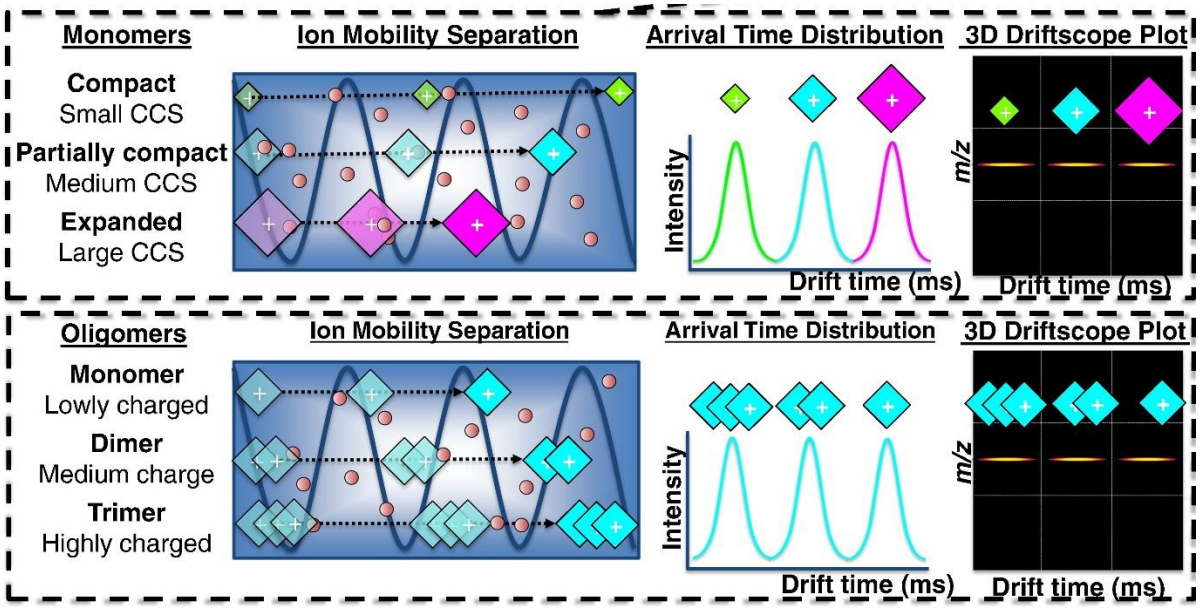

Figure 7: The principle of Ion mobility chromatography. *Ions are passed through a drift tube filled with buffer gas, thereby separating molecules both by charge and size. On top is depicted the situation of different conformations of the same mass and charge. Here, large molecules (with a large CCS) will have lower mobility, as they experience more collisions with the gas molecules, and will have larger drift times. On the bottom is depicted the situation with different oligomers with different charges. Usually, the highly charged ions have a shorter drift time, as they experience more ionic forces. Image adapted from Woods et al.*(60)

More recently, Weerasinghe and co-workers used IM-MS to study the dynamic properties of the enzyme lanthipeptide synthetase. Due to its high mass (mass > 100.000 kDa), it is difficult to study the dynamic interactions of lanthipeptide synthetase using NMR; it will lead to poor signal dispersion, which makes spectral assignment difficult. It's dynamic properties have been investigated with HDX-MS (61,62). Weerasinghe *et al.* show that IM-native MS provides additional information on the dynamics as both inter- and intramolecular interactions and conformational dynamics. Native MS was able to resolve both the free, bound, and double bound form of lanthipeptide synthetase with its precursor (Figure 8A). The free enzyme was examined in depth by IM-native, and two major enzyme conformations were found with different CCS values, which correspond to an open and closed form (Figure 8B). Then, the interaction of the enzyme with its precursor peptide was investigated, also revealing two conformations in the bound state, however with a smaller difference in CCS. This suggest that the conformational equilibrium shifts towards a more homogenous population of structures (63).

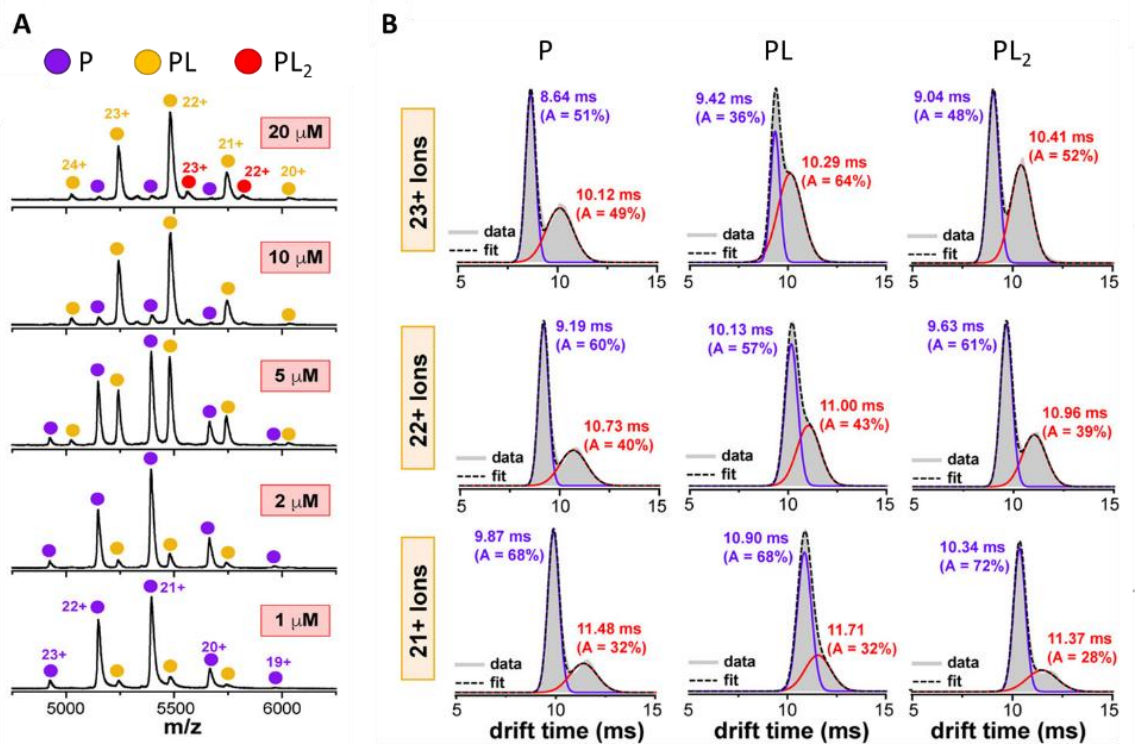

Figure 8: Binding of lanthipeptide synthetase (P) to its precursor (L). *A) The binding of protein and ligand upon different ligand concentrations to quantify binding parameters. B) IM revealed two different bound species, purple and red, with different CCS, corresponding to an open and closed conformation. Image adapted from Weerasinghe et al. 2021* (63).

Furthermore, Butler et al. provided detailed investigations of the acid induced unfolding of SOD1, a dimeric metalloprotein of interest in several neurodegenerative conditions. Each monomer binds one copper ion, and one zinc ion. Authors employ two different commercially available IM-MS instruments, namely a drift tube IM-MS and trapped IM-MS. Association of the dimer was prompted by increasing concentrations of acetonitrile and formic. Evoking dissociation by increasing acidity is common practice in other analytical methods, such as NMR and spectroscopy, and authors show that this is also applicable in IM-MS(64).

Fragmentation

In addition to IM-MS, collision induced unfolding (CIU) can be performed to measure ion stabilities, and to provide information on condensed-phase folding, domain structure and ligand binding(65). By increasing the collision voltage, a protein or protein complex will unfold, which results in a different CCS and a different arrival time(66). Data derived from these experiments are often plotted in a 3D plot of the intensity, the collision voltage, and the drift time, which is called the CIU fingerprint (Figure 9A). CIU can also be combined with collision induced dissociation (CID), which may create a more discriminating fingerprint, as shown for several kinase inhibitors(67). This combination was pioneered by Rabuck *et al.* who investigated different kinase inhibitors. There are different types of kinase inhibitors, the most important two being type I, that binds ATP within the kinase domain, and type 2, that binds ATP outside of the binding pocket. Because of the small difference in CCS, it was difficult to distinguish between the two types by IM-MS alone. However, with addition of CID, key patterns could be identified for each type (Figure 9B).

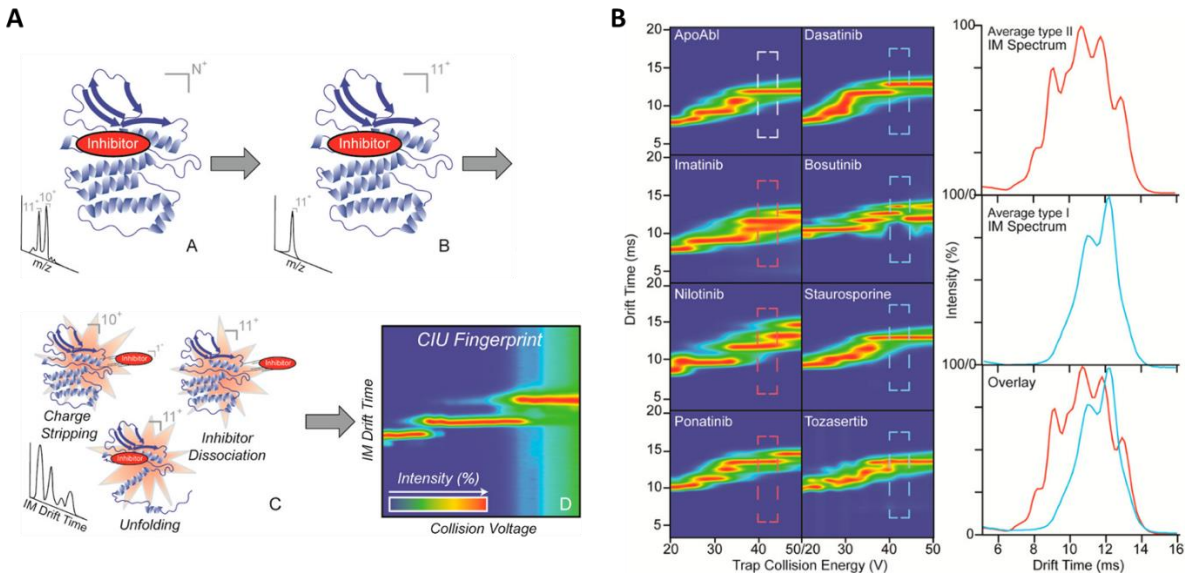

Figure 9: CIU fingerprint upon protein-ligand binding. *A) a schematic description of the steps in an CIU assay of a kinase inhibitor. The protein-ligand bound complex is analysed (a) and a single charge species is selected for collisional activation (b). With increasing collision energy, the complex will undergo charge stripping, unfolding and dissociation (c), this data is then converted into the CIU 'fingerprint'(d). B) This process has been applied to a variety of kinase inhibitors and their fingerprints are shown in the left panel. The intensities are plotted against the drift time between 40 and 44 V, which shows a distinct pattern for type I (blue) and type II (red) inhibitors. Images adapted from Rabuck et al. 2013*(67)*.*

To enable the detection of even larger molecules, an in-house modified IM-mass spectrometer was recently reported (detection limit of $m/z > 20.000$). The performance of the instrument was showcased by performing CIU experiments of concanavalin A, a 102 kDa tetrameric protein, and the aldolase complex, a 156 kDa complex(68). One of the drawbacks of the analysis of higher mass protein complexes is that they require higher collision energy for efficient unfolding. The addition of a new capillary exit lens was able to solve this, enabling a maximum collision energy of 400 V, as opposed to 230V(69).

In addition to dissociating polymeric protein complexes in CIU, collision energy and additional fragmentation techniques can be used to give additional structural information. Without fragmentation, native MS gives the total mass of a protein or protein complex. When introducing different types of fragmentation, these complexes can be dissociated, or the protein can be broken down in fragment ions to give more residue specific information.

This can be used for example for determining binding sites of peptides, proteins, and complexes. Which was demonstrated for example by Lermyte and colleagues, who identified binding regions for different metal ions in amyloid-beta by native top-down MS(70). Tree different fragmentation techniques were used: electron capture dissociation (ECD), infrared multiphoton dissociation (IRMPD), and collision-induced dissociation (CID). The binding region could be identified and results between different fragmentation techniques were consistent, thus neither fragmentation technique led to dissociation or translocation of the metal ion. However, these fragmentation parameters cannot be applied to all interactions and fragmentation types and parameters should be optimized for each individual interaction, which can be a time-consuming process.

Another use for fragmentation in the study of protein interactions are to resolve heterogeneity of species with the same mass, determine flexible protein regions and it can be combined with IM which provides additional conformational information. Recently, an interesting review on this and other uses of top-down native mass spectrometry in protein complexes has been published(8).

Discussion

Molecular processes and signalling are driven by protein interactions, as proteins interact with other proteins, small molecules, and metal ions. Understanding these interactions and reactions might aid in the understanding of diseases and developments of treatment options, diagnostic systems, and biocatalysts. With the development of ESI, native MS has emerged as tool to study these interactions. Several advantages of native MS compared to traditional methods to study protein interactions is that it requires only picomoles of sample, data acquisition is quick, and it does not require isotopic or fluorescent labelling. This makes native MS especially suitable for the analysis of donor or patient derived samples. But more importantly, native MS is particularly useful when examining heterogenous samples as individual binding events can be resolved.

In the last few years, many innovations have been done to increase sensitivity and sample handling, for example the use of nano emitters, online buffer exchange and direct MS. Additionally, the use of IM-MS and different fragmentation techniques have shown to provide information on protein dynamics and identification of binding sites and flexible regions, similar to NMR data. Additionally, techniques like NMR face high signal-to-noise ratios and tedious assignment of peaks as the protein mass increases, but with native MS complexes of almost 500 kDa have been measured and analysed(71). Lastly, several groups have engineered online temperature control systems to study thermodynamics, which demonstrate that there is no limit to the application of native MS.

We believe native MS can be exploited much more to understand the heterogenous nature of proteins, for example in the study of post-translational modifications (PTMs). PTMs are known to regulate various biological processes by changing protein activity, localization and binding partners(56). For example: phosphorylation, which can act as an on- and off-switch in signalling pathways; ubiquitination, which can regulate proteasomal degradation; and acetylation, which regulates protein function. Another important PTM is glycosylation, which is heterogenous by itself as multiple glycan building blocks can lead to infinite glycostructures. Native MS has already been used to demonstrate that the interactions and binding kinetics of α1-acid glycoprotein and haptoglobin are highly dependent on their glycosylation structure(72,73).

The PTM profile of proteins can be influence by a variety of factors, for example by disease, and are often identified as biomarkers(74,75). We hypothesize that native MS can be used to study patient derived proteins and their activity while distinguishing between different proteoforms. Thereby linking proteoforms to function. This principle can also be used to study glycan related differences in binding of protein complexes. It is for example known that overall fucosylation of proteins is increased in cancer patients(76), it might be mixed with its binding partner and followed over time using native MS. Then, you can see at glance whether the extend of fucosylation affects complex formation.

Another opportunity to further use native MS is to combine it with IM to study how PTMs change the conformation of proteins. This can even be extended by adding an online temperature control system to study the effect of PTMs on thermodynamics.

Of course, native MS has certain drawbacks, and it will not replace other high-resolution techniques to study protein interactions and complexes. Native MS, by itself cannot resolve the exact structure and location of glycans without the use of fragmentation techniques, which parameters can require extensive optimization. Additionally, tandem MS will increase data complexity and thereby increase time necessary for data analysis. Also, the exact dynamic regions of the protein cannot be readily resolved as is done by NMR. But even though NMR is also advancing to study more heterogenous samples and glycoproteins, it still requires significant more sample and time, and isotopic labelled proteins(77). Additional techniques can be necessary to supplement native MS, for example using crosslinking and hydrogen deuterium exchange to gain more information about binding and/or dynamic regions within the protein.

Conclusion

When examining protein interactions, native MS is a valuable tool in the toolbox. It requires little sample, and little sample preparation; especially when using online buffer exchange or direct-MS. But most importantly, using native MS, different isoforms can readily be resolved, making this technique especially suitable for analysing heterogenous samples. The possibilities and implications of native MS are constantly growing, thereby enabling the analysis of bigger and more complex systems. There is always room for more innovation, for example online pH control to study unfolding or online titration systems. However, we believe that the existing platform already allows for the study of many unknowns, especially in the study of PTMs and their functions.

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