A Guide to Native Mass Spectrometry to determine complex interactomes of molecular machines

Rita Puglisi1, Elisabetta Boeri Erba2 and Annalisa Pastore1

1 UK Dementia Research Institute at the Wohl Institute of King’s College London, UK
2 Univ. Grenoble Alpes, CEA, CNRS, Institut de Biologie Structurale (IBS), Grenoble, France

Keywords
Isc operon; FeS cluster; native mass spectrometry; proteins; structural biology

Correspondence
A. Pastore, UK Dementia Research Institute at the Wohl Institute of King’s College London, 5 Cutcombe Rd, SE59RT London, UK
E-mail: Annalisa.pastore@crick.ac.uk

Native mass spectrometry is an emerging technique in biology that gives the possibility to study noncovalently bound complexes with high sensitivity and accuracy. It thus allows the characterization of macromolecular assemblies, assessing their mass and stoichiometries and mapping the interacting surfaces. In this review, we discuss the application of native mass spectrometry to dynamic molecular machines based on multiple weak interactions. In the study of these machines, it is crucial to understand which and under which conditions various complexes form at any time point. We focus on the specific example of the iron–sulfur cluster biogenesis machine because this is an archetype of a dynamic machine that requires very specific and demanding experimental conditions, such as anaerobicity and the need of retaining the fold of marginally folded proteins. We describe the advantages, challenges and current limitations of the technique by providing examples from our own experience and suggesting possible future solutions.

Introduction
Mass spectrometry (MS) is a powerful technique that can measure the mass of molecules with high accuracy, sensitivity, resolution and speed [1]. When samples are analysed by MS, they are ionized, separated according to their mass-to-charge ratio ($m/z$) and detected. MS was first developed as early as in 1912 [2] and then applied for a long time mostly for analytic purpose to establish or confirm the identity of a compound or a mixture of molecules. Biological applications were allowed only in the late 1980s thanks to the development of soft ionization methods as electrospray ionization (ESI) [3,4] and matrix-assisted laser desorption/ionization (MALDI) [5,6]. ESI in particular led to the possibility, in the ‘90s and around the turning of the millennium, of performing experiments under native conditions (for an early classical review, see Last and Robinson, 1999 [7]). What is now usually referred to as ‘native MS’ thus enables the characterization of macromolecular assemblies without their disassembly [8].

Using native MS, it is in principle possible to determine with high resolution and selectivity the stoichiometry of assembly components [9-13], map direct interactions between subunits and provide information about the mechanism of formation of macromolecular assemblies [14]. Native MS can also provide information on the dynamic behaviour of complexes and assess the presence of distinct forms that could detect allostery [15,16]. Another exciting application is the possibility to follow the kinetics of a reaction by incubating mixtures of differently labelled forms of noncovalent complexes (e.g. labelled with $^2$H or $^{13}$C and $^{15}$N) [17,18]. As for all MS applications, native MS has the additional advantage to be undemanding...
about sample quantities: few picomoles of sample are sufficient for obtaining the mass of even large macromolecular assemblies with an error of a few daltons.

Investigating macromolecular complexes requires customized mass spectrometers, because assemblies with molecular masses above 60 kDa generate ions with \( m/z \) ratios above 4000 that exceed the detection limit of standard instruments. Around the 2000, major hardware modifications allowed the detection of noncovalent assemblies using nano-ESI quadrupole time-of-flight (Q-TOF) mass spectrometers ([19,20]). Besides nano-ESI-Q-TOF instruments, Fourier transform (FT)-based mass spectrometers have also successfully been used to study noncovalent interactions. In particular, Orbitrap instruments, which were introduced in the year 2000 ([21,22]), show excellent resolution and accuracy that allows transmission of heavy ions up to an \( m/z \) of 40 000 ([23]).

Native MS has been applied to complex machines such as the ribosome [24-27], viruses [28-33] and membrane proteins [34-40]. These examples concern systems that are relatively well defined and are in some sense ‘static complexes’. However, native MS can also be applied to study dynamical molecular machines formed by multiple weak and mutually exclusive interactions [41,42].

Here, we discuss, as a representative example, the application of native MS to the core machine for iron–sulfur (FeS) cluster biogenesis in *E. coli*. This specific example was not chosen by chance: one of the early native MS studies of a molecular assembly concerns precisely a FeS protein, *E. coli* biotin synthetase [43]. This protein was demonstrated to exist in solution as a mixture of monomer, dimer and tetramer and to be bound to a FeS cluster. We thus thought FeS clusters appropriate as a paradigm of the more general theme. Recently, native MS has been applied to the study of FeS binding proteins by an increasing number of groups who have confirmed its applicability [44,45] and its great value to extract mechanistic details [46].

We analyse the advantages, problems and challenges proposed by native MS for the investigation of FeS cluster biogenesis based on our own experience in view of a wider perspective that goes beyond the specific application. We demonstrate that, while uniquely suited for the general purpose, further developments may be needed to make native MS fully applicable to the study of this challenging pathway.

**General overview of the FeS cluster biogenesis machine**

Iron–sulfur cluster biogenesis is a representative example of a dynamic biological machine, which strongly relies on the formation and dissociation of different interactions. Also present in the inorganic world, FeS clusters are essential components of the cell since they are attached to proteins and have structural or redox roles [47]. In bacteria, the components are encoded in the *isc* operon and have high sequence homology to the eukaryotic ones (Fig. 1) [48]. The Isc machine comprises a number of essential proteins present among a promoter, a desulfurase IscS, a scaffold protein IscU, a putative alternative scaffold protein IscA, a ferredoxin that provides electrons FdX and two chaperone proteins HscB and HscA.

The IscS is a pyridoxal phosphate (PLP) binding enzyme that catalyses the conversion of L-cysteine into L-alanine and generates sulfur subsequently transferred to IscU [49] or to other proteins belonging to other metabolic pathways [50,51]. It forms a complex with IscU, a small protein that transiently binds the cluster and passes it on to further acceptors. IscA is an alternative scaffold. Ferredoxin provides electrons for the FeS formation process. The two chaperones HscA and HscB are thought to assist cluster transfer [52]. Also involved in the machine is CyaY (frataxin in eukaryotes), an iron binding protein that is outside the operon. This protein has been widely studied because in humans it is associated with Friedreich’s ataxia [53]. It was firstly associated with the *isc* operon through interaction with HscB [54] and later on confirmed by pull-down experiments [55,56]. CyaY participates in FeS cluster formation as a regulator of the reaction speed [56,57].

Other ancillary proteins are selectively present in prokaryotes or in eukaryotes. Among them is YfhJ (also called IscX) that is specific for prokaryotes. This protein was suggested to be with frataxin, a regulator of the IscS activity depending on the iron concentration [58] (Fig. 2). Intriguingly, YfhJ, Fdx and CyaY compete for the same site on IscS [57,59-61] and the binding affinity to IscS is modulated by the presence of iron cations.

**Strengths of using native MS for the study of the Isc system**

Several are the challenges in the study of the FeS cluster core machine. While the Isc proteins and their interaction affinities are relatively well known, proving the existence and characterizing transient intermediates is a major challenge. Yet, this knowledge is indispensable for reconstructing the sequence of events occurring in cluster assembly and determining possible allosteric regulations. These multiple interactions are more complex also because many of the components
can be in more than one state (e.g. metal-bound/free, cluster-bound/free, ATP/ADP-bound/free and reduced/oxidized) (Fig. 3 and Table 1). The analysis of all the species formed thus requires a technique able to detect at the same time all the possible complexes in a range of molecular weights of 100-200 KDa (Table 2) and having different affinities ranging from 1 to 30 l[m] [62] (Fig. 4). It is here where native MS appears superior to most alternative techniques since it is able to retain protein structure, folding and interactions and detect the formation of transient complexes up to a molecular weight of MDa with a typical accuracy of a few Da. Only small amounts of samples are needed (5–10 μl), different ratios of the partners can be investigated, and no specific labels are necessary thus making the technique noninvasive. We also noticed that it is so sensitive to permit observation of minor species. We could, for instance, detect not only the IscS dimer, which is the dominant species in solution, but also the presence of an IscS tetramer and of a minor hexameric form (Fig. 5). The IscS tetramer could be detected also in size-exclusion chromatography and by small-angle scattering. Since the concentrations used in these studies are in the same order of magnitude observed for the same proteins in the cell [63], it is possible that these species may form under specific conditions, but their biological relevance is probably limited.

**An essential control: retention of the enzymatic activity of the Isc system**

Native MS experiments are usually performed using volatile buffers such as ammonium acetate [64,65], ethylenediammonium diacetate or alkylammonium acetate buffers [66]. Unlike other types of ESI-MS analysis, neither acidic conditions nor organic solvents are used. Typically, the buffer is exchanged immediately prior to native MS analysis [67]. The use of a volatile buffer might nevertheless represent a challenge for proteins that are marginally stable or not monodispersed as some of the components of the Isc system. Therefore, an essential control is to assess whether the enzymatic activity and cluster formation are affected by the experimental conditions dictated by the technique such as the presence and absence of ammonium acetate. This must be done under strict anaerobic conditions in a chamber kept under nitrogen atmosphere. The reaction can be followed by absorbance...
spectroscopy following absorbance variations at 458 nm or 406 nm and/or circular dichroism in the range 300-600 nm as a function of time. Typical conditions for the reactions are 3 mM DTT, 50 lM IscU, 1 lM IscS and 25 lM Fe(NH₄)₂(SO₄)₂ preincubated for 30 min in 20 mM Tris/HCl at pH 8 and 150 mM NaCl [56]. The reaction is then initiated by adding 250 lM of the substrate L-cysteine. The addition of CyaY (typically between 3 lM and 10 lM) inhibits the reaction [57]. In our hands, the system containing IscS, IscU and CyaY remains active in ammonium acetate provided that the buffer concentration does not exceed 50 mM (Fig. 6). Retention of the enzymatic activity also confirms that the experimental conditions do not alter protein structure.

Fig. 3. Summary of the main components of the Isc system in bacteria. Most of the Isc proteins involved in the FeS cluster biogenesis exist in different free and complexed forms bound to cofactors and/or ions that are essential for folding or function. When the structure of a specific form from *Escherichia coli* was not present in PDB, we modelled it from close orthologues to provide a visual impression of where the cofactor would be bound. The figure was produced using the Pymol software (https://pymol.org/2/).

| Table 1. Summary of the molecular weights of Isc proteins and their cofactors |
|--------------------------|--------------------------|--------------------------|
| **Protein** | **MW (Da)** | **Metal/Cofactor** | **Metal/Cofactor MW (Da)** |
| IscR | 17 337 | 2Fe2S | 176 |
| IscS | 45 089 | PLP | 247 |
| IscU | 13 849 | Zn²⁺ | 65 |
| IscA | 11 556 | 2Fe2S | 176 |
| HscA | 66 652 | ADP | 427 |
| HscB | 20 138 | ATP | 507 |
| FdX | 12 331 | 2Fe2S | 176 |
| YfhJ | 7732 | Fe²⁺/Fe³⁺ | 56 |
| CyaY | 12 231 | Fe²⁺/Fe³⁺ | 56 |

| Table 2. Isc complexes and molecular weights expected by mixing the desulfurase IscS with the scaffold protein IscU and frataxin CyaY, as example of the complexity of the system |
|--------------------------|--------------------------|--------------------------|
| **Mixture** | **Complexes expected MW (Da)** |
| IscS | (IscS)₂ | 90 178 |
| IscS + IscU | (IscS)₂(IscU) | 104 027 |
| IscU | (IscS)₂(IscU)₂ | 117 876 |
| IscS + IscU + CyaY | (IscS)₂(IscU)₂(CyaY) | 116 258 |
| | (IscS)₂(IscU)₂(CyaY)₂ | 128 489 |
| | (IscS)₂(IscU)₂(CyaY) | 130 107 |
| | (IscS)₂(IscU)₂(CyaY)₂ | 142 338 |
What can native MS report on the Isc system?

In this section, we shall discuss various aspects of the study of the FeS cluster biogenesis machine that native MS can assist.

Using native MS to assess stoichiometry and symmetry

The IscS forms an obligate symmetric dimer composed of two identical 45 kDa subunits. At the interface between them, there is a cleft characterized by a positively charged surface due to the presence of three conserved arginines. This surface is a privileged site of interaction for protein negatively charged such as CyaY [57], YfhJ [58,59] and Fdx [61]. Furthermore, it was shown that IscS is able to host HscB in the same pocket [68]. The same binding site is present on the symmetric site; thus, IscS can bind two molecules (of the same protein or two different proteins) at the same time. This opens the likely possibility of an allosteric regulation of the IscS desulphurase activity. In addition, each subunit of IscS is also able to bind IscU forming a complex with the stoichiometry of 1:1, meaning that the dimer binds two molecules of IscU. Native MS was applied to investigate whether IscS and other partners (e.g. CyaY) could form heterocomplexes in the presence or absence of IscU. In our studies, we typically injected a mixture of 5 μM IscS, 5 μM IscU and increasing molar ratios of CyaY ranging from 5 to 15 μM. We observed signals typical of the single components together with signals corresponding to noncovalent assemblies (Fig. 7). Data analyses indicated the presence of binary species, which could be identified as IscS2-IscU, IscS2-IscU2, IscS2-CyaY, IscS2-CyaY2 and CyaY-IscU together with the heterocomplexes IscS2-IscU-CyaY, IscS2-IscU2-CyaY, IscS2-IscU-CyaY2 and IscS2-IscU2-CyaY2. This evidence also confirmed that binding of IscU to IscS does not compete with CyaY binding confirming previous studies [57].

Validating unexpected interactions

In our investigation of the Isc system by native MS, we also detected unexpected species such as complexes not previously described. For instance, we observed an IscU-CyaY interaction (Fig. 8A): this has been
repeatedly described between eukaryotic IscU and frataxin but not for the prokaryotic proteins [57,69]. A key question is thus whether these interactions are biologically significant. In these doubtful cases, it is essential to use alternative techniques to validate the interactions independently. Approaches complementary to native MS may include NMR, ITC and/or cross-linking. Each of these techniques has drawbacks, but consistent results obtained by different approaches may together validate the significance of newly disclosed interactions.

Native MS preserves the binding of prosthetic groups

Some of the Isc components have constitutive binding partners such as prosthetic groups or ions. They are either essential for function (as it is PLP for IscS or ATP for HscA) or for fold stability (as it is zinc for IscU). It is widely accepted, for instance, that the reaction mechanism catalysed by IscS follows the rules proposed for the A. vinelandii homologue NIFS. In this mechanism, PLP would be essential for substrate activation through binding to the cysteine and forming an external aldimine base of Schiff prone to undergo a nucleophilic attack to liberate sulfur [70,71]. Additionally, it was described that IscS remains enzymatically active only as long as it is bound to PLP [72]. Therefore, observing PLP on the desulfurase is essential to ensure correct folding of the enzyme and its activity.

IscU can bind a zinc metal ion in the same active site, which is responsible for hosting the cluster. This cation is thought to stabilize the folded form [73]. The presence of zinc does not alter the interaction with IscS and increases FeS cluster formation [74]. Eukaryotic frataxin and bacterial CyaY have been shown to form stable, although relatively weak, Fe$^{2+}$ and Fe$^{3+}$ complexes with a stoichiometry of up to six to seven iron ions [75,76]. Also, YfhJ binds to iron in both its forms Fe$^{2+}$ and Fe$^{3+}$, through a negatively charged surface [77], and the surface responsible for the iron binding is the same which recognizes IscS.

We could prove that native MS preserves binding of all of these prosthetic groups to the respective protein: we clearly observed two molecules of PLP bound to the IscS dimer (Fig. 8B). We were also able to discriminate between iron-loaded and iron-free CyaY species (Fig. 8C). In other cases, especially when more than one protein was injected, many signals were present in a limited m/z range, making the data interpretation arduous. In these cases, it was not possible to determine whether loaded species or free ones were involved in binding (Fig. 8D).

The challenge of working under anaerobic conditions

FeS clusters are a special type of prosthetic groups that may require specific experimental setting. They can be highly sensitive to oxygen [78] as oxygen species can convert them to unstable forms that rapidly decompose. This is particularly relevant for proteins of the core machine such as IscU that is able to bind FeS clusters but only in a weak and transient way. Under aerobic conditions, the cluster on IscU disassembles in a few minutes as it is testified by the absorbance spectra. Iron–sulfur clusters have a typical absorbance spectrum whose appearance demonstrates cluster formation. The enzymatic assay requires the presence of a source of sulfur (Cys), iron (iron ammonium sulfate), a desulfurase enzyme (IscS), a scaffold protein on which the cluster forms (IscU) and a reducing agent (DTT). The experiment was carried out using 50 μM IscU in the presence of 1 μM IscS, 250 μM Cys, 25 μM Fe(NH$_4$)$_2$(SO$_4$)$_2$ and 3 mM DTT in 20 mM Tris/HCl at pH 8, 150 mM NaCl at pH 8 (blue) or in 50 mM ammonium acetate at pH 8 (red).

Fig. 6. Kinetics of FeS cluster formation followed by absorbance spectra. Iron–sulfur clusters have a typical absorbance spectrum whose appearance demonstrates cluster formation. The enzymatic assay requires the presence of a source of sulfur (Cys), iron (iron ammonium sulfate), a desulfurase enzyme (IscS), a scaffold protein on which the cluster forms (IscU) and a reducing agent (DTT). The experiment was carried out using 50 μM IscU in the presence of 1 μM IscS, 250 μM Cys, 25 μM Fe(NH$_4$)$_2$(SO$_4$)$_2$ and 3 mM DTT in 20 mM Tris/HCl at pH 8, 150 mM NaCl at pH 8 (blue) or in 50 mM ammonium acetate at pH 8 (red).

Fig. 7. Potential artefacts of the technique. The MS spectrum shows species observed by native MS by mixing IscS 5 μM with IscU 5 μM and increasing concentration of CyaY up to 15 μM in 250 mM ammonium acetate at pH 8.
spectrum or, more simply, by the disappearance of the dark red colour that characterizes the cluster-loaded protein [72]. This is the reason why all the studies on the FeS cluster formation on this scaffold are carried out under strict anaerobic conditions. Unfortunately, the achievement of these conditions is often impractical or difficult with certain biophysical techniques. We could probably state that O2 exposure during the MS experiments may not be a problem because when the sample is infused into the ionization source, there is insufficient time for reaction to occur with any O2 present prior to ionization/introduction of low pressure.

So far, we studied the interactions among Isc proteins in an aerobic environment and thus in the absence of the FeS clusters.

An alternative solution is to carry out the measurements on mutants that have higher affinity for the cluster. A mutant of IscU, for instance, that replaces a conserved aspartate to alanine (IscUD39A in E. coli IscU) loses the cluster slowly and can thus be used for the purpose [79]. This behaviour was explained by the elimination of a competition between the aspartate and a close-by histidine: since both aspartate and histidine can chelate the cluster in a mutually exclusive fashion, mutation of one of the two residues leads to cluster stabilization [80]. In the past, we have indeed been able to observe a 2Fe2S cluster on the IscU mutant within hours from its anaerobic purification. In the future, we aim to build a dry box around the
injection entrance of the spectrometer to be able to load the sample on the ESI needle and mount it on the source without exposure to oxygen. This will undoubtedly require some skill, but this step, even if delicate and cumbersome, will increase the potential applications of the instrument to oxygen-sensitive proteins.

Observing marginally stable protein conformations by MS

Proteins can assume multiple conformations depending on interactions, environmental conditions or intrinsic marginal stability [81]. Interconversion between conformations may be at the heart of their function. This is the case for at least one of the proteins involved in the core FeS assembly, IscU. When produced by overexpression in E. coli, IscU is present in solution as an equilibrium between two distinct forms: a structured conformation (S-state) and a partially dynamically disordered form (D-state) [82]. The S-state is stabilized by zinc, which binds in the same site that coordinates the cluster. When bound to IscS, IscU is in the S-state [59,83] but the D-state was suggested to be relevant for cluster release. In the constitutive presence of zinc, a higher rate of cluster formation was observed, consistent with the presence of a more stable fold on which the cluster is assembled with the lower energetic cost [74]. In our hands and in our instrumental conditions, it was hard to distinguish between the two forms by native MS: we observed only one Gaussian distribution of signals (Fig. 8E).

More sophisticated experiments should be designed to probe for the presence of different conformations, such as coupling native MS with ion mobility (IM), which separates ions based on their differential mobility through a buffer gas [84,85]. Another approach is to use hydrogen/deuterium exchange (HDX) MS [86,87]. In this approach, folded conformations would have comparably fewer labile hydrogens readily accessible to the solvent and available for exchange than the unfolded conformation [88]. This generates differences between two populations, which could be assessed by MS.

Conclusions

We have reviewed how native MS, a technique in use for several years but still relatively underexploited, can be adapted to the study of the FeS cluster biogenesis core machine. We discussed in detail the advantages and limitations of the technique applied to this specific system. We found that native MS offers several unique advantages over other techniques that range from the exquisite sensitivity, to the possibility to observe multiple states of the same protein within the instrumental resolution, to the possibility of looking at complex mixtures over a large mass range. This is of paramount importance for transient machines in which identification of the different complexes formed at different stages of the pathway matters, introducing a temporal dimension to the investigation.

In the context of a specific problem discussed here, a main current limitation remains certainly the difficulty to carry out the whole experiment anaerobically. Overcoming this aspect will require either ad hoc local solutions or a more general policy of the manufacturers, which could however not currently be justified given the limited numbers of users who might need these conditions. This might easily change in the future with the spreading of the technique. Another aspect that will require some consideration is the biological significance of minor species observed in some spectra, which will require comparison with measurements carried out by other techniques such as NMR or ITC. This step should anyway always be carried out as no technique can be trusted by its own. Despite these limitations, native MS undoubtedly constitutes an effective and powerful tool to be added to the already large range of biophysical techniques in place for the study of FeS cluster biogenesis. Its wider application to other similarly challenging machines may lead to a considerable step forward in our understanding of complex molecular processes in near future.

Acknowledgements

This work was supported by INSTRUCT and the UK Dementia Research Institute (REI 3556) that is funded by the Medical Research Council, Alzheimer UK and Alzheimer Foundation. We acknowledge the platforms of the Grenoble Instruct-ERIC Center (ISBG; UMS 3518 CNRS-CEA-UGA-EMBL) within the Grenoble Partnership for Structural Biology (PSB). Platform access was supported by FRISBI (ANR-10-INBS-05-02) and GRAL, a project of the University Grenoble Alpes graduate school (Ecoles Universitaires de Recherche) CBH-EUR-GS (ANR-17-EURE-0003). RP acknowledges EMBO for a fellowship.

Author contributions

RP carried out and wrote the first draft of the manuscript. EBE provided her expert knowledge in MS. AP finalized the manuscript.
Conflict of interest

The authors declare no conflict of interest.

References

1. De Hoffmann E & Stroobant V (2007) Mass Spectrometry: Principles and Applications. Wiley.
2. Thomson JJ. (1913) Rays of Positive Electricity and their Applications to Chemical Analysis. Longmans, Editor. Greens and Co.
3. Fenn JB, Mann M, Meng C, Wong S & Whitehouse C (1989) Electrospray ionization for mass spectrometry of large biomolecules. Science 246, 64–71.
4. Chowdhury SK, Katta V & Chait BT (1990) Electrospray ionization mass spectrometric peptide mapping: a rapid, sensitive technique for protein structure analysis. Biochem Biophys Res Commun 167, 686–692.
5. Tanaka K, Waki H, Ido Y, Akita S, Yoshida Y, Yoshida T & Matsuo T (1988) Protein and polymer analyses up to m/z 100 000 by laser ionization time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 2, 151–153.
6. Karas M & Hillenkamp F (1988) Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons. Anal Chem 60, 2299–2301.
7. Last AM & Robinson CV (1999) Protein folding and interactions revealed by mass spectrometry. Curr Opin Chem Biol 3, 564–570.
8. Leney AC & Heck AJ (2017) Native Mass Spectrometry: What is in the Name? J Am Soc Mass Spectrom 28, 5–13.
9. Rostom AA & Robinson CV (1999) Disassembly of intact multiprotein complexes in the gas phase. Curr Opin Struct Biol 9, 135–141.
10. Tito MA, Tars K, Valegard K, Hajdu J & Robinson CV (2000) Electrospray time-of-flight mass spectrometry of the intact MS2 virus capsid. J Am Chem Soc 122, 3550–3551.
11. Ilag LL, Viderer H, McKay AR, Sobott F, Fucini P, Nierhaus KH & Robinson CV (2005) Heptameric (L12) 6/L10 rather than canonical pentameric complexes are found by tandem MS of intact ribosomes from thermophilic bacteria. Proc Natl Acad Sci U S A 102, 8192–8197.
12. Boeri Erba E & Petosa C (2015) The emerging role of native mass spectrometry in characterizing the structure and dynamics of macromolecular complexes. Protein Sci 24, 1176–1192.
13. Lossl P, van de Waterbeemd M & Heck AJ (2016) The diverse and expanding role of mass spectrometry in structural and molecular biology. EMBO J 35, 2634–2657.
14. Aquilina JA, Benesch JL, Bateman OA, Slingsby C, Robinson CV (2003) Polydispersity of a mammalian chaperone: mass spectrometry reveals the population of oligomers in alphaB-crystallin. Proc Natl Acad Sci USA 100, 10611–10616.
15. Patrick JW, Boone CD, Liu W, Conover GM, Liu Y, Cong X & Laganowsky A (2018) Allostery revealed within lipid binding events to membrane proteins. Proc Natl Acad Sci USA 115, 2976–2981.
16. Dyachenko A, Gruber R, Shimon L, Horovitz A & Sharon M (2013) Allosteric mechanisms can be distinguished using structural mass spectrometry. Proc Natl Acad Sci USA 110, 7235–7239.
17. Yee AW, Moulin M, Breteau N, Haertlein M, Mitchell EP, Cooper JB, Boeri Erba E & Forsyth VT (2016) Impact of Deuterium on the Assembly Kinetics of Transthreycin Monitored by Native Mass Spectrometry and Implications for Amyloidoses. Angew Chem Int Ed Engl 55, 9292–9296.
18. Yee AW, Aldeghi M, Blakeley MP, Ostermann A, Mas PJ, Moulin M, de Sanctis D, Bowler MW, Mueller-Dieckmann C, Mitchell EP et al. (2019) A molecular mechanism for transthreycin amyloidogenesis. Nat Commun 10, 925.
19. Sobott F, Hernández H, McCammon MG, Tito MA & Robinson CV (2002) A tandem mass spectrometer for improved transmission and analysis of large macromolecular assemblies. Anal Chem 74, 1402–1407.
20. van den Heuvel RH, van Duijn E, Mazon H, Synowsky SA, Lorenzen K, Versluis C, Brouns SJJ, Langridge D, van der Oost J, Hayes J et al. (2006) Improving the performance of a quadrupole time-of-flight instrument for macromolecular mass spectrometry. Anal Chem 78, 7473–7483.
21. Makarov A (2000) Electrostatically aligned quadrupole axisymmetric ion trapping: a high-performance technique of mass analysis. Anal Chem 72, 1156–1162.
22. Makarov A, Denisov E, Kholomeev A, Balschun W, Lange O, Strupat K & Horning S (2006) Performance evaluation of a hybrid linear ion trap/orbitrap mass spectrometer. Anal Chem 78, 2113–2120.
23. Snijder J & Heck AJ (2014) Analytical approaches for size and mass analysis of large protein assemblies. Annu Rev Anal Chem (Palo Alto Calif) 7, 43–64.
24. Grepa P, Krokowski D, Gordiyenko Y, Krowarsch D, Robinson CV, Otlewski J, Grankowski N & Tchórzewski M (2010) Biophysical properties of the eukaryotic ribosomal stalk. Biochemistry 49, 924–933.
25. Thoma C, Fraterman S, Gentzel M, Wilm M & Hentze MW (2008) Translation initiation by the c-myc mRNA internal ribosome entry sequence and the poly(A) tail. RNA 14 (8), 1579–1589.
26. van de Waterbeemd M, Tamara S, Fort KL, Damoc E, Franc V, Bieri P, Itten M, Makarov A, Ban N & Heck AJR (2018) Dissecting ribosomal particles throughout the kingdoms of life using advanced hybrid mass spectrometry methods. Nat Commun 9, 2493.
Native mass spectrometry in iron-sulfur cluster biogenesis

27 van de Waterbeemd M, Fort KL, Boll D, Reinhardtszyha M, Routh A, Makarov A & Heck AJR (2017) High-fidelity mass analysis unveils heterogeneity in intact ribosomal particles. Nat Methods 14, 283–286.

28 Weiss VU, Pogan R, Zoratto S, Bond KM, Boulanger P, Jarrold MF, Lykten N, Pahl D, Puffer N, Schelhaas M et al. (2019) Virus-like particle size and molecular weight/mass determination applying gas-phase electrophoresis (native nES GEMMA). Anal Bioanal Chem 411, 5951–5962.

29 Veesler D, Khayat R, Krishnamurthy S, Snijder J, Huang RK, Heck AJR, Anand GS & Robinson CV (2011) Mass spectrometry of intact V- complexes studied by native mass spectrometry. J Biol Chem 286, 2320–2337.

30 van de Waterbeemd M, Snijder J, Tsvetkova IB, Dragnea BG, Cornelissen JH & Heck AJR (2016) Examining the Heterogeneous Genome Content of Multipartite Viruses BMV and CCMV by Native Mass Spectrometry. J Am Soc Mass Spectrom 27, 1000–1009.

31 Veesler C & Heck AJ (2011) Modern biomolecular mass spectrometry and its role in studying virus structure, dynamics, and assembly. Angew Chem Int Ed 50, 8248–8262.

32 Kondylis P, Schlicksup CJ, Zlotnick A & Jacobson SC (2019) Analytical Techniques to Characterize the Structure, Properties, and Assembly of Virus Capsids. Anal Chem 91, 622–636.

33 Bereszczak JZ, Barbu IM, Tan M, Xia M, Jiang X, van Duijn E & Heck AJR (2012) Structure, stability and dynamics of norovirus P domain derived protein complexes studied by native mass spectrometry. J Struct Biol 177, 273–282.

34 Zhou M, Morgner N, Barrera NP, Politis A, Isaacson SC, Matak-Vinkovic D, Murata T, Bernal RA, Stock D & Robinson CV (2011) Mass spectrometry of intact V-type ATPases reveals bound lipids and the effects of nucleotide binding. Science 334, 380–385.

35 Barrera NP, Di Bartolo N, Booth PJ & Robinson CV (2008) Micelles protect membrane complexes from solution to vacuum. Science 321, 243–246.

36 van Dyck JF, Konijnenberg A & Sobott F (2017) Native Mass Spectrometry for the Characterization of Structure and Interactions of Membrane Proteins. Methods Mol Biol 1635, 205–232.

37 Robinson CV, Roehas T & Hansen SB (2019) Tools for Understanding Nanoscale Lipid Regulation of Ion Channels. Trends Biochem Sci 44, 795–806.

38 Laganowsky A, Reading E, Allison TM, Ulmschneider MB, Degiacomi MT, Baldwin AJ & Robinson CV (2014) Membrane proteins bind lipids selectively to modulate their structure and function. Nature 510, 172–175.

39 Gupta K, Li J, Liko I, Gault J, Bechara C, Wu D, Hopper JTS, Giles K, Benesch JLP & Robinson CV (2018) Identifying key membrane protein lipid interactions using mass spectrometry. Nat Protoc 13, 1106–1120.

40 Bechara C & Robinson CV (2015) Different modes of lipid binding to membrane proteins probed by mass spectrometry. J Am Chem Soc 137, 5240–5247.

41 Snijder J, RoseRJ, VeeslerD, JohnsonJE, HeckAJR (2013) Studying 18 MDa virus assemblies with native mass spectrometry. Angew Chem Int Ed Engl 52, 4020–4023.

42 Shoemaker GK, van Duijn E, Crawford SE, Uetrecht JP, Bassam MM, Kamali S, Holland AA, Cramer SP, Hamilton CJ, Johnson MK et al. (2015) NsrR from Streptomyces coelicolor is a nitric oxide-sensing [4Fe-4S] cluster protein with a specialized regulatory function. J Biol Chem 290, 12689–12704.

43 Rao G, Pattenaude SA, Alwan K, Blackburn NJ, Britt RD, Rauchfuss TB (2019) The binuclear cluster of [FeFe] hydrogenase is formed with sulfur donated by cysteine of an [Fe(Cys)(CO)2(CN)] organometallic precursor. Proc Natl Acad Sci U S A 116, 20850–20855.

44 Parent A, Elduque X, Cornu D, Belot L, Le Caer J-P, Urbina HD, Silberg JJ, Hoff KG & Vickery LE (2001) Mammalian frataxin directly enhances sulfur transfer of NFS1 persulfide to both ISCU and free thiols. Nature 410, 568–572.

45 Melber A & Wingé DR (2018) Steps Toward Understanding Mitochondrial Fe/S Cluster Biogenesis. Methods Enzymol 599, 265–292.

46 Roche B, Aussel L, Ezratty B, Mandin P, Py B & Barras F (2013) Iron/sulfur proteins biogenesis in prokaryotes: formation, regulation and diversity. Biochim Biophys Acta 1827, 455–469.

47 Urbina HD, Silberg JG, Hoff KG & Vickery LE (2001) Transfer of sulfur from IscS to IscU during Fe/S cluster formation. J Biol Chem 276, 44521–44526.

50 Kambampati R & Lauhon CT (2000) Evidence for the transfer of sulfane sulfur from IscS to ThiII during the in vitro biosynthesis of 4-thiouridine in Escherichia coli (RNA). J Biol Chem 275, 10727–10730.

51 Lauhon CT & Kambampati R (2000) The iscS gene in Escherichia coli is required for the biosynthesis of 4-thiouridine, thiamin, and NAD. J Biol Chem 275, 20096–20103.

52 Puglisi R & Pastore A (2018) The role of chaperones in iron-sulfur cluster biogenesis. FEBS Lett 592, 4011–4019.
Pastore A & Puccio H (2013) Frataxin: a protein in search for a function. *J Neurochem* **126** (Suppl 1), 43–52.

Huynen MA, Snel B, Bork P, Gibson TJ (2001) The phylogenetic distribution of frataxin indicates a role in iron-sulfur cluster protein assembly. *Hum Mol Genet* **10**, 2463–2468.

Gerber J, Muhlenhoff U & Lil R (2003) An interaction between frataxin and Isu1/Nfs1 that is crucial for Fe/S cluster synthesis on Isu1. *EMBO Rep* **4**, 906–911.

Adinolfi S, Iannuzzi C, Prischi F, Pastore C, Iametti S, Martin SR, Bonomi F & Pastore A (2009) Bacterial frataxin CyaY is the gatekeeper of iron-sulfur cluster formation catalyzed by IscS. *Nat Struct Mol Biol* **16**, 390–396.

Prischi F, Konarev PV, Iannuzzi C, Pastore C, Adinolfi S, Martin SR, Svergun DI & Pastore A (2010) Structural bases for the interaction of frataxin with the central components of iron-sulfur cluster assembly. *Nat Commun* **1**, 95.

Adinolfi S, Puglisi R, Crack JC, Iannuzzi C, Dal Piaz F, Konarev PV, Svergun DI, Martin S, Le Brun NE & Pastore A (2017) The Molecular Bases of the Dual Regulation of Bacterial Iron Sulfur Cluster Biogenesis by CyaY and IscX. *Front Mol Biosci* **4**, 97.

Shi R, Proteau A, Villarroya M, Moukediri I, Zhang L, Trempe J-F, Matte A, Armengod ME & Cygler M (2010) Structural basis for Fe-S cluster assembly and tRNA thiolation mediated by IscS protein-protein interactions. *PLoS Biol* **8**, e1000354.

Kim JH, Bothe JR, Frederick RO, Holder JC, Markley J (2014) Role of IscX in iron-sulfur cluster biogenesis in *Escherichia coli*. *J Am Soc Chem* **136**, 7933–7942.

Yan R, Konarev PV, Iannuzzi C, Adinolfi S, Roche B, Kelly G, Simon L, Martin SR, Py B, Barras F et al. (2013) Ferredoxin competes with bacterial frataxin in binding to the desulfurase IscS. *J Biol Chem* **288**, 24777–24787.

Prischi F & Pastore A (2017) Hybrid Methods in Iron-Sulfur Cluster Biogenesis. *Front Mol Biosci* **4**, 12.

Schmidt A, Kochanowski K, Vedelaar S, Ahmèr E, Volkmer B, Callipo L, Knoops K, Bauer M, Aebersold R & Heinemann M (2016) The quantitative and condition-dependent *Escherichia coli* proteome. *Nat Biotechnol* **34**, 104–110.

Konermann L (2017) Addressing a Common Misconception: Ammonium Acetate as Neutral pH "Buffer" for Native Electrospray Mass Spectrometry. *J Am Soc Mass Spectrom* **28**, 1827–1835.

Hernandez H & Robinson CV (2007) Determining the stoichiometry and interactions of macromolecular assemblies from mass spectrometry. *Nat Protoc* **2**, 715–726.

Zhuang X, Gavrilidou AFM & Zenobi R (2017) Influence of Alkalynammonium Acetate Buffers on Protein-Ligand Noncovalent Interactions Using Native Mass Spectrometry. *J Am Soc Mass Spectrom* **28**, 341–346.

Boeri Erba E, Signor L, Oliva MF, Hans F, Petosa C (2018) Characterizing Intact Macromolecular Complexes Using Native Mass Spectrometry. *Methods Mol Biol* **1764**, 133–151.

Puglisi R, Yan R, Adinolfi S & Pastore A (2016) A New Tessera into the Interactome of the isc Operon: A Novel Interaction between HscB and IscS. *Front Mol Biosci* **3**, 48.

Layer G, Ollagnier-de Choudens S, Sanakis Y & Fontecave M (2006) Iron-sulfur cluster biosynthesis: characterization of *Escherichia coli* CyaY as an iron donor for the assembly of [2Fe-2S] clusters in the scaffold IscU. *J Biol Chem* **281**, 16256–16263.

Zheng L, White RH, Cash VL & Dean DR (1994) Mechanism for the desulfurization of L-cysteine catalyzed by the nifS gene product. *Biochemistry* **33**, 4714–4720.

Fujii T, Maeda M, Mihara H, Kurihara T, Esaki N, Hata Y (2000) Structure of a NifS homologue: X-ray structure analysis of CsdB, an *Escherichia coli* counterpart of mammalian selenocysteine lyase. *Biochemistry* **39**, 1263–1273.

Prischi F, Pastore C, Carroni M, Iannuzzi C, Adinolfi S, Temussi P & Pastore A (2010) Of the vulnerability of orphan complex proteins: the case study of the *E. coli* IscU and IscS proteins. *Protein Expr Purif* **73**, 161–166.

Markley JL, Kim JH, Dai Z, Bothe JR, Cai K, Frederick RO & Tonelli M (2013) Metamorphic protein IscU alternates conformations in the course of its role as the scaffold protein for iron-sulfur cluster biosynthesis and delivery. *FEBS Lett* **587**, 1172–1179.

Iannuzzi C, Adrover M, Puglisi R, Yan R, Temussi PA & Pastore A (2014) The role of zinc in the stability of the marginally stable IscU scaffold protein. *Protein Sci* **23**, 1208–1219.

Yoon T & Cowan JA (2003) Iron-sulfur cluster biosynthesis. Characterization of frataxin as an iron donor for assembly of [2Fe-2S] clusters in ISU-type proteins. *J Am Chem Soc* **125**, 6078–6084.

Bou-Abdallah F, Adinolfi S, Pastore A, Laue TM & Dennis Chasteen N (2004) Iron binding and oxidation kinetics in frataxin CyaY of *Escherichia coli*. *J Biol Chem* **284**, 605–615.

Pastore C et al. (2006) YfhJ, a molecular adaptor in iron-sulfur cluster formation or a frataxin-like protein? *Structure* **14**, 857–867.

Imlay JA (2003) Pathways of oxidative damage. *Annu Rev Microbiol* **57**, 395–418.

Foster MW et al. (2000) A mutant human IscU protein contains a stable [2Fe–2S]2+ center of possible functional significance. *J Am Chem Soc* **122**, 6805–6806.

Adrover M, Howes BD, Iannuzzi C, Smulevich G & Pastore A (2015) Anatomy of an iron-sulfur cluster scaffold protein: Understanding the determinants of [2Fe-2S] cluster stability on IscU. *Biochim Biophys Acta* **1853**, 1448–1456.
81 Pastore A, Martin SR & Temussi PA (2019) Generalized View of Protein Folding. In Medio Stat Virtus. J Am Chem Soc 141, 2194–2200.
82 Kim JH, Füzéry AK, Tonelli M, Ta DT, Westler WM, Vickery LE & Markley JL (2009) Structure and dynamics of the iron-sulfur cluster assembly scaffold protein IscU and its interaction with the cochaperone HscB. Biochemistry 48, 6062–6071.
83 Yan R, Kelly G & Pastore A (2014) The scaffold protein IscU retains a structured conformation in the Fe-S cluster assembly complex. ChemBioChem 15, 1682–1686.
84 Lanucara F, Holman SW, Gray CJ & Eyers CE (2014) The power of ion mobility-mass spectrometry for structural characterization and the study of conformational dynamics. Nat Chem 6, 281–294.
85 Ben-Nissan G & Sharon M (2018) The application of ion-mobility mass spectrometry for structure/function investigation of protein complexes. Curr Opin Chem Biol 42, 25–33.
86 Mistarz UH & Rand KD (2018) Installation, validation, and application examples of two instrumental setups for gas-phase HDX-MS analysis of peptides and proteins. Methods 144, 113–124.
87 Kostyukevich Y, Acter T, Zherebker A, Ahmed A, Kim S & Nikolaev E (2018) Hydrogen/deuterium exchange in mass spectrometry. Mass Spectrom Rev 37, 811–853.
88 Katta V & Chait BT (1991) Conformational changes in proteins probed by hydrogen-exchange electrospray-ionization mass spectrometry. Rapid Commun Mass Spectrum 5, 214–217.