Trends in
Biochemical Sciences

CellPress

Review

Characterizing metal–biomolecule interactions by mass spectrometry

Samuel E. Janisse,[1] Rebeca L. Fernandez,[1] and Marie C. Heffern [1,*]

Metal micronutrients are essential for life and exist in a delicate balance to maintain an organism's health. The labile nature of metal–biomolecule interactions clouds the understanding of metal binders and metal-mediated conformational changes that are influential to health and disease. Mass spectrometry (MS)-based methods and technologies have been developed to better understand metal micronutrient dynamics in the intra- and extracellular environment. In this review, we describe the challenges associated with studying labile metals in human biology and highlight MS-based methods for the discovery and study of metal–biomolecule interactions.

Highlights

Over one-third of proteins bind metals and are involved in a variety of biological functions, ranging from catalysis to hormone regulation.

Although several powerful spectroscopic techniques exist to study the static, tightly bound metal pool (e.g., NMR, electron paramagnetic resonance, circular dichroism, UV-visible absorption spectroscopy), there is a dearth of robust throughput analytical techniques to evaluate labile and transient metal interactions.

Mass spectrometry (MS)-based techniques and workflows are being developed to address the challenges that traditional spectroscopic methods face when studying labile metal binding in the intra- and extracellular space.

Continual development of MS-based tools is instrumental; insights into the dynamic interactions of loosely bound metal biomolecules in the extracellular space (i.e., plasma and blood) will improve disease monitoring and diagnostics.

Paradigms of metal-binding interactions in biology

Metal ions are associated with 30–50% of proteins, serving diverse functions that include structural cofactors, catalytic centers, and metabolite transporters [1–4]. To maintain a state of equilibrium, a network of proteins and molecules has evolved that regulates metal micronutrients via chaperones, transporters, storage proteins, and metal-binding small molecules like chelators [5–8]. Early identification of metal-binding molecules stemmed from isolated discoveries of tightly bound ions. As the field has progressed, increasing work has not only diversified the known subset of metalloproteins and peptides but has also introduced the concept that the biological metal population is subdivided into two distinct pools: the static pool (i.e., tightly bound metal ions that are typically buried in a protein) and the more loosely bound or surface-exposed 'labile' pool [9–11]. While existing analytical and protein isolation tools are frequently able to preserve the metal–biomolecule interaction of the static pool, the labile pool may evade detection by traditional methods due to their weaker affinity and exchangeable nature. Therefore, it is crucial to develop techniques that enable studies of the metal interactions of species that can readily exchange metal micronutrients with their environment. In this review, we discuss how existing and emerging mass spectrometry (MS) technologies are leveraged to address questions regarding dynamic metal interactions in biological systems. We highlight how MS, under biologically relevant conditions, can directly probe binding interactions of spectroscopically challenging molecules as well as capture the dynamics of metalloprotein interactions that otherwise evade discovery by classical biophysical and biochemical methods. We also discuss how pairing advances in chromatographic enrichment and separation with MS analysis can identify metal-binding information in complex biological mixtures. The featured examples seek to underscore the potential for parallel development in these areas to bring forth new paradigms for metal–biomolecule interactions.

Challenges in identifying metal-binding interactions beyond known structural motifs

Almost 30 years ago, it was estimated that approximately one-third of all purified proteins and around half of all enzymes need a metal ion for their biological function [12], but only a subset of metalloproteins had been isolated and validated. One classical approach to identify proteins by their function was by generating an alignment of consensus sequences. The sequencing of

[1]Department of Chemistry, University of California, Davis, One Shields Drive, Davis, CA 95616, USA

*Correspondence:
mcheffern@ucdavis.edu (M.C. Heffern).

the human genome [13] and subsequent purification and structural characterization of newly identified proteins provided scientists with a wealth of information relating sequence to structure. As many of these proteins lacked known functions, bioinformatic analyses became common to identify relationships between structure and function in a high-throughput manner [14]. The foundational work by Andreini *et al.* describes numerous databases that connected available structural information to enzymes with known metal cofactors and their functions [15,16]. In this way, predictive tools can be employed to the **metalloproteome** (see Glossary) using known **metal-binding motifs** as search parameters in protein, family, and domain databases to yield predictions of proteins that bind metals with follow-up experimental structure/function characterization.

While this approach has been powerful in identifying motifs within the same protein region, several metalloproteins that have been discovered would have evaded these bioinformatics-driven predictions. Although canonical motifs exist, such as the copper-binding ATCUN motif [17] and the iron-binding 2-histidine-1-carboxylate motif [18], additional factors that influence the specific role of amino acid residues on metal interactions are not strongly accounted for in sequence-based analysis. First, the same metal-binding motif that constitutes the primary coordination sphere of a metalloenzyme is likely to have a unique second coordination sphere that supports the enzyme function; one amino acid sequence motif can have divergent functions as a result of its outer coordination sphere and secondary structure. In the case of a protein with no reported structure, it is difficult to extrapolate amino acid positions from a sequence; residues that are sequentially distant may come together in a folded protein to form a metal coordination site. Finally, it is likely that noncanonical metal-binding motifs exist that remain to be identified and investigated. The many challenges that arise with bioinformatics-based prediction highlight the importance of using a multifaceted approach to discover and elucidate the function and dynamics of metal-binding species in tandem. Bioinorganic chemists leverage a host of spectroscopic techniques such as UV-visible, NMR, and electron paramagnetic resonance (EPR) spectroscopy to gain crucial information regarding the metal-binding environment. While powerful, many of these techniques require high sample concentrations and/or present challenges in complex mixtures, limiting studies in many biological samples. MS offers promise in filling this gap. The remaining sections of this review focus on advances in MS to address the study of metal-binding species.

Beyond classical motifs: MS approaches to characterize spectroscopically challenging metal–biomolecule interactions

MS-based techniques are appealing for studies of metal complexation to biomolecules due to their sensitivity, low sample volume requirement, and potential for high-throughput formats (Table 1). A consideration when adapting and applying MS-based techniques to metal-binding interactions is the weaker nature of coordination bonds relative to covalent interactions. **Electrospray ionization (ESI)**-based MS techniques are attractive for the study of interactions like metal coordination, as it preserves structure, post-translational modifications, and noncovalent interactions during ionization. Metal adducts may be rapidly screened and assessed by the co-incubation of solutions of metal salts and biomolecules (Figure 1A). ESI-MS-based profiling can be further coupled with a multitude of **peptide fragmentation** techniques such as collision-induced dissociation (CID), rapid multiphoton-induced dissociation (RMPID), and electron capture/transfer dissociation (ECD/ETD) to identify the regions of metal binding within a larger peptide or protein.

MS-based techniques also fill an important gap in the study of metal interactions with peptides and small proteins that lack either chromophoric residues or higher-order structure, which renders studies by traditional spectroscopic methods difficult [19–21]. One such example is C-peptide, a hormone-like peptide co-secreted with insulin and zinc by pancreatic beta cells. Initially

Glossary

Bottom-up proteomics: the process of analyzing proteins through enzymatic digestion followed by MS-based fragmentation of resulting peptides for protein identification and quantification.
Electrospray ionization (ESI): a mode of ionization that generates highly charged molecular ions from solution into the gas phase without fragmentation.
Metal-binding motifs: amino acid sequences arranged in a manner that promotes metal coordination.
Metallomics: the study of metals and their biological roles.
Metalloproteome: all proteins in an organism with biologically relevant metal interactions.
Native mass spectrometry (NMS): spectroscopic analysis of analytes in their endogenous form by maintaining physiologically relevant pH.
Peptide fragmentation: the process of fragmenting a peptide along the backbone, producing smaller segments.
Size exclusion chromatography (SEC): analyte separation based on molecular weight.
Top-down mass spectrometry (TDMS): method of analyzing proteins by MS through fragmentation without the use of enzymatic digestion to generate peptides.

Table 1. MS techniques used to assess metal binding, summarizing the advantages, disadvantages, and nuances of methods that can be used to study metal-binding species

	Method	Advantage	Disadvantage	Couple with…
Direct	ESI-based techniques	• Sensitivity, low sample volume requirement, potential for high-throughput format • Impressive analyte size range, provides data on conformational dynamics and metal stoichiometry • Preserves structure, post--translational modifications, and noncovalent interactions (when using NMS)	• Information should be validated via other solution-based methods as metal-adduct formation may occur during desolvation of the charged droplets • Oxidation state may change during ionization process • Limited sensitivity and size capabilities (m/z range constrained by preservation of large complexes)	• CID, RMPID, or ECD/ETD to identify regions of metal binding in an amino acid sequence • IM to obtain biologically relevant structural information and observe metal-induced conformational changes
	ICP-MS	• Quantify total metal levels • Assess distribution shifts of metal binders in different physiological states	• Complex sample matrix preparation limits throughput • Unable to determine the identity of the metal--binding species	• SEC-ICP-MS to simultaneously quantify metal levels and differentiate metal-binding species; workflow can be further paired with anion exchange chromatography • Gel electrophoresis followed by laser ablation (LA--ICP-MS)
Indirect	IMAC	• Inferred metal binding prior to MS identification • Easily adapted for throughout sample processing • Enrichment is not limited to volatile buffers • Useful for discovery experiments	• Reliance on ternary complex formation limits enrichment to specific metal coordination geometry • Strong metal binders may 'strip' metal from chelating resin	• 'Top-down' and 'bottom-up' proteomics to identify putative metal binders • IM-MS
	Differential labeling	• Localize residue-specific metal binding • Isolate labile metal-binding regions • Maintenance of metal interaction post-labeling is not required	• Limited to specific chemical labels for residues • Necessitates optimization of label concentration and reaction time • Requires prior knowledge of metal binding	• Top-down and bottom-up proteomics

regarded as inert, a host of studies have shown it to be linked to a multitude of improved biological outcomes independent of insulin [22]. The exact mechanism of C-peptide's bioactivity is unclear and investigations into inconsistencies with experimental results led to the finding that metal interactions with C-peptide had notable impacts on its activity [23]. Direct coordination was initially thought to be unlikely due to the lack of canonical metal binding sequence motifs in C-peptide, but a series of MS experiments by the Spence grouped revealed the formation of metal adducts with a number of divalent metal ions. in particular, the zinc adduct ion was visible at high abundance relative to the unmetallated species [24]. Tandem MS studies of C-peptide–Zn^{2+} determined the binding location to be near the N terminus [24]. Later work by our group further extended these findings, showing that C-peptide also forms an adduct with Cu^{2+}. On co-incubation of Cu^{2+} and Zn^{2+}, the presence of only the Cu^{2+} adduct was shown by high-resolution MS, suggesting competitive binding of Cu^{2+} over Zn^{2+} when both species are present [25]. The data were corroborated by additional spectroscopic studies (EPR, NMR, and Fourier-transform infrared spectroscopy) [25], validating the use of the technique to assess solution-phase dynamics.

Complementing the fragmentation-based ESI-MS techniques, ion mobility MS (IM-MS) provides structural information to probe metal-induced conformational changes (Figure 1B). The Bowers group has demonstrated the strengths of IM-MS by characterizing direct metal binding to the reproductive neurohormone oxytocin [26,27]. MS studies indicate that binding of oxytocin to metals is dependent on pH: Zn^{2+}, Mg^{2+}, Ni^{2+}, Ca^{2+}, Mn^{2+}, Co^{2+}, and Cu^{2+} coordinate at pH ~3, while at higher pH only Cu^{2+} and Ni^{2+} metal complexes can be observed with several deprotonation states. By contrast, no deprotonation was seen at lower pH, perhaps indicating that metal coordination facilitated the deprotonation of three or four amide hydrogens at higher pH.

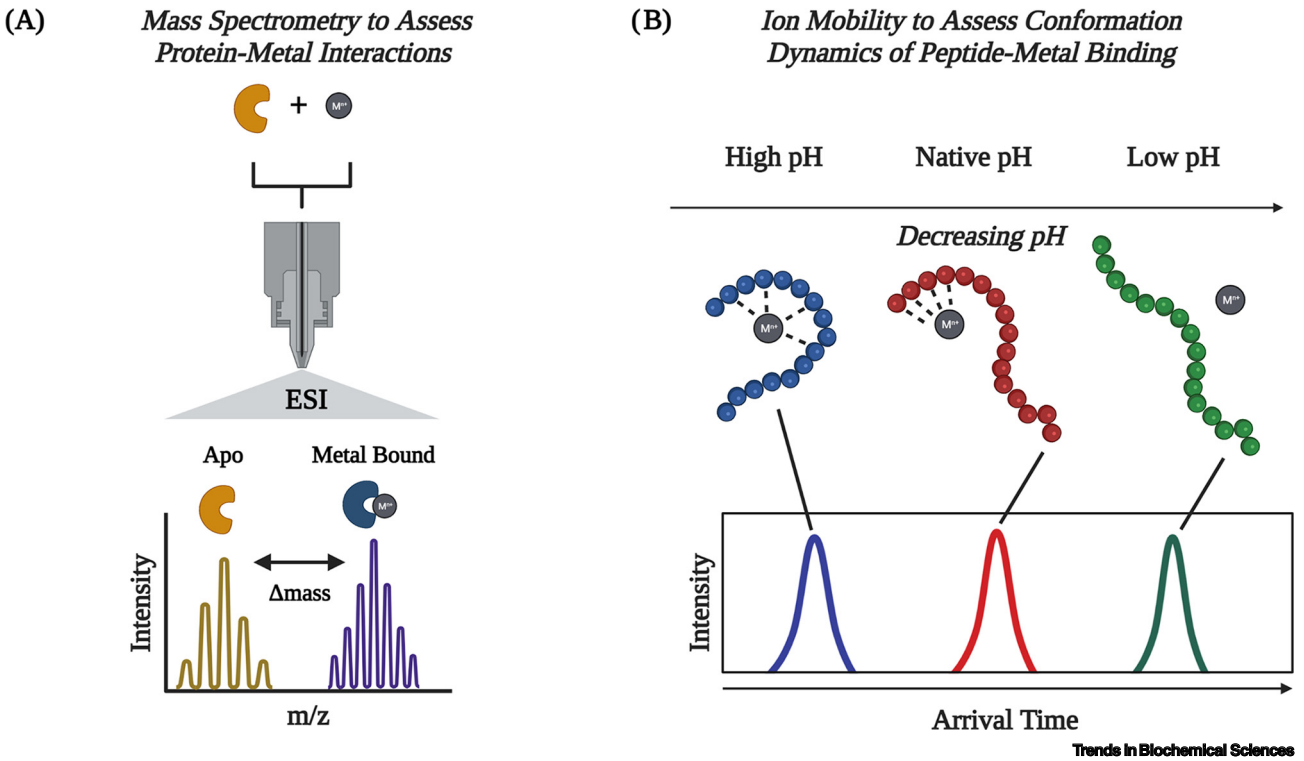

Figure 1. Mass spectrometry (MS)-based methods to assess metal binding and metal-induced structural mobility. (A) Native MS can be used to assess metal interactions by monitoring mass shifts and distinct isotopic patterns. (B) Differences in pH change the propensity to and location of metal interactions and can be monitored by ion mobility. Created with BioRender.com. Abbreviation: ESI, electrospray ionization.

CID, IM-MS, and computational experiments pointed to the most probable site of metal binding by four nitrogen atoms at residues 1, 6, 8, and 9, making a $4N_{1689}$ complex. Conversely, experiments conducted at lower pH suggested an octahedral 6O coordination by the carbonyl group or a trigonal bipyramidal 4O-1N coordination with an amine group at the N terminus [26,27]. Metal-containing biomolecules exist in multiple conformations in the extracellular space, confounding understanding of their function. The application of MS-based techniques has elucidated the importance of probing metal–hormone adduct formation.

Resolving metal-binding dynamics with native MS (NMS) of metalloproteins

Given the nature and intermediate strength of metal–ligand coordination bonds, metal–biomolecule interactions are sensitive to pH, redox environment, and, in the case of proteins, 3D structure. These properties pose challenges to conventional sample preparation methods for MS analysis, which often use low- or high-pH solvents that can influence metal interactions. **NMS** is a rapidly developing MS technique used to study intact biomolecules in their native state. The term 'native' refers to the analyte state prior to ionization wherein the analyte most closely resembles its biological state. NMS has been developed to tackle an impressive size range of analytes from small molecules to large (hundreds of kDa) protein complexes [28,29]. Importantly for metalloproteins and peptides, the approach can leave noncovalent interactions, including metal coordination bonds, intact (Figure 1A). NMS can be used in conjunction with IM to retain biologically relevant structural information, including metal-induced conformational dynamics.

A distinction of NMS from non-native MS techniques is the inclusion of a buffer to maintain biologically relevant pH and ionic strength prior to ionization. Buffer choice can strongly influence the

quality and sensitivity of data, as many common buffers (phosphate, Tris-HCl, Bis-Tris, etc.) are nonvolatile and are incompatible with ESI [30]. Common volatile buffers, such as ammonium acetate and triethyl ammonium bicarbonate, are useful in providing proper ionic strength but have little to no buffering capacity at physiologically relevant pH (~7) [31,32]. The lack of adequate buffering renders them susceptible to pH changes during the electrospray process; however, the timescale of pH-induced protein unfolding may be longer than spectral acquisition, and high concentrations (>100 mM) may aid in mitigating pH changes [32]. When studying large complexes, the instrument mass-to-charge (m/z) range can be a limiting factor, and this limitation can be further exacerbated due to the preservation of structure in NMS, which typically generates lower-charge states; however, many modern instruments and techniques are being developed to have extended m/z options allowing large (kDa) complexes to be studied.

In the context of traditional structural biology methods, which frequently require structural rigidity and homogeneity, NMS fills an important need in studies of the conformational dynamics of intrinsically disordered proteins (IDPs) and peptides. In the event where multiple conformers are present, such as in the case where the metal can bind at multiple locations in a semi-disordered system, NMS data can provide conformational differentiation while simultaneously reporting on metal stoichiometry. This ability was recently showcased in an assessment of metal binding by the antiangiogenic plasmin-derived histidine-rich glycoprotein peptide (HRP) fragment by NMS [33]. Studies have suggested that Zn^{2+} binds to the histidine-rich region of HRP, influencing its antiangiogenic properties [33]. Martin *et al.* used NMS to quantify the binding of five to six Zn^{2+} ions to a peptide mimetic of the HRP proteolytic fragment comprising amino acids HRP330–364 (HRP330). IM-MS experiments showed a decrease in arrival time when HRP330 was complexed to zinc, indicative of a more compact cross-section. Increasing Zn^{2+} concentration led to arrival time peak broadening, suggesting the presence of multiple structural conformers [34]. ETD and CID showed reduced fragmentation in select regions of the peptide, providing additional evidence of multiple locations of zinc binding [34].

Recent studies have shown the promise of extending NMS to native **top-down MS** (nTDMS) workflows to reveal key insights into metal interactions beyond classical cytosolic proteins to include recent studies of membrane metalloproteins via incorporation into nanodiscs and the characterization of alpha synuclein's metal-induced aggregation propensities [35–38]. Another exciting application of NMS called native ambient MS imaging is providing opportunities to directly study metalloproteins in tissues with little to no pretreatment; recent work by Hale and coworkers, for instance, showed that coupling NMS with MS imaging allows the direct assessment and localization of metallated forms of GTPase Ran, paralbumin-alpha, and carbonic anhydrase localized in rat brains [39]. While the method is currently limited in its sensitivity and size capabilities, further development of these technologies should provide exciting opportunities to observe tissue-specific differences in metalloprotein states.

Considerations for MS analysis

While MS-based techniques are promising for the probing of metal–biomolecule interactions, the information to be gleaned from ESI-MS should be interpreted with caution and paired with other solution-based validation methods. For instance, due to the nature of the electrospray process and ion generation, Cu^{2+} adduct formation may occur more readily during the desolvation of the charged droplets generated by ESI. A thorough study conducted by Bal *et al.* further substantiated these concerns by illustrating key differences between solution-phase metal-binding equilibrium and gas-phase equilibrium [40]. Moreover, when analyzing transition-metal complexes such as those with Cu^{+} and Fe^{2+} via ESI-MS, the oxidation state may change during the ionization process depending on the source conditions, therein reducing or increasing metal-biomolecule

formation. While the oxidation state of the bound metal post-ionization can be determined by careful examination of the isotopic distribution of the metal complex with a mass spectrometer with high-resolution capability, the true endogenous oxidation state of the complex needs to be validated through follow-up experiments or prior knowledge of the oxidation state.

In addition, there is often uncertainty associated with the use of fragmentation-based studies to localize a metal-binding site. The difficulty arises from potential 'metal hopping', which is akin to the 'mobile proton theory' where the proton is transferred and facilitates the fragmentation process [41]. This phenomenon has been studied in peptides with several acidic residues in which, during fragmentation, chromium was inserted into the peptide backbone, occluding accurate localization [42]. The Spence group proposed a similar event when identifying the zinc-binding site of C-peptide [24]. Therefore, care should be taken when suggesting metal-binding locations based on fragmentation spectra alone. Nevertheless, these studies highlight the power of MS to quickly gain insight into metal binding and to elucidate the complex nature of metal-binding dynamics.

Classifying metal-protein populations in biological mixtures

Profiling metal populations based on size

While the field of metalloproteomics has provided key insights into metal–biomolecule interactions, there remains a subset of this population that is uncharacterized due to challenges in metal coordination preservation [11]. The uncharacterized population is a mixture of large and small proteins and peptides as well as small-molecule metabolites like citrate. Separating metal-binding species via molecular weight cutoff (MWCO) filters is appealing due to the experimental ease, efficiency, and range of cutoff sizes. Efforts have been made to classify clinical copper status in terms of populations bound to the major carrier ceruloplasmin (CP) [43–45]. To achieve this, MWCO filtration has been applied alongside total metal and/or immunoassay quantification of CP [46]. Wilson disease, a genetic copper-trafficking disorder, is typically monitored via comparison of CP- and non-CP-bound copper levels [43,47]. Furthermore, MWCO filtration methods were recently leveraged on mice missing the copper importer Ctr1 to identify a novel copper carrier of <2 kDa, termed small copper carrier (SCC) [48]. SCC was proposed to be responsible for increased urinary copper excretion in the mutant mice, indicating that low-molecular-weight metal-binding species in the blood may influence and represent an organism's metal status, warranting further investigation into their identities.

Understanding not only total metal levels but also how the distribution shifts in disease states holds promise in diagnostics. The limitation of using strict MWCO approaches for this purpose is that they bin the copper pool into simply high or low molecular weight rather than a spectrum of sizes. Indirect methods like chelator/ultrafiltration methods also suffer from the inability to directly analyze speciated metals and rely on approximations and assumptions of metal binding [45,46]. Other multidimensional separation methods involving the use of NMS can be used to probe how speciation occurs in metalloproteins but currently lack the ability to profile metal distribution in biofluids in a high-throughput manner. To this end, research has incorporated a combination of chromatographic separation techniques like anion exchange, size exclusion, and 2D gel electrophoresis followed by laser ablation coupled with inductively coupled plasma MS (ICP-MS) (Table 1) [49,50]. **Size exclusion chromatography (SEC)** separates analytes based on molecular weight while ICP-MS enables accurate metal quantitation (Figure 2).

When combined, changes in metal speciation and distribution among molecular-weight classes can be accurately measured and contrasted across conditions. SEC-ICP-MS has found utility in the classification of Wilson disease based on copper distribution, monitoring of zinc redistribution

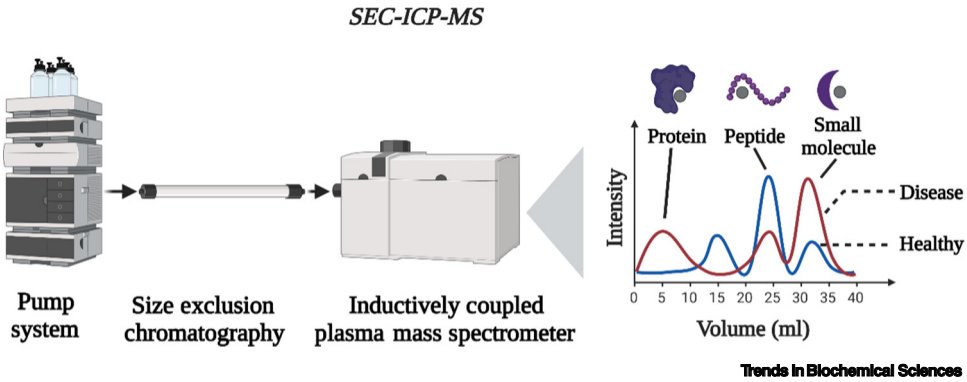

Figure 2. Profiling metal distribution by size exclusion chromatography–inductively coupled plasma mass spectrometry (SEC-ICP-MS). The diversity of metal-binding species is monitored by SEC-ICP-MS. Changes in metal distribution are used to assess disease states and progression. Created with BioRender.com.

in serum with high lipid content, and metalloprotein determination in human breast milk, serum, and cerebrospinal fluid [51–57]. Another instance of the application of SEC-ICP-MS in diagnostic settings is monitoring the redistribution of iron post-intravenous injection of iron-containing compounds used to treat iron deficiency. Using custom SEC-ICP-MS, Neu *et al.* monitored the increase of iron-binding species at different molecular weights after an injection of Ferrlecit (sodium ferric gluconate). Clear differences were observed for both protein-bound (higher molecular weight) and non-protein-bound (lower molecular weight) species 3 h post-injection [58]. While still in the early stages of development, SEC-ICP-MS used in conjunction with proteomics-based MS techniques will provide a deeper picture of the complexities of metal dynamics and speciation.

Enriching metal-binding species from biological mixtures with immobilized metal affinity chromatography (IMAC)

Complementary to developments in chromatography-paired ICP-MS, chromatographic enrichment of protein populations based on metal-binding propensities has the potential to identify new metal-binding populations [59]. **Bottom-up proteomics** enables both the identification of proteins and a snapshot of the protein abundance landscape in a biological mixture. Despite its utility, sample preparation involves harsh denaturing conditions and enzymatic digestion leading to loss of metal coordination. Thus, in addition to investigating the tightly bound pool of metals found buried in the center of a protein, it becomes important to purposefully identify labile metal-bound pools of biomolecules. This limit on sample preparation may be circumvented by preserving metal-interaction information prior to the application of bottom-up proteomics. IMAC has the potential to fill this gap (Figure 3 and Table 1).

IMAC was first introduced in the literature as a method for fractionating histidine- and cysteine-containing proteins [60]. While its most widespread use in current research is the purification of histidine-tagged proteins, researchers have demonstrated its potential to identify putative metal-binding species that are difficult to predict based on sequence information alone [60,61]. IMAC comprises three main components: an immobilizing resin, an extending linker, and a metal chelating group. Biomolecules with metal-binding propensities will interact with the immobilized metal creating a ternary complex, whereas non-metal-binding species will not interact with the resin, allowing their removal. In the early 2000s, several studies focused on the use of IMAC to understand the metalloproteome illustrated the potential application of IMAC to gain biological insights. For example, She *et al.* described cell-line-dependent differences between

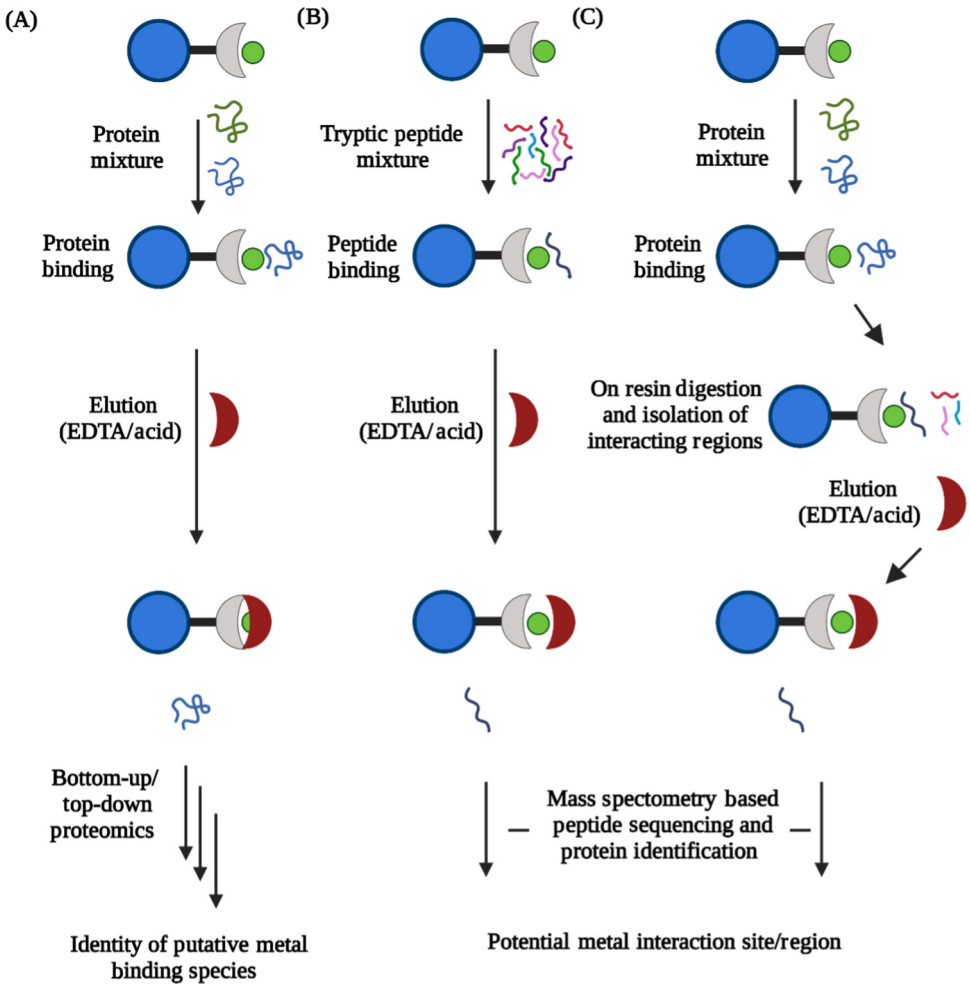

Figure 3. Varying metal-binding species enrichment methods via immobilized metal affinity chromatography (IMAC). (A) Traditional enrichment of proteins by IMAC. (B) Identification of metal interaction sites of enzymatically digested proteins. (C) On-resin protein digestion to identify putative metal interaction sites. Created with BioRender.com.

proteins enriched on Cu^{2+}- and Zn^{2+}-IMAC resin [62]. Among those proteins enriched on the Cu^{2+}-loaded IMAC resin was protein disulfide isomerase (PDI), which had been only recently determined to bind Cu^{2+}. Smith *et al.* followed by separating microsomal and cytosolic components and applying each to a Cu^{2+}-IMAC resin. A total of 67 proteins were enriched, with several not previously reported to associate with copper [62,63].

Additionally, IMAC has been uniquely applied to understand metal binding and regulation in extracellular proteins in blood-based samples. Wang *et al.* applied serum to IMAC resins loaded with Zn^{2+}, Cd^{2+}, Ca^{2+}, Ni^{2+}, and Pb^{2+}, resulting in over 700 protein identifications with several proteins showing metal-specific enrichment levels. For example, transthyretin was moderately enriched on Ni^{2+} and Cu^{2+} resins whereas Zn^{2+}-IMAC enriched transthyretin to a lesser extent [64]. Extending IMAC's application to study metal-binding dynamics, our laboratory explored the utilization of IMAC to study the relative strength of the Cu^{2+}–IDA interaction by incubating a fixed amount of resin with increasing percentages of serum [65]. Both 1D gel electrophoresis and

MS-based proteomics revealed key differences in protein populations with respect to increasing serum. The workflow was further extended to study differences in copper-interacting proteins in Wilson disease and healthy controls. In addition to producing differentially abundant proteins, the IMAC workflow acted as a pseudo-clean-up step while preserving key protein abundance information in the non-IMAC-based proteomics workflow [65].

Identifying metal interaction sites

Although the typical IMAC workflow leaves the metal interaction site ambiguous, this challenge can be addressed by including protein digests in the method. For instance, an initial IMAC enrichment followed by trypsin digestion and reapplication of digested proteins to IMAC can point to the specific sequences that interact with the resin. Smith *et al.* demonstrated this approach with *Streptococcus pneumoniae* lysates applied to Co^{2+}- and Ni^{2+}-IMAC, resulting in over 200 putative metal-binding proteins. Reapplication of tryptic peptides revealed the sequence motifs H(X)nH, M(X)nH, and H(X)nM [66]. To streamline this approach, She and coworkers showed that digestion-paired IMAC can be performed with a single enrichment step via 'on-column protein digestion' [62]. In on-column digestion, the sample is first enriched on column, but prior to elution of the bound species the enzyme is applied directly to the resin. In theory, the remaining peptides will be the sections of proteins interacting with the immobilized metal and can be eluted and analyzed. The approach was used to find Bi^{3+}-interacting sites of proteins from *Helicobacter pylori* to provide insight into the antibacterial mode of Bi-containing gastrointestinal drugs [67].

An additional method to identify putative protein metal-binding sites identified by IMAC is differential labeling (Table 1). In this approach, native and denatured proteins are modified with a residue-specific label (i.e., iodoacetamide for cysteine alkylation) followed by MS-based proteomics analysis (bottom up or top down). Metal interaction sites are correlated with residues showing differential modification as metal coordination is likely to inhibit reaction with the chemical label [68,69]. Using metallothionine, a cysteine-rich metalloprotein, as a model, Krezel and colleagues illustrated that a dual labeling strategy was more effective in assessing metal interaction sites of moderate to low binding affinities than the traditional single-label workflow [70]. Similar to the traditional workflow, the native protein is first modified with a residue-specific label. However, after the initial labeling the same protein is denatured, the metal is removed, and it is modified with a second chemical label with similar residue specificity. Residues with the second chemical label are probable locations of metal interactions. While differential labeling is powerful, it requires careful optimization of the labile concentration as well as labels that are specific to metal-coordinating residues under the protein's native conditions. Nevertheless, this is an important tool to elucidate metal interaction sites. These examples highlight the diversity of applications of IMAC paired with MS-based analysis and should remain in the toolkit of bioinorganic chemists as new technologies emerge.

Concluding remarks

The increasing diversity of MS-based tools and emerging technologies described herein will enable cutting-edge breakthroughs in the field of **metallomics**. The expansion of MS applications will provide vital insight complementary to other established spectroscopic approaches routinely used in the bioinorganic field. While we have highlighted recent advances and applications toward understanding metal-binding dynamics, the breadth of available tools remains narrow. For example, we lack accurate methods to monitor the metal loading of large proteins that require several metal cofactors for proper function. Despite recent developments, these methods are currently limited to small (<20 kDa) metalloproteins and need further refinement. In addition, there is a particular deficit in tools that explore the extracellular space; study has been hindered not only by its complexity, but also by the dynamic range of analytes (see Outstanding questions). Combining the power of computational chemistry, chromatography systems, and

Outstanding questions

What are the labile metal interactions in the extracellular space?

Can MS-based methods be developed to monitor metal-mediated complex formation and used to gain insight into solution-phase dynamics?

What is the influence of the various fragmentation techniques on metal binding and the ability to capture endogenous metal interaction sites?

To what degree does metal speciation allow differentiation of diseases and disease progression?

MS will facilitate the characterization of metal-binding-site dynamics with respect to changes in post-translational modifications, mutations, and ternary complex formation. Importantly, this research will investigate links between metal regulation, health, and disease. While these relationships have been suggested, there exists a large barrier to improving human health and developing disease diagnostic tools. An understanding of metal biomolecules in the intra- and extracellular space will usher in a new frontier in our understanding of the functional role metals have in biology.

Declaration of interests

No interests are declared.

References

1. Baradaran, R. *et al.* (2013) Crystal structure of the entire respiratory complex I. *Nature* 494, 443–448
2. Putignano, V. *et al.* (2018) MetalPDB in 2018: a database of metal sites in biological macromolecular structures. *Nucleic Acids Res.* 46, D459–D464
3. Jiang, B. *et al.* (2016) Hephaestin and ceruloplasmin facilitate iron metabolism in the mouse kidney. *Sci. Rep.* 6, 39470
4. Cassandri, M. *et al.* (2017) Zinc-finger proteins in health and disease. *Cell Death Discov.* 3, 17071
5. Xiao, Z. *et al.* (2011) Unification of the copper(I) binding affinities of the metallo-chaperones Atx1, Atox1, and related proteins: detection probes and affinity standards. *J. Biol. Chem.* 286, 11047–11055
6. Lee, V.J. and Heffern, M.C. (2022) Structure–activity assessment of flavonoids as modulators of copper transport. *Front. Chem.* 10, 972198
7. Massai, L. *et al.* (2021) Direct detection of iron clusters in L ferritins through ESI-MS experiments. *Dalton Trans.* 50, 16464–16467
8. Yanatori, I. and Kishi, F. (2019) DMT1 and iron transport. *Free Radic. Biol. Med.* 133, 55–63
9. Ackerman, C.M. and Chang, C.J. (2018) Copper signaling in the brain and beyond. *J. Biol. Chem.* 293, 4628–4635
10. Ge, E.J. *et al.* (2021) Connecting copper and cancer: from transition metal signaling to metalloplasia. *Nat. Rev. Cancer* 22, 101–113
11. Lutsenko, S. (2016) Copper trafficking to the secretory pathway. *Metallomics* 8, 840–852
12. Holm, R.H. *et al.* (1996) Structural and functional aspects of metal sites in biology. *Chem. Rev.* 96, 2239–2314
13. Collins, F.S. *et al.* (1998) New goals for the U.S. Human Genome Project: 1998–2003. *Science* 282, 682–689
14. Dupont, C.L. *et al.* (2010) History of biological metal utilization inferred through phylogenomic analysis of protein structures. *Proc. Natl. Acad. Sci. U. S. A.* 107, 10567–10572
15. Andreini, C. *et al.* (2009) Metalloproteomes: a bioinformatic approach. *Acc. Chem. Res.* 42, 1471–1479
16. Andreini, C. *et al.* (2008) Metal ions in biological catalysis: from enzyme databases to general principles. *J. Biol. Inorg. Chem.* 13, 1205–1218
17. Gonzalez, P. *et al.* (2018) N-terminal Cu-binding motifs (Xxx-Zzz-His, Xxx-His) and their derivatives: chemistry, biology and medicinal applications. *Chemistry* 24, 8029–8041
18. Bruijnincx, P.C.A. *et al.* (2007) Modeling the 2-His–1-carboxylate facial triad: iron-catecholato complexes as structural and functional models of the extradiol cleaving dioxygenases. *J. Am. Chem. Soc.* 129, 2275–2286
19. Stevenson, M.J. *et al.* (2019) Metal-dependent hormone function: the emerging interdisciplinary field of metalloendocrinology. *Metallomics* 11, 85–110
20. Stevenson, M.J. *et al.* (2019) Analysis of metal effects on C-peptide structure and internalization. *Chembiochem* 20, 2447–2453
21. Kulprachakarn, K. *et al.* (2016) Copper(II) binding properties of hepcidin. *J. Biol. Inorg. Chem.* 21, 329–338
22. Wahren, J. *et al.* (2007) C-peptide is a bioactive peptide. *Diabetologia* 50, 503–509
23. Meyer, J.A. *et al.* (2008) Metal-activated C-peptide facilitates glucose clearance and the release of a nitric oxide stimulus via the GLUT1 transporter. *Diabetologia* 51, 175–182
24. Keltner, Z. *et al.* (2010) Mass spectrometric characterization and activity of zinc-activated proinsulin C-peptide and C-peptide mutants. *Analyst* 135, 278–288
25. Stevenson, M.J. *et al.* (2020) Elucidation of a copper binding site in proinsulin C-peptide and its implications for metal-modulated activity. *Inorg. Chem.* 59, 9339–9349
26. Wyttenbach, T. *et al.* (2008) Interactions of the hormone oxytocin with divalent metal ions. *J. Am. Chem. Soc.* 130, 5993–6000
27. Liu, D. *et al.* (2005) Oxytocin-receptor binding: why divalent metals are essential. *J. Am. Chem. Soc.* 127, 2024–2025
28. Leney, A.C. and Heck, A.J.R. (2017) Native mass spectrometry: what is in the name? *J. Am. Soc. Mass Spectrom.* 28, 5–13
29. Zhou, M. *et al.* (2020) Higher-order structural characterisation of native proteins and complexes by top-down mass spectrometry. *Chem. Sci.* 11, 12918–12936
30. Iavarone, A.T. *et al.* (2004) Buffer loading for counteracting metal salt-induced signal suppression in electrospray ionization. *Anal. Chem.* 76, 3944–3950
31. Lemaire, D. *et al.* (2001) Stabilization of gas-phase noncovalent macromolecular complexes in electrospray mass spectrometry using aqueous triethylammonium bicarbonate buffer. *Anal. Chem.* 73, 1699–1706
32. 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
33. Magrì, A. *et al.* (2018) Peptides derived from the histidine-proline rich glycoprotein bind copper ions and exhibit anti-angiogenic properties. *Dalton Trans.* 47, 9492–9503
34. Martin, E.M. *et al.* (2018) Native electrospray mass spectrometry approaches to probe the interaction between zinc and an anti-angiogenic peptide from histidine-rich glycoprotein. *Sci. Rep.* 8, 8646
35. Wongkongkathep, P. *et al.* (2018) Native top-down mass spectrometry and ion mobility MS for characterizing the cobalt and manganese metal binding of α-synuclein protein. *J. Am. Soc. Mass Spectrom.* 29, 1870–1880
36. Fields, C.R. *et al.* (2019) Targeting alpha-synuclein as a therapy for Parkinson's disease. *Front. Mol. Neurosci.* 12, 299
37. Moons, R. *et al.* (2020) Metal ions shape α-synuclein. *Sci. Rep.* 10, 16293
38. Ro, S.Y. *et al.* (2019) Native top-down mass spectrometry provides insights into the copper centers of membrane-bound methane monooxygenase. *Nat. Commun.* 10, 2675
39. Sisley, E.K. *et al.* (2022) Native ambient mass spectrometry imaging of ligand-bound and metal-bound proteins in rat brain. *J. Am. Chem. Soc.* 144, 2120–2128
40. Plonka, D. *et al.* (2021) Electrospray-induced mass spectrometry is not suitable for determination of peptidic Cu(II) complexes. *J. Am. Soc. Mass Spectrom.* 32, 2766–2776
41. Wysocki, V.H. *et al.* (2000) Mobile and localized protons: a framework for understanding peptide dissociation. *J. Mass Spectrom.* 35, 1399–1406

42. Pu, D. *et al.* (2008) The effects of chromium(III) coordination on the dissociation of acidic peptides. *J. Mass Spectrom.* 43, 773–781
43. Ferenci, P. *et al.* (2012) EASL clinical practice guidelines: Wilson's disease. *J. Hepatol.* 56, 671–685
44. Walshe, J.M. (2010) Monitoring copper in Wilson's disease. *Adv. Clin. Chem.* 50, 151–163
45. Twomey, P.J. *et al.* (2008) Non-ceruloplasmin-bound copper in routine clinical practice in different laboratories. *J. Trace Elem. Med. Biol.* 22, 50–53
46. Woimant, F. *et al.* (2019) New tools for Wilson's disease diagnosis: exchangeable copper fraction. *Ann. Transl. Med.* 7, S70
47. Członkowska, A. *et al.* (2018) Wilson disease. *Nat. Rev. Dis. Primers* 4, 21
48. Gioilli, B.D. *et al.* (2022) Secretion and uptake of copper via a small copper carrier in blood fluid. *Metallomics* 14, mfac006
49. Chen, J. *et al.* (2022) Laser ablation–inductively coupled plasma–mass spectrometry (LA-ICP-MS)-based strategies applied for the analysis of metal-binding protein in biological samples: an update on recent advances. *Anal. Bioanal. Chem.* 414, 7023–7033
50. Sariego Muñiz, C. *et al.* (2001) Speciation of essential elements in human serum using anion-exchange chromatography coupled to post-column isotope dilution analysis with double focusing ICP-MS. *J. Anal. At. Spectrom.* 16, 587–592
51. Acosta, M. *et al.* (2018) Novel method for metalloproteins determination in human breast milk by size exclusion chromatography coupled to inductively coupled plasma mass spectrometry. *J. Pharm. Biomed. Anal.* 158, 209–213
52. Michalke, B. *et al.* (2021) Simultaneous quantification and speciation of trace metals in paired serum and CSF samples by size exclusion chromatography–inductively coupled plasma–dynamic reaction cell–mass spectrometry (SEC-DRC-ICP-MS). *Int. J. Mol. Sci.* 22, 8892
53. Latorre, M. *et al.* (2019) Size exclusion chromatography–inductively coupled plasma–mass spectrometry for determining metal-low molecular weight compound complexes in natural wines. *Talanta* 195, 558–565
54. Kirsipuu, T. *et al.* (2020) Copper(II)-binding equilibria in human blood. *Sci. Rep.* 10, 5686
55. Coverdale, J.P.C. *et al.* (2019) A metalloproteomic analysis of interactions between plasma proteins and zinc: elevated fatty acid levels affect zinc distribution. *Metallomics* 11, 1805–1819
56. Quarles, C.D. *et al.* (2020) LC-ICP-MS method for the determination of "extractable copper" in serum. *Metallomics* 12, 1348–1355
57. del Castillo Busto, M.E. *et al.* (2022) A fit-for-purpose copper speciation method for the determination of exchangeable copper relevant to Wilson's disease. *Anal. Bioanal. Chem.* 414, 561–573
58. Neu, H.M. *et al.* (2019) Snapshots of iron speciation: tracking the fate of iron nanoparticle drugs via a liquid chromatography-inductively coupled plasma-mass spectrometric approach. *Mol. Pharm.* 16, 1272–1281
59. Zhou, Y. *et al.* (2022) Metalloproteomics for biomedical research: methodology and applications. *Annu. Rev. Biochem.* 91, 449–473
60. Gaberc-Porekar, V. and Menart, V. (2001) Perspectives of immobilized-metal affinity chromatography. *J. Biochem. Biophys. Methods* 49, 335–360
61. Chang, Y.Y. *et al.* (2017) Immobilized metal affinity chromatography (IMAC) for metalloproteomics and phosphoproteomics. In *Inorganic and organometallic transition metal complexes with biological molecules and living cells*, pp. 329–353, Academic Press
62. She, Y.M. *et al.* (2003) Identification of metal-binding proteins in human hepatoma lines by immobilized metal affinity chromatography and mass spectrometry. *Mol. Cell. Proteomics* 2, 1306–1318
63. Smith, S.D. *et al.* (2004) Using immobilized metal affinity chromatography, two-dimensional electrophoresis and mass spectrometry to identify hepatocellular proteins with copper-binding ability. *J. Proteome Res.* 3, 834–840
64. Wang, F. *et al.* (2013) Immobilized metal affinity chromatography and human serum proteomics. *J. Chromatogr. B* 934, 26–33
65. Janisse, S.E. *et al.* (2022) Systematic evaluation of copper(II)-loaded immobilized metal affinity chromatography for selective enrichment of copper-binding species in human serum and plasma. *Metallomics* 14, mfac059
66. Sun, X. *et al.* (2013) Putative cobalt- and nickel-binding proteins and motifs in *Streptococcus pneumoniae*. *Metallomics* 5, 928–935
67. Wang, Y. *et al.* (2015) Bio-coordination of bismuth in *Helicobacter pylori* revealed by immobilized metal affinity chromatography. *Chem. Commun. (Camb.)* 51, 16479–16482
68. Chen, S.H. *et al.* (2013) Combining chemical labeling, bottom-up and top-down ion-mobility mass spectrometry to identify metal-binding sites of partially metalated metallothionein. *Anal. Chem.* 85, 3229–3237
69. Chen, S.H. and Russell, D.H. (2015) Reaction of human Cd7metallothionein and N-ethylmaleimide: kinetic and structural insights from electrospray ionization mass spectrometry. *Biochemistry* 54, 6021–6028
70. Peris-Díaz, M.D. *et al.* (2020) Metal- and affinity-specific dual labeling of cysteine-rich proteins for identification of metal-binding sites. *Anal. Chem.* 92, 12950–12958