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Tools to study distinct metal pools in biology

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Current research interests in the field of metals in biology are very diverse, but are underpinned by a common need to study how stimuli affect metal homeostasis. This perspectives article argues for the need to combine information from multiple technical approaches to characterise the different metal pools inside living cells.

Introduction

The field of bioinorganic chemistry saw great advances in the latter half of last century, motivated by the development of technologies that enabled more thorough study of the interactions of metal ions with biological systems. Developments in chromatography and spectrometry facilitated the detection of trace elements;[1] protein crystallography permitted the identification of metals at the active site of enzymes;[2] protein engineering enabled separation of proteins and allowed study of the role of metal ions;[3] and advances in genome sequencing revealed new metalloproteins.[4] This led to a deep appreciation of the vast array of metal-containing proteins, and the roles of essential transition metals in biology.

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Over the past ten years, the field of metallomics has gained particular momentum,[5] with the development of many sophisticated techniques to study the speciation of metals, whether taken up naturally or administered pharmaceutically. Notably, research in the subfield of metalloproteomics has resulted in the more rapid discovery of metalloproteins,[6] while highly sensitive spectroscopic methods enable their more detailed study.[7]

The recent advances in metallomics technology have been accompanied by rapidly increasing knowledge about the interactions of metal ions with biological systems. As a result, the pertinent questions in bioinorganic chemistry are very different from those of ten years ago. It is timely, therefore, to re-examine the current issues of greatest interest and the tools we have at hand, to assess how best they can be used to further our understanding of metals in biology. This perspectives article considers current challenges in studying metals in biology and the tools we have to study them, arguing for the need to combine information from various techniques to gain greatest appreciation of the biological roles of metal ions.

New challenges in the study of transition metals in biology

While the importance of transition metals as enzyme cofactors is well-established, much remains to be learnt about their roles in health and disease, with research following a number of directions.

1. *Identifying new roles for transition metals.* It is accepted that iron levels are maintained at static levels within cells, and are perturbed only by long-term supplementation or starvation,[4a] with a possible exception in the signalling role of iron in macrophages.[4b] In contrast, recent studies of other transition metals have demonstrated mobilisation in response to specific biological stimuli. For example, there is a redistribution of copper upon depolarisation of hippocampal neurons,[8] while the release of zinc from intracellular stores is considered to be a signal, effecting physiological changes in tissue such as the pancreas[9] and prostate.[10] These results may indicate other, more dynamic roles for transition metals, rather than solely as

static cofactors. Further study of the potential signalling roles of metals requires techniques to study their movement within and between cells.

2. *Understanding the roles of new metalloproteins.* Recent years have seen advances in both proteomics and metallomics research that have enabled the identification of many new metalloproteins.[11] This must be matched by the development of clear and efficient methodologies to elucidate the roles of these newly-identified proteins in metal homeostasis. Essential to this is an understanding of the effect of genetically manipulated protein levels on metal distribution throughout the cell.

3. *A role for transition metals in disease.* Recent studies have indicated that misregulation of transition metals is related to disease genesis and progression. For example, increased levels of iron, copper and zinc ions in the brain are believed to play a role in the genesis of neurodegenerative diseases such as Alzheimer's, evidenced by observations of altered metal homeostasis in disease patients.[12] In order to better understand the potential role of metals in disease, it is important to be able to assess cellular metal status in health and disease.

4. *Manipulation of metal homeostasis.* Genetic modification of metal levels in bacteria or plants has a number of potential technological applications. For example, increased metal accumulation by plants is of interest in cleaning up metal toxicity in soils as well as in increasing the dietary availability of essential metals.[13] The activity of cyanobacterial hydrogenase is limited by poor availability of Ni(II) inside these cells, and increased intracellular Ni(II) levels are predicted to enhance H_2-generating ability.[14] Simple and reliable methods for measuring metal concentrations within cells are essential in studying the effects of genetic modification on intracellular metal levels.

Current research interests are therefore very broad, ranging from bacteria to humans; from studying new metalloproteins to investigating the effects of biological stimuli on metal speciation. Common to all these studies is the need to understand how the alteration of conditions, whether by knocking down a metalloprotein or activating a signalling pathway, can affect metal distribution within a cell. To better appreciate the roles of transition metals in biology, it is essential to develop methodologies that can provide an overview of metal homeostasis.

While only the essential transition metals, notably iron, copper, nickel and zinc, will be considered here, the considerations highlighted would equally apply to the study of non-essential or toxic metal ions in biology. This review also focuses primarily on techniques to study transition metals in single cells, rather than in tissue samples.

Pools of metals in biology

Transition metals play essential roles in proteins, whether as structural elements (*e.g.* zinc in zinc finger motifs), electron carriers (*e.g.* copper in cytochrome C oxidase), facilitators of oxygen transport (*e.g.* iron in haemoglobin) or as sites at which enzyme catalysis can take place (*e.g.* nickel in hydrogenase). Availability of these metals to newly-forming proteins is therefore essential for protein function and cellular activity. Cells have a complex array of proteins which maintain metal homeostasis and deliver metals to metalloproteins.[15] These include metal import and export proteins, metal storage proteins,[16] metal chaperones,[17] metal sensing proteins[18] and metal inserting enzymes[15] (Fig. 1).

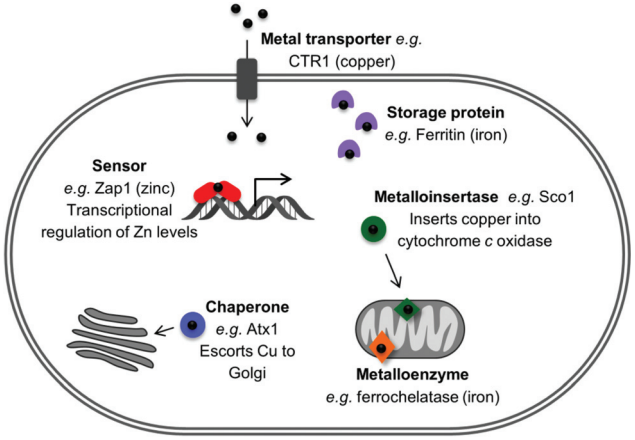

Fig. 1 A number of different protein types are involved in the trafficking and regulation of metal levels in the cell.

These proteins bind to metals with high but widely varying affinities, each passing the metal ion on to another protein with even higher binding affinity;[19] the metalloenzyme, the typical end-point of this exchange, has the highest metal-binding affinity. The necessary exchange of metal ions between different metalloprotein pathways, and the requirement for a supply of metal ions to nascent metalloproteins mean that in addition to the strongly-chelated metal in proteins, there must also be a labile pool of metal that is less tightly bound, and can be sequestered upon demand.[20] This pool, also called the bioavailable pool or the exchangeable pool, comprises not only free metal ions (which are negligible for metals such as copper)[21] but also metal ions bound relatively weakly to cytosolic ligands such as glutathione. The concentration of metal in this pool is regulated by metal-sensing proteins that enable cells to respond to chronic depletion or over-supply of metal.[18a] The total metal pool of the cell can therefore be considered as the sum of the protein-bound metal pool and the labile metal pool (Fig. 2).

Stimuli can therefore be expected to affect these pools in different ways: increased metallation of a protein might cause a complementary decrease in the labile metal pool without affecting the total metal content, for example; or a stimulus might cause an increase in all three pools. Without assessing the impact of stimuli on all three pools, it is impossible to gain a clear picture of metal changes in the cell. This perspectives article will consider current methods for studying each of these pools.

Requirements for measurement of metal content

Current research interests find a common basis in understanding the effects of an external signal (whether a genetic

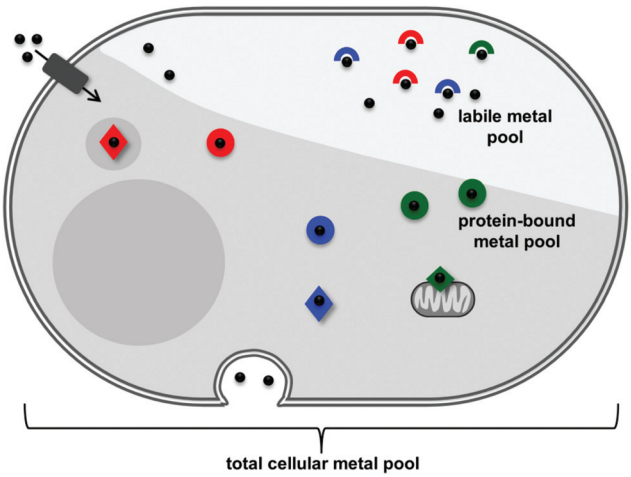

Fig. 2 The total metal content of the cell can be considered to comprise the pool of metals that are tightly bound to proteins, and the labile pool of bioavailable metal.

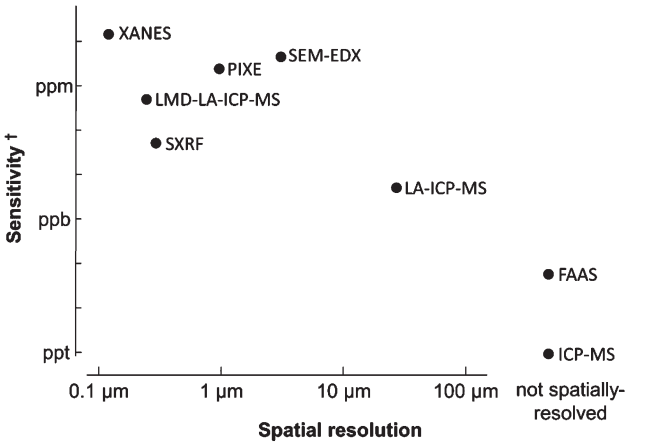

Fig. 3 Current techniques for measuring the total metal pool vary in their sensitivity and spatial resolution.[22] †Detection limits for a given technique will vary for different metals; values here represent approximate mean sensitivities.

manipulation or a biological stimulus) on metal levels. Techniques that study metal content must therefore be sensitive to changes in metal levels following the signalling event, but need only measure relative changes in metal pools rather than absolute metal concentrations. While this can obviate the need for laborious calibration, it is nevertheless important to perform rigorous controls, particularly in distinguishing baseline metal levels.

A further characteristic of current studies is the interest in observing the redistribution of metal pools within a cell, necessitating spatially-resolved techniques. For cellular studies, there must be sufficient resolution to discriminate between distinct sub-cellular regions, and this can be achieved by microscopy techniques. While not all techniques discussed below enable derivation of spatial data, this is nonetheless an important consideration. Some relative localisation information can also be gained by other techniques, such as sub-cellular fractionation prior to analysis.

Total metal content

Measurements of total metal content have formed the basis of bioinorganic studies for more than half a century, particularly through absorption spectroscopy and mass spectrometry. There have been vast improvements in these methods, as well as the development of new techniques for the study of total metal content. While studies of the total metal pool have traditionally been hindered by low sensitivity and spatial resolution, current technology has more than met these challenges (Fig. 3).

The total metal pool can be defined as all metal ions within the cell, regardless of oxidation state or coordination environment. Quantification of the total metal pool is therefore of particular value in reporting movement of metal into or out of a cell in response to a stimulus.

Traditional techniques for the measurement of total metal content, such as flame atomic absorption spectroscopy (FAAS) or inductively-coupled plasma mass spectrometry (ICP-MS), require the digestion of cells prior to analysis, and can therefore report only on total changes in intracellular metal concentration for a population of cells, without sub-cellular spatial information. Amongst these techniques, ICP-MS, in particular, has advanced to the stage where it can now detect concentrations below 1 ppt for most elements.[23] For a less abundant transition metal such as Cu, which is found in total concentrations of approximately 3×10^5 atoms per cell,[21] the use of one million cells per experiment will give final copper concentrations of 10 ppt for standard ICP-MS experiments, sufficiently above detection limits, to enable meaningful studies of basal and even depleted metal levels. Despite their lack of spatial resolution, ICP-MS and FAAS have proved valuable in the study of various systems such as the effect of the herpes simplex virus on intracellular metal levels[24] and the distribution of copper in trout hepatocytes.[25]

Recently, laser ablation ICP-MS (LA-ICP-MS) has emerged as a technique to measure metal concentrations with some degree of spatial discrimination. In this method, ionisation is achieved by laser irradiation. By controlling the region that is ablated, measurements of metal concentrations can be made for very specific regions of a tissue sample. While LA-ICP-MS is currently limited to resolutions above 50 μm and therefore cannot be used for studies on a single cell, it has enabled investigations of metal distribution in small tissue sections such as Alzheimer's plaques,[26] mouse spinal cord,[27] mouse cerebrum and brain stem[28] and mouse substantia nigra.[29] Recent developments have seen the achievement of 10 μm resolution by coupling of a laser microdissection (LMD) system to LA-ICP-MS,[30] while the use of a near-field set-up, working at distances smaller than the wavelengths involved, enables less than 200 nm resolution.[31] Spatially-resolved ICP-MS techniques therefore have great potential for cellular studies of the total metal pool.

Other techniques for studying metal distribution with spatial resolution have been helpfully reviewed elsewhere,[22]

and include scanning electron microscope–energy-dispersive X-ray spectroscopy (SEM–EDX), in which each element generates a unique X-ray emission pattern upon interaction with electromagnetic radiation[32] and particle-induced X-ray emission spectrometry (PIXE), which measures the X-ray fluorescence resulting from the filling of inner shell vacancies created by photon bombardment.[33]

Spatially-resolved information about the metal pool has perhaps been most effectively collected to date by synchrotron X-ray fluorescence (SXRF) microscopy, in which the fluorescence of an atom following synchrotron-induced X-ray irradiation can be calibrated against a standard to calculate its concentration.[34] An array of metals can be measured concurrently in this manner, with spatial resolutions of 0.1 μm.[22a] While SXRF is unable to distinguish between oxidation states or coordination environments, such information can be gained from other synchrotron X-ray techniques[35] such as extended X-ray absorption fine structure (EXAFS), which can provide information on neighbouring atoms.[36] Measurement of the incident X-ray energy across the absorption edge (XANES) enables determination of oxidation state,[37] and has been used to successfully study copper oxidation state in cells.[38]

Labile metal pool

The labile metal pool comprises cytosolic metal ions that are accessible to chelators. Accordingly, the metal must be in ionic form, or bound relatively weakly. The labile pool is believed to be maintained at homeostasis by metal-sensing proteins that can induce the movement of metals into the cell, and from intracellular stores.[18a,b]

Since it was first identified in 1946,[39] there has been sustained interest in studying the labile iron pool, and recent interest in studying the labile pool of other metals has arisen from various angles, including the putative role of transition metals as transient signals. In contrast to the total metal pool, studies of the labile metal pool must take into account the oxidation state and coordination environment. While the absolute concentration of metal in the labile pool is difficult to measure, relative changes provide valuable information.

Requirements for a sensor of the labile metal pool

A good sensor of labile metal concentrations must exhibit:

1. High selectivity for the metal of interest.[40]

2. Metal binding must result in a measurable change in the fluorescence spectrum of the probe that can be readily detected by common spectroscopic techniques.

3. Metal binding affinity that is appropriate for the study of the buffered metal pool. An affinity that is too high will result in the probe sequestering metal from proteins, while a probe with too low an affinity will not show any spectroscopic changes at physiological metal concentrations. Perhaps the best indication of suitable affinities may be gleaned from the metal-binding affinities of metal sensing proteins, which act as intracellular probes of the same pool.[41] Sensors for iron, which is the most abundant transition metal in the cell and is weakly bound in the cytoplasm, have affinities[42] between 10^5 and 10^7 M^{-1}; zinc, with relatively high concentrations but stronger binding is sensed with affinities[43,44] of 10^{12} to 10^{15} M^{-1}, and the less-abundant and more strongly bound copper,[45,46] with affinities of 10^{18} to 10^{21} M^{-1}. These concentrations therefore provide approximate binding affinities that should be sought in the development of new probes for the labile metal pool. The development of sets of probes with varying metal-binding affinities to further enhance information about the labile metal pool, and its bioavailability is a particularly valuable goal for future research.[47]

4. Reversibility of binding is often desired, as it will enable the probe to report on cycles of metal binding, and to monitor dynamic changes in metal distribution over time.

5. Cellular compatibility of the probe requires that it is sufficiently taken up by the cell, is targeted to the region of interest, minimally perturbs cellular homeostasis, and does not cross-react with other sub-cellular species. The localisation of the probe must also be carefully considered – it is only possible to draw conclusions about labile metal concentrations in compartments to which the sensor localises.

Measuring the labile iron pool

Due to sustained interest in the labile iron pool, many techniques have arisen for its study. These have been comprehensibly reviewed.[48] Early methods involved the tracking of radio-labelled iron in the form of transferrin,[39] complexation of iron with desferrioxamine and detection by electron paramagnetic resonance (EPR) spectroscopy,[49] and fractionation of low molecular weight cytosolic species and identification of iron-containing complexes.[50]

A need to study the labile iron pool non-destructively led to the development of fluorescence techniques for the purpose. The most common protocol uses calcein-AM, the membrane-permeable and non-fluorescent acetoxymethyl ester of calcein, a fluorescein derivative.[51] Calcein AM is hydrolysed by intracellular esterases to calcein, which binds to iron in the labile pool. The resultant fluorescence quenching is used to calculate overall iron concentration (Fig. 4). Calcein AM and various analogues[51,52] continue to be the method of choice for many studies of the labile iron pool (Fig. 5), but they are relatively insensitive to oxidation state, and require the subsequent addition of a chelator for quantification, precluding use in real-time imaging of live cells to study dynamic changes in the labile iron pool. Furthermore, this technique cannot be generalised to the study of any other metal ion, as the poor selectivity of calcein must be offset by the enormous excess of iron over other metal ions in the cytosol.

A number of new fluorescent probes have been recently developed to enable more sophisticated live cell imaging of the labile iron pool, including some which are selective for either Fe(II) or Fe(III) over other metal ions, and which can report concentrations of labile iron in living cells (Fig. 5).[52,53] These probes have been developed with similar strategies to fluorescent sensors for other metal ions, discussed below.

Measuring the labile pool of other metal ions

The past decade has seen extensive research into the development of sensors for the labile pool of other metal ions. Since these ions are less abundant than iron, selectivity is of much greater concern. The broad range of available probes, primarily for Zn(II), but also for other transition metals ions, have been extensively reviewed elsewhere.[54] A particularly helpful recent review by Palmer *et al.* provides a detailed discussion of fluorescent sensors for the labile metal pool.[9] For the sake of this discussion, we can divide sensors into several general classes.

A number of fluorescent proteins have been developed that can report on the presence of metal ions.[55] These proteins are generally based on naturally-occurring subunits, and signal the presence of metal by a FRET change (Fig. 6). An alternative strategy, which has found success for Ca^{2+} sensing, involves the use of a circularly-permuted fluorescent protein for which fluorescence is restored by metal binding.[56] This strategy is likely also to have success for the study of transition metal ions. Although genetically-encoded metal sensors are limited by their poor dynamic range, metal-binding affinity can be readily modified, a property that has found great utility in the measurement of the concentration of the labile copper pool in yeast.[47b]

By far the largest group of sensors of the labile metal pool is the small molecule fluorescent sensors.[54] Generally containing a ligand set and a fluorophore whose emission is modulated by metal-binding, these sensors have found extensive application in biological studies. These simple, turn-on sensors are limited by their potential for background interference or hypersensitivity to probe environment and their relatively short wavelengths of excitation and emission, and consequently much current research is focused on new classes of probes that address these issues (Fig. 7). Ratiometric probes report on metal concentration by a change in the ratio of two emission peaks, independent of probe concentration, and are therefore particularly useful for the measurement of mobile metal pools.[57] Metal sensors based on luminescent lanthanoids can also serve as ratiometric probes, and lend the further advantage of long luminescence lifetimes for use in time-gated imaging, which can eliminate interference from cellular autofluorescence.[58] The limitations of short excitation wavelengths can be overcome by the use of two-photon probes, which enable excitation with two photons of half the energy,[59] or by employing fluorophores that emit in the near-infrared.[60] The latter are particularly attractive for their potential use in whole animal studies. While these various strategies have yielded a broad array of valuable probes, sensors for

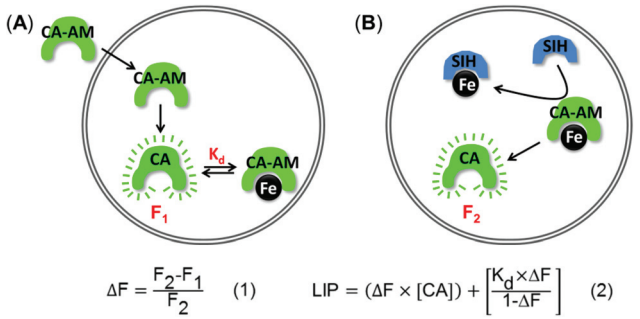

Fig. 4 The calcein method for measuring the labile metal pool. (A) Cells are treated with the acetomethoxy derivative of calcein (CA-AM), which is non-fluorescent and membrane-permeable. In the cell, CA-AM is hydrolysed to calcein (CA), which is highly fluorescent but is quenched upon the binding of iron. The fluorescence from unbound CA, F_1, is measured. (B) The membrane-permeant iron chelator, salicylaldehyde isonicotinoylhydrazone (SIH) is added to the cells, which binds to iron and releases fluorescent CA. The fluorescence from CA, F_2, is measured, and can be calibrated to measure total intracellular [CA]. The labile iron pool (LIP) can be calculated by eqn (1) and (2). Adapted from Kakhlon *et al.*[48a]

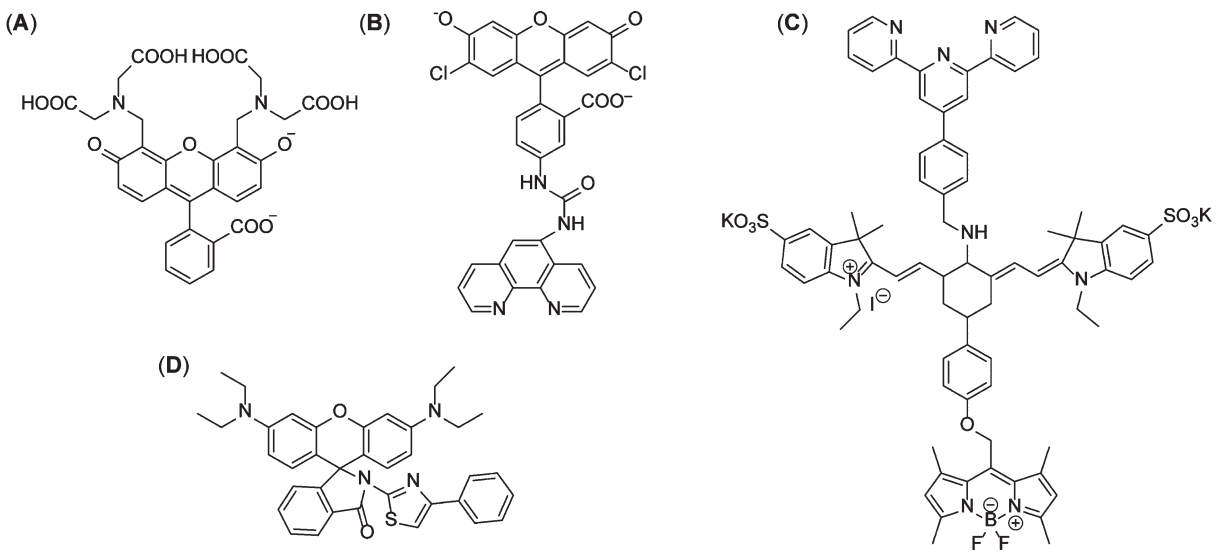

Fig. 5 A number of fluorescent sensors have been developed to study the labile iron pool. (A) The most commonly-used fluorescein derivative, calcein, (B) an early commercial alternative, Phen Green SK, and more recent selective probes, (C) Fe(II)-sensor BDP-Cy-Tpy[53d] and (D) Fe(III)-sensor S1.[53e]

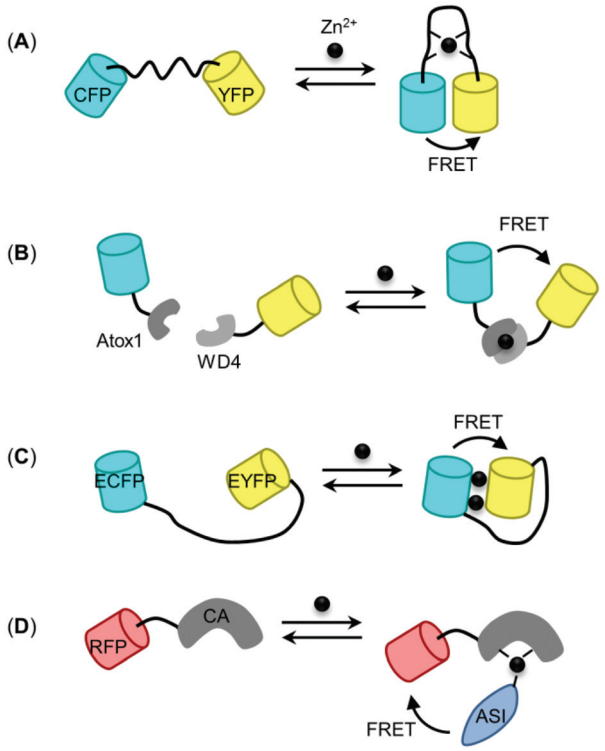

Fig. 6 Genetically-encoded fluorescent sensors have been developed that signal the presence of Zn(II) by various FRET-based mechanisms: (A) a Zn(II)-binding site in the linker region brings together two fluorescent proteins;[55a] (B) incorporation of a pair of copper-binding proteins enables coupling of two fluorescent proteins upon Zn(II)-binding;[55b] (C) Zn(II) binding sites were incorporated on the surfaces of the fluorescent proteins[55c] and (D) fluorescent aryl sulfonamide inhibitors selectively bind to zinc-bound carbonic anhydrase.[55d] Abbreviations: CFP = cyan fluorescent protein; YFP = yellow fluorescent protein; ECFP = enhanced cyan fluorescent protein; EYFP = enhanced yellow fluorescent protein; RFP = red fluorescent protein; CA = carbonic anhydrase; ASI = aryl sulphonamide inhibitor.

paramagnetic metal ions such as Cu(II), high spin Fe(II), Fe(III) and Mn(II) remain a challenge as they tend to quench the fluorescence of nearby fluorophores.

To overcome these limitations, a recent solution has been the use of reaction-based sensors, demonstrated by the development of sensors for Co(II)[63] and Fe(III).[65] These probes, which rely on a metal-catalysed cleavage reaction to yield a fluorescent product, are irreversible, and therefore cannot be used for the study of dynamic changes in living cells. Future development of these probes, which provide an elegant alternative to traditional fluorescent probes, will require rigorous testing to distinguish whether they are reporting on the same metal pool as chelation-based probes, or whether it is only unchelated metal ions that are able to undergo reactions.

While the overwhelming majority of fluorescent studies of the labile metal pool measure relative changes in metal content under stimulation or in disease, there is also interest in absolute quantification of this pool. Krężel and Maret report the use of the FluoZin zinc sensor to measure the labile zinc pool in adenocarcinoma cells.[10] Recognising the interaction of the probe itself with the labile pool, they make measurements at various probe concentrations and extrapolate to a zero probe concentration to quantify zinc in cells under various conditions. Despite the merits of such an approach, the most robust method to measure concentrations of the labile pool is the use of a ratiometric probe, as discussed earlier.

The examples discussed to this point have been of fluorescence or luminescence-based sensors, but there is interest in measuring the labile metal pool using other imaging modalities, most notably magnetic resonance (MR) imaging. Responsive MR metal sensors exhibit divergent abilities to relax the nuclei of water molecules in the presence and absence of metal, and have been developed for a range of metal ions.[66] MR techniques enjoy exceptional spatial resolution and can be used for whole animal imaging, but are limited by their poor sensitivity and the high concentrations of probe required.

Protein-bound metal pool

The protein-bound metal pool refers to all metal that is tightly chelated to proteins, whether in metalloenzymes, metal sensing proteins, metal chaperones or metal transport proteins. In general, studies tend to focus on an individual metal-binding protein, or a group of inter-related proteins, rather than the protein-bound metal pool as a whole. Various sophisticated techniques have been developed for studying changes in metallation of proteins.[7b] Such methods typically involve separation of proteins by gel electrophoresis, capillary electrophoresis or liquid chromatography followed by spectrometric analysis, whether by ICP-MS,[67] MS/MS[68] or X-ray techniques[69] (Fig. 8). The requirement for these techniques is that they resolve the apoprotein from the holoprotein protein, enabling measurement of relative levels of metallation. The necessary separation of proteins by chromatography or electrophoresis prior to analysis may not proceed with retention of the metal ion, and will result in a loss of spatial resolution. Immunostaining with antibodies for metalloproteins can provide this information, but it is essential to note that antibodies not be sensitive to the metallation state of the protein, and therefore localisation changes must be interpreted with caution.

These various biochemical techniques do allow for the simultaneous study of a number of metal-binding proteins, but can only be used for known proteins, as they require matching to a known protein mass. There appears to be no reported method for studying changes to the metal-bound protein pool as a whole; the most straight-forward solution would be to subtract the labile metal from the total-metal pool, but this is not practicable as absolute concentrations (particularly of the labile metal pool) cannot be measured. But perhaps such analysis is not necessary – the discussion explores the proposition that current techniques of studying individual metalloproteins in conjunction with measurement of the labile and total metal pools can be profitably used to increase understanding of the interactions of metal ions with biological systems.

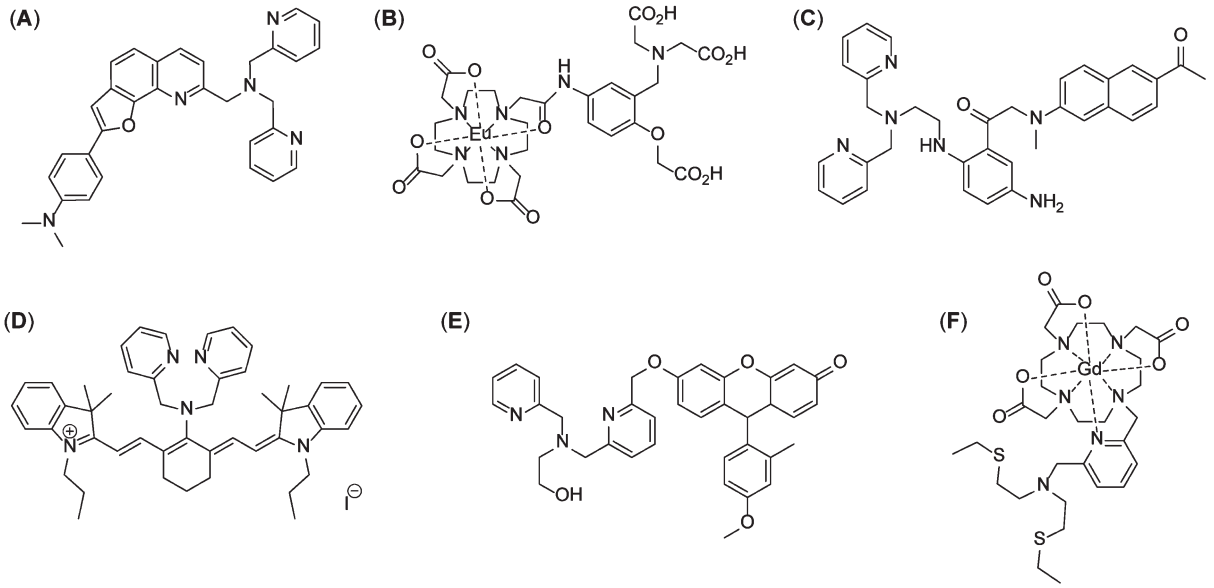

Fig. 7 A range of new types of sensors have emerged to complement the extensive field of turn-on fluorescent probes, with representative structures shown here. (A) FQ1, a ratiometric fluorescent Zn^{2+} probe,[57d] (B) EuL^2, a luminescent lanthanoid Zn^{2+} probe,[58a] (C) AZn1, a two-photon Zn^{2+} probe,[61] (D) DPA-Cy, a near-IR Zn^{2+} probe,[62] (E) CP1, a reaction-based Co^{2+} probe[63] and (F) CG2, an MR-based Cu^{+} sensor.[64]

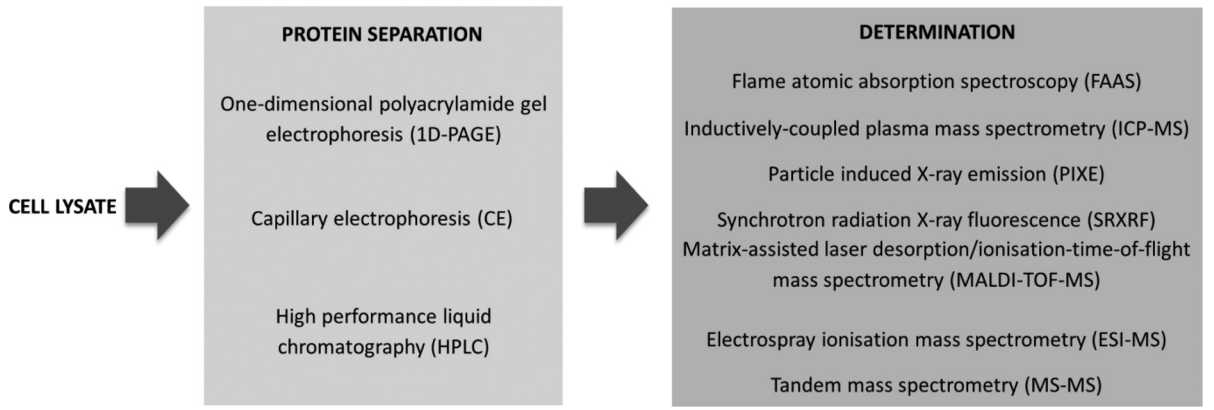

Fig. 8 Experimental approaches to studying metalloproteins. Mixtures of proteins can be separated by various methods and analysed by spectrometric techniques.

Combining the information from multiple techniques

Research in the past decade has seen the development of sophisticated techniques to study each of the three cellular pools of metal, so it is important to consider what these methods can produce in concert. Studies of the total metal content report on changes in overall metal efflux or influx. Measurement of the labile metal pool indicates how much metal is immediately available for the cell to use, with long-term changes in metal levels suggesting a shift in the homeostasis normally controlled by metal sensing proteins. Changes to the protein-bound metal pool, on the other hand, can be indicative of changed transcription of metal-binding proteins, or of an altered ability of a protein to acquire the necessary metal; in cases where there is a low labile metal concentration, for example, metal export proteins are likely to exist in apo form while metal import proteins are more likely to be metallated. The three pools therefore provide distinct, yet complementary, information regarding metal homeostasis, and concurrent measurement of each pool will maximise the conclusions which can be drawn. These advantages are demonstrated in the emerging studies which have examined each cellular metal pool.

Studies of iron in biology have traditionally measured all three pools concurrently, with recent examples being the study of liver iron levels in ischaemia/reperfusion,[70] altered iron metabolism in glomerular endothelial cells following treatment with angiotensin II[71] and irradiation-induced iron changes in rat brain embryos.[72] This methodology has enabled the development of a clear understanding of the roles and regulation of iron in biology. In the case of the other transition metals, such studies are much less common, and it is more

usual to find examples where just two of these pools are studied in conjunction. Much can therefore be learnt from the study of iron for application to other transition metal ions.

A number of studies of other essential transition metals have measured total metal content by ICP-MS concurrently with quantification of emission output from fluorescent probes.[63,73] This can be valuable in validating that new probes are able to measure real changes in metal levels, but it is important to remember that the two techniques are measuring distinct metal pools. This combination of techniques has proven to be powerful when spatially-resolved information about the total metal pool is gained: a novel fluorescent sensor for the labile Cu(I) pool was used in conjunction with a X-ray fluorescence microscopy to reveal that neuronal copper pools were redistributed upon depolarisation of the cells, a valuable piece of evidence for copper's putative signalling role.[8] In contrast, combined studies of the total metal content and the protein-bound metal pool are relatively more common,[74] and are principally concerned with correlating total metal levels with protein function or extent of metallation.

There are far fewer studies of non-ferrous transition metals that investigate all three cellular pools, but the wealth of results that they yield is evidence of the value of such a methodology, and two notable recent examples are discussed here. In a study of the importance of mitochondrial zinc, Atkinson *et al.*,[75] report the concurrent use of a mitochondrially-targeted Zn(II) sensor, RhodZin-3 dipotassium salt to study the labile zinc pool, inductively-coupled plasma-optical emission spectrometry (ICP-OES) and atomic absorption spectroscopy (AAS) to study the total metal content, and Western blot and blue native PAGE protein analysis, which led to the discovery of a labile mitochondrial pool of Zn(II) that is important for respiration, and the identification of a protein that controls the levels of this pool. In another study of mitochondrial metal pools, Dodani *et al.*[76] used MitoCS1, a novel Cu(I) probe that localises to the mitochondria and ICP-OES analysis of whole cell and mitochondrial copper levels in conjunction with studies of the copper proteins, SCO1, SCO2 and COX, in order to understand how copper homeostasis is perturbed in patients bearing mutations in SCO1 and SCO2. These studies highlight the wealth of information that can be gained from investigating the three metal pools within a single system, and certainly provide impetus for future studies of a similar type.

Conclusions

The last decade has therefore seen the advancement in technologies to study each of the cellular metal pools, to the extent that highly sophisticated information can now be derived about distribution and localisation within a single cell. Particularly valuable future improvements lie in the development of sensors of the labile metal pool for paramagnetic metal ions, and in spatially-resolved techniques for studying the protein-bound metal pool. Future improvements in the design of sensors of the labile metal pool for paramagnetic metal ions, and in spatially-resolved techniques for studying the protein-bound metal pool would be particularly valuable.

The emerging studies which simultaneously investigate all three metal pools demonstrate the power of such a methodology, and provide compelling evidence for the adoption of multiple techniques in future studies. Employing a strategy of studying total, labile and protein-bound metal pools is likely to have a significant impact on the information that can be derived about metal ions in biology; far greater than can be achieved by incremental improvements to the investigation of a single pool. A particularly important consequence of studying the three metal pools is that enables the identification of unexpected metal perturbation. Where a stimulus might traditionally be understood to decrease the total metal pool, for example, an unexpected increase of the labile metal pool would herald new interactions between the metal ion and cellular systems.

These tools enable us to study how signals, whether physiological or pathological, can affect transition metal homeostasis. Equally important, although beyond the scope of this review, will be studies of the new roles that metal ions themselves might play on physiological processes. The combination of such studies will see greater appreciation of the interactions of metal ions with biological processes.

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