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Metalloproteomics: Challenges and Prospective for Clinical Research Applications

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Abstract

Metals are essential cofactors, utilized in many critical cellular processes. For example, zinc is important in insulin biosynthesis, and may play a role in Alzheimer's disease, but much of how this happens remains unknown. Knowing which metal is in which protein at a given point in time would lead to new insights into how they do their work. New tools are being developed to investigate this, with potential for biomedical applications. In this report, we consider the promise and limitations of these techniques. We provide a brief overview of the techniques available, and a discussion of the technical challenges to biomedical applications, with particular focus on what must be overcome for the potential of these approaches to be achieved.

Keywords

metalloproteomics; metals; metalloproteins; molecular pathology; elemental analysis

Emergence of Metalloproteomics, and importance of metals in human health

Metals are essential cofactors, utilized in respiration, metabolism, development, nerve transmission, signal transduction, and many other critical processes (Figure 1) [1]. Also, metals introduced into our systems artificially, either through environmental contaminants or as part of drugs, also have many effects on biochemistry. An intricate network of protein interactions ensures metals are in the right locations, at the right time, and in the right amount, for human health [2].

As one example of the biological roles of metals, and their pathways within the cells, let us consider those of the metal zinc (Zn(II)). Zinc proteins play key roles in many biological processes, including carbon fixation, biosynthesis and redox regulation [3]. Zinc is abundant in all living organisms. The zinc proteome represents about 10% of the entire proteome, and the total cellular zinc content is in the submillimolar range [4]. Although zinc is an essential metal, excess Zn(II) can be toxic (at about 300mg elemental zinc by ingestion in humans) [5], because it can competitively bind to the wrong nascent metalloproteins destined for

other essential metal ions, such as Fe(II), Mn(II) and Co(II) [6]. Accordingly, cells exert tight control over Zn(II) by over-expression of zinc binding proteins to limit the free zinc concentration in the cytosol [2]. The over-capacity for zinc binding renders the cytosol essentially devoid of unbound Zn(II) [7]. Because of this, a leading hypothesis is that once a zinc ion enters the cells, it is thought to pass from one protein zinc site to another, probably like copper trafficking down a gradient of increasing affinities [8], to reach its destination site. We speculate that a myriad of cellular zinc trafficking routes evolved to deliver zinc ions to a great variety of zinc-binding proteins. If zinc handling mirrors copper, these zinc trafficking routes would interlink to form a zinc trafficking 'interactome', or an intricate network of protein interactions that govern selective zinc distribution among competitive protein sites in cells. Fidelity of specific protein recognitions would be 'hard-wired' to ensure proper metallation of a large number of zinc binding proteins while minimizing zinc toxicity [6].

While this is our hypothesis, our current knowledge of zinc biology, like that of other metals, is largely grounded on experimental analysis of individual zinc proteins with their active sites filled with zinc ions. How zinc ions selectively populate their protein targets is still a mystery. Bound zinc ions provide enzymes with powerful catalytic groups, serve as an effective control ion for cellular signaling, and have a great impact on protein structures [9]. To fulfill these structure-function roles, zinc ions must compete with a repertoire of similar transition metal ions for their protein sites. While the zinc trafficking 'interactome' affords a conceptual framework, the molecular constituents of this protein interaction network has yet to be defined. Zinc uptake and efflux transporters represent the entry and exit points of the zinc interactome [10]. Beyond the membrane transporters, the intermediate steps along the zinc trafficking pathways are entirely unknown.

While the zinc trafficking pathways remain elusive, their physiological or pathological significances are highlighted by a few well-studied terminal zinc recipients at the end of various zinc trafficking pathways. One example is insulin, which is stored in the form of zinc-coordinated hexamers in the secretory granules of pancreatic β -cells [11]. Zinc is required for multiple steps in insulin biosynthesis and secretion in response to the fluctuation of blood glucose level [12]. Abnormal insulin secretion associated with β -cell impairments is an essential element in the progression of patients from a state of glucose tolerance to the disease type-2 diabetes. At present, the zinc trafficking pathway leading to the zinc-enriched insulin granules is unclear. This knowledge gap hinders our understanding of insulin biogenesis and its regulated secretion, which is essential to understanding why insulin secretion fails to compensate for insulin resistance during the progression of type-2 diabetes. Aberrant zinc trafficking pathways may move zinc to unintended high-affinity zinc sinks with pathological consequences. For example, a key factor in Alzheimer's disease etiology is the enrichment of zinc in senile plaques (we are also aware of a role for copper in Alzheimers, but are focused in our discussion here on zinc) [13]. The primary constituent of the plaques is the aggregated amyloid beta peptides ($A\beta$). Zinc is a potential amyloid seeding factor because its binding to $A\beta$ was found to trigger rapid amyloid aggregation [14]. Understanding how cerebral zinc finds its way to $A\beta$ may shed light on the neuropathogenesis of Alzheimer's disease, thus providing new drug targets for therapeutic

interventions. These examples of the role of zinc in diabetes and Alzheimer's disease underscore the importance of this metal physiologically and make clear that a better delineation of zinc's pathways would help us better understand the etiology of these diseases. But, few approaches exist that allow us to pinpoint, at a given point in time where a metal such as zinc is within the cell, - in what protein, in what stoichiometry, with what consequence?

To better understand how these metals play a role in human health, we need new tools. Imaging is one such tool with the potential to provide information about the quantity and spatial distribution of metals within cells. Over the past few decades, imaging has become an increasingly powerful tool, and the direct approaches it enables have shed new light on the biology of metals. One of the primary examples is the development of optically-fluorescent, selective metal-ion sensors. Of these, calcium indicators have the longest history, and most widespread awareness [15]. And sensors for copper, zinc, iron, and other metals are adding ever more to our picture of cellular metals [16-18]. Direct imaging of total metals in cells, without regard for their bioavailability, has also become possible more recently. As early as the 1980's, advances in microanalysis were enabling the development of electron microscopy based compositional analysis – or the ability to distinguish the chemical composition of samples at the cellular or subcellular level [19]. The development of hard x-ray fluorescence microprobes at synchrotron x-ray sources, which took place in the early 2000's, provided simpler sample preparation requirements, and has made this work more accessible [20]. Yet, to relate this information to its context, to the library of information we have about metal-binding proteins, there is in turn an imminent need for tools that relate new images of metal-ion homeostasis to the proteins responsible for changing it.

As new techniques for cellular imaging of metals provide us with more insight as to their quantity, and spatial distribution, it raises new questions about their speciation. So much of their activity depends on the protein partner of the metal. Approaches to identify and quantify these metal-protein complexes, dubbed metalloproteomics, seek to identify the metal-binding proteins, and determine the quantity of metal present in the proteins at various points. Among the techniques developed are those that couple liquid chromatography with inductively-coupled-plasma mass-spectrometry (ICP-MS) [21-23]. This has a particular advantage for pulse chase experiments, utilizing isotopic sensitivity of mass spectrometry to identify newly-formed metalloproteins pools. Others have utilized laser-ablation ICP-MS to study gels [24-27], and many other approaches continue to be explored. There are advantages and limitations to each, as will be discussed in this report.

Practical Considerations for Metalloproteomics

Metalloproteomics differs from proteomics in the nature of the analyte. Metalloproteins are, fundamentally, coordination complexes. As such, the metals are not covalently bound, but only coordinatively-complexed, to the proteins. There is great variety in the nature, and strength, of this interaction among the vast array of metal cofactors, which includes iron-sulfur cluster, heme cofactors, and other types of metal centers. Yet the technical challenge for metalloproteomics is that, at least for a subset of these metalloproteins, they exist in a

dynamic exchange with their apo- proteins, and the ionic metals themselves (or other metal-containing species), which can be described in the most simplistic sense,

where K is the binding constant of the metal (M) to the protein chain (P) to form the metalloproteins complex (MP) [1]. Lysing the cell, and breaking cell membranes, alone could expose the protein to pools of previously unavailable metals and possibly change the metal to protein ratio. Beyond that, any separation technique applied to the sample, in producing conditions that separate the proteins of different sizes, could also potentially separate metal ions from proteins, driving this equilibrium further towards the un-metallated protein state. Clearly, avoiding perturbation of metal-protein complexes is an important, and challenging design parameter for these techniques.

Further, metalloproteins are of relatively low abundance in a given natural or clinical sample, and the analyte of interest, the metal atom, is present in much lower quantities than the protein itself, with often one metal atom (perhaps 55 Daltons in mass) present in a protein molecule that may be 30,000 Daltons large. So, in the face of all these challenges, one might ask, what has been achieved?

Survey of approaches to Metalloproteomics

Liquid Chromatography/spectroscopy-based approaches

X-ray spectroscopy has been of great historical importance in characterizing metal-binding sites, through the use of methods such as X-ray absorption (XAS), which can be used to identify metals in solution through their characteristic electron binding energies. As early as 2005, high-throughput X-ray absorption spectroscopy (HTXAS) was being developed as a technology for investigating the metalloproteome [28]. More recently, High-throughput X-ray absorption spectroscopy was used to measure transition metal content based on quantitative detection of X-ray fluorescence signals for 3879 purified proteins from several hundred different protein families generated by the New York SGX Research Center for Structural Genomics [29]. The results of this study must be carefully interpreted – since all the proteins, regardless of organism of origin, were expressed in *E. coli*. Without doubt, only by examining proteins expressed in their native organism can one know whether any observed metal binding is physiologically relevant. And, a much smaller fraction, only roughly 10%, of the proteins were determined to bind metals – much smaller than estimates elsewhere of one third. Further, this technology is only particularly useful for purified protein samples of reasonable high metal concentration, since a significant amount of analyte is needed for XAS. Yet, this approach has some benefit, - thousands of samples were assessed and the presence of some metal in many of them lays the groundwork for much future, hypothesis-driven research.

Liquid Chromatography/MS –based approaches

Other metal-detecting spectroscopies have also been applied to liquid separations of proteins. For example, protein solutions separated by liquid chromatography can then be subjected to inductively mass-spectrometry (ICP-MS) for metal identification [30]. A particular advantage of this approach is that ICP-MS can detect isotopes, which allows pulse-chase labeling for the identification of newly-formed proteins. In one example of this work, periplasmic proteins from *Synechocystis* PCC 6803 were resolved by native two-dimensional liquid chromatography and analyzed for metals by ICP-MS [23]. This work led to the conclusion that the compartment where the protein folds can determine which metal is bound, at least in this organism. In another example, cytoplasmic metalloproteins from *Pyrococcus furiosus* were analyzed by liquid chromatography and ICP-MS to identify 343 metal peaks in chromatography fractions, 158 of which did not match any predicted metalloproteins [31]. Other work using variations on this approach have also been described [21,22,32]. In a variation on this approach, the ICP-MS can be performed on a two-dimensional solid separation, such as is generated by gel electrophoresis, using laser ablation vaporization of the sample followed by ICP-MS detection. Several examples of this work exist [26,27,33-35].

Unlike other detection approaches which may require a special facility, ICP-MS systems are commercially available, which is often an advantage. The isotope sensitivity, which enables pulse-chase labeling as described above may be another advantage. The detection method is also quite sensitive, and the way samples are introduced into an ICP-MS by pumped flow pairs very naturally with liquid chromatography. However, the trade-off is that metals are not detected simultaneously, requiring dedicated sample volume for each metal analyzed. Also, the detection method, namely the inductively coupled plasma used to ionize the sample, is destructive, which prohibits further analysis of the sample. Some arrangements split the flow of analyte, so that some parallel sample is preserved. However this, in turn, requires larger quantities of sample. The method is also sensitive to matrix effects as well, that can make some determinations more difficult than others. Couple this with the large datasets that are obtained as results and it can be very non-intuitive to understand and interpret the output. Still, particularly as ICP-MS technology itself improves, there is certainly much promise for this technique.

Electrophoresis/spectroscopy-based approaches

The metals in the same gel-electrophoresis-separation we just described can be detected in a different, direct way - via spectroscopy. As described earlier, XAS has long been used to identify metals. Using similar principles, X-ray fluorescence spectroscopy can be used to create images of electrophoresis separations. In one example of this work, a previously unknown zinc-binding protein was identified as being important in the homeostasis of this metal in *Pseudomonas aeruginosa* (Figure 2) [36]. It has also been used to show that, in the same organism, deletion of copper transporters results in up-regulation of the copper protein azurin [37]. Several other examples of the application of this approach exist [38-44].

This approach affords potentially higher detection sensitivity as compared to ICP-MS and it is possible to detect multiple metals simultaneously from the same sample volume. With

multiple metals detected simultaneously, it may be the detection method of choice for samples that are difficult to obtain. Because it is non-destructive, it can be followed by mass spectrometry to identify proteins, and it is possible to determine information about the oxidation state of the metal (through X-ray Absorption Near Edge Spectroscopy, XANES) [45]. On the other hand, this approach usually requires access to an x-ray synchrotron, making it a bit less accessible. And the electrophoresis format itself, which is particularly compatible with x-ray fluorescence detection because the samples are solid and flat, often requires large amounts of sample, and does not provide the best resolution. If the separations approach for this improves, it may be the method of choice for rare, or difficult to obtain, biomedical samples.

Electrophoresis/autoradiography approach

In another approach, researchers have used the radioactive isotopes of some metals to identify the proteins that bind them. This work was performed by first labeling the proteins with a short-lived isotope, separating them, detecting them by autoradiography, and then identifying the proteins by mass spectrometry. This approach has been used to work to identify the major copper-binding proteins in the periplasm of *E. coli*, and in work to identify the soluble iron and zinc proteins in the same organism [46,47]. An advantage of this technique, as with liquid chromatography/ICP-MS approaches, is that pulse-chase experiments to identify newly-formed protein are possible. Also, the images obtained in this approach are very intuitive to interpret. However, particularly for the study of higher organisms, such as human samples, it may be more of a concern to introduce the radioisotopes. Furthermore, unless one is situated next to a radioisotope production facility, these radioisotopes may be difficult to obtain. Because of the short half-life of some isotopes, and the relatively long electrophoresis separations, false negatives are possible, and should not be over-interpreted. Yet, the results so far continue to provide the basis for much hypothesis-driven work.

Expert commentary and five year view

It is increasingly possible to analyze samples where large quantities of material are available – bacterial cultures, yeast cultures. It remains much more challenging to analyze more limited samples, such as human tissues. Part of the challenge is limitations imposed by detection. Some of the factors that affect this include: a) not just the ultimate sensitivity of the technique, but quantity/volume of sample required, b) issues of relative abundance, which make it difficult to prepare a sample with sufficient analyte, and c) challenges in detecting multiple elements simultaneously. The other half of the challenge is limitations imposed by separation. Some of the considerations there include: a) do compatible separations technologies exist that provide for detection-compatible separation at very small scale, such as those required for studying very limited samples, including human tissue? b) Are the separations technologies developing likely to strip native metals from the proteins? The challenges imposed by limited sample availability may be partly addressed by combining nano-scale separations technologies with non-destructive detection methods, such as x-ray fluorescence. Also, the information volume of single cell metal imaging could be greatly expanded by integration with *in-situ* protein tagging and detection techniques. Co-

localization of subcellular metal distribution and protein expression would allow for a correlation of protein functions and intracellular metal trafficking. Technological breakthroughs such as this will unquestionably lead to many paradigm-shifting discoveries in an uncharted research field at the interface of metallochemistry and cell biology. Metalloproteomics is a rapidly evolving methodology, enabled by many recent technological advances. Many of the approaches have already increased our knowledge, and many have unique capabilities for providing special information (such as isotope pulse-chase by ICP-MS, or oxidative state by XANES). Challenges exist for this being routinely useful for rare or clinical samples (such as samples taken directly from humans). The potential payoff for technological progress is a better understanding of physiology.

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Key issues

- An intricate network of protein interactions ensures metals are in the right locations, at the right time, and in the right amount, for human health.
- Over the past few decades, imaging has become an increasingly powerful tool, and the direct approaches it enables have shed new light on the biology of metals
- There is an imminent need for tools that relate new images of metal-ion homeostasis to the proteins responsible for changing it.
- Some tools have emerged, combining separations techniques such as electrophoresis and liquid chromatography with detection techniques such as mass spectrometry, x-ray fluorescence, and autoradiography.
- It remains a challenge to work with biomedical samples, but the pay-off is great, and the field is rapidly evolving.

Figure 1.

Figure 2.