Metallomics as a Junction between Life Sciences

Yaşam Bilimleri Arasında Bir Bağlantı Noktası Olarak Metallomik

Review Article / Derleme

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ABSTRACT

Metallomics is a rapidly growing research area investigating the interaction of metals with biological molecules (e.g. DNA, proteins and metabolites) in living systems. It aims the understanding of all metal dependent metabolic processes such as uptake, transport, storage and excretion at molecular level. In order to access the qualitative and quantitative information of metals, which mostly occur at trace amounts and in the form of non-covalent complexes with biological ligands in a highly complex biological matrix, and to elucidate the metal-dependent life processes, metallomics utilizes the state of the art analytical and spectroscopic techniques. The mostly used approach for the analysis of metal complexes by preserving native metal species is the hyphenation of a chromatographic or an electrophoretic technique for high resolution separation with an elemental (e.g., ICP-MS) or molecular (e.g., ESI-MS or MALDI-MS) spectrometry technique for detection and/or identification. X-ray absorption and X-ray fluorescence spectrometry and in-silico approaches with bioinformatics are among other main techniques/methodologies contributing the research activities in metallomics. This study highlights the basic terms, primarily used analytical approaches, state-of-the art instrumental techniques and very representative recent applications in the field.

Key Words
Metallomics, hyphenated techniques, metalloproteomics, metallometabolomics.

ÖZET

Metallomik, canlılarda metallerin biyolojik moleküllerle (örn.,DNA, proteinler ve metabolitler) etkileşimini inceleyen ve hızla gelişen bir araştırma alanıdır. Metallerin biyolojik sistemde geçirdiği tüm metabolik süreçlerin (alım, taşınma, depolanma ve atılım gibi) moleküler düzeyde anlaşılmasını hedefler. Metaller, biyolojik sistemlerde eser düzeylerde, genellikle kovalent olmayan koordinasyon kompleksleri halinde ve oldukça karmaşık bir biyolojik çevrede bulunur. Bu nedenle metallomik, metallere ilişkin nitel ve nicel bilgi edinilmesi ve metal-bağımlı yaşamsal süreçlerin aydınlatılması amacıyla, ileri analitik ve spektroskopik tekniklerden yararlanır. Metalik türlerin fizyolojik ortamdaki doğal formunu korumak suretiyle metal komplekslerinin analizi için en yaygın yaklaşım, Hibrit Tekniklerin kullanılmasıdır; bu sistemde, bileşenlerin yüksek çözünürlükte ayrımı için kromatografik veya elektroforetik bir teknik ile tayin ve/veya tanımlama için element (örn., ICP-MS) veya moleküle (örn., ESI-MS veya MALDI-MS) özgül bir spektrometrik teknik birlikte kullanılmaktadır. X-ışınları absorpsiyon ve X-ışınları floresans spektroskopisi teknikleri ile bilgisayar-destekli teknolojileri içeren biyoinformatik yaklaşımlar da metallomik alanındaki araştırmalara katkı sağlayan diğer araçlar arasındadır. Bu çalışmada alanla ilgili temel kavramlar, kullanılan başlıca analitik yaklaşımlar ve ileri aletsel teknikler ele alınmış ve yakın zamanda yapılmış çalışmalardan örnekler seçilerek derlenmiştir.

Anahtar Kelimeler
Metallomik, hibrit teknikler, metalloproteomik, metallometabolomik.

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Abbreviations

2-DE	Two dimensional gel electrophoresis;
AAS	Atomic Absorption Spectrometry;
AES	Atomic Emission Spectrometry;
CE	Capillary electrophoresis;
CID	Collision induced dissociation;
DNA	Deoxyribonucleic acid;
ESI	Electrospray ionization;
EXAFS	Extended X-ray absorption fine structure;
FT-ICR	Fourier transform-ion cyclotron resonance;
ICP	Inductively coupled plasma;
INAA	Instrumental neutron activation analysis;
IT	Ion trap;
LMWCr	Low molecular weight chromium binding substance;
MALDI	Matrix-assisted laser desorption/ionization;
MS	Mass spectrometry;
MT	Metallothionein;
oMALDI	Orthogonal MALDI;
PC	Phytochelatin;
PIXE	Particle induced X-ray emission;
PSD	Post source decay;
PTM	Post-translational modification;
SCID	Source collision induced dissociation;
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis;
SEC	Size-exclusion chromatography;
SR-XRF	Synchrotron radiation X-ray fluorescence;
SIMS	Secondary ion mass spectrometry;
XAS	X-ray absorption spectrometry;
XANES	X-ray absorption near-edge structure.

INTRODUCTION

Tremendous advancements have been realized in the life sciences in our age. With the genome sequencing projects of a variety of organisms including human, research in life sciences entered a new period and a series of -omics has emerged dedicated to the studies of a particular set of components (Box 1). As systems biology has supplanted the reductionist approach, the central dogma of molecular biology has been re-evaluated to a new paradigm in which the genome gives rise to the transcriptome which is then translated to produce the proteome. While the genome is accepted as a static information resource with a defined gene content which remains the same (with few exceptions) regardless of cell type or environmental conditions; both transcriptome and proteome are dynamic entities which change in response to external and internal events [1]. Although data at the preceding level is informative, it is never adequate enough to entirely explain the next level; therefore must it be examined to complete the whole picture. For instance, the post-translational modifications (PTMs) through the addition of covalent modifiers or through proteolytic cleavage affect the activity, binding interactions, turnover or localization of many proteins [2].

The proteins that can be potentially expressed in an organism and their amino acid sequences could be predicted by the knowledge of entire genome DNA sequence. Therefore, the knowledge acquired by genomics has become a treasure for the area of proteomics to develop however the actual advancement has come through the invention and use of soft ionization techniques (MALDI and ESI) in mass spectrometry [3].

The life processes are maintained by not only organic molecules but also inorganic constituents [9]. Metalloproteins are one of the subclasses of the proteins [10] and differs from metal binding proteins. The discrimination is attributed to the case that metal provides a function to the protein (metalloprotein) rather than the strength of non-covalent metal bond which is stable enough to survive in sample preparation, separation or identification steps [11]. Whereas in metal binding proteins, the binding of metals to protein is nonspecific and does not provide any specific function to the protein. It is believed that one third of the all proteins require a metal cofactor for their structural, catalytic or regulatory functions. Metalloproteomics is a proteomics derived area exploring the metals associated to a protein ligand [10,12]. Beside of proteins, metal associated metabolites and other non-protein metal containing molecules should be included into analytical targets and the interaction of these constituents with their biota should be necessarily investigated for a clear understanding of the metal dependent life processes.

The metallome term was first coined by Williams [13] and then metallomics by Haraguchi [14,15]. More current and extended definition of the metallome is "the entirety of metal or metalloid species present in a cell or tissue type, their identity, quantity and location" by Mounicou et al. [16], and metallomics "the study of the metallome, interactions, and functional connections of metal ions and other metal species with genes, proteins, metabolites, and other biomolecules in biological systems" in IUPAC technical report [8] by Lobinski et al.

Box 1. Definition of some terms related to "omics".

Term	Definition
-omics	Suffix in biological terms: "Large-scale study of" biological entities such as DNA, RNA, protein or other molecular complement of cells, tissues or organisms [4].
Genomics	The study of all of the nucleotide sequences, including structural genes, regulatory sequences, and noncoding DNA segments, in the chromosomes of an organism [5].
Transcriptomics	Deals with transcriptome which represents the full set or a specific subset of messenger RNA (mRNA) molecules produced by a given cell under a specific condition [6].
Proteomics	The study of proteome which is the protein complement of a given cell (tissue or organism) including the set of all protein isoforms and protein modifications under a defined set of conditions [1,2].
Metabolomics	Deals with metabolome which is described as the qualitative and quantitative collection of all low molecular weight molecules (metabolites) present in a cell that are participants in general metabolic reactions and that are required for the maintenance, growth and normal function of a cell [7].
Metallome	Entirety of metal- and metalloid species present in a biological system, defined as to their identity and/or quantity [8].
Metallomics	The study of the metallome, interactions, and functional connections of metal ions and other metal species with genes, proteins, metabolites, and other biomolecules in biological systems [8].
Ionomics	Based on the building up of a large collection of mutants (differing by deletion of a particular gene) of a model organism, analyzing them for a considerable number of elements and linking the set of element concentrations with DNA sequences in order to detect metal-regulated genes [8].

Several other -omics (e.g. Degradomics, Epigenomics, Glycomics, Interactomics, Lipidomics, Microbiomics, Peptidomics, Steroidomics) are not described in detail since they are not within the scope of the present review.

The objective of this study is to provide a brief overview of the current analytical methodologies and very representative and recent applications of metallomics.

Analytical Methodologies and Instrumentation in Metallomics

Living organisms use strictly controlled systems to sense and uptake metals from their biota and further transport, incorporate into specific targets, store and excrete. The understanding of biological systems requires the screening of the metallic species through the metabolic processes. These can be fulfilled by in vivo, in vitro and even in silico approaches and require selective and sensitive analytical and instrumental methods. Most of the metabolic processes are maintained by proteins however the study of metallome covers the non-protein biomolecules as well. While investigation of metalloproteome utilizes the relevant genome and protein databases, metallometabolome search requires the de novo identification methods thus MS techniques with high accuracy and resolution. The instrumental techniques primarily used for the reliable detection and identification of metals, metalloids and their species in biological samples are given below.

Elemental Analysis Techniques

Reliable determination of elemental composition of a living organism, organ or tissue even in a cell and cell compartment is an important and challenging task. Diverse of species selective methods, which use of radioactive (autoradiography, INAA), atomic spectroscopic (AAS and AES), X-ray spectrometric (XAS, XRF) and atomic mass spectrometric (ICP-MS) techniques, are utilized for this purpose. However, the need of radioisotopes in autoradiography, the relatively high cost and the radioactive effect to the specimens requiring handling and disposal procedures in INAA, single element capacity of AAS and relatively low sensitivity of AES and X-ray techniques limit the both qualitative and quantitative analysis of metals which are present at trace/ultra trace amounts and occur in a highly complex matrix in biological samples.

Advances in plasma sources as an atomization and ionization source and application to mass

spectrometry have made the ICP-MS the method of choice. The technique provides a robust, selective, sensitive and multi-elemental analysis of metal (e.g., Cu, Zn, Fe, Mo), metalloid (e.g., Se) and some non-metals (e.g., S, P, I) in biosamples. ICP-MS can be coupled to a chromatographic or an electrophoretic system at on-line or off-line manner [17], hence gives information of abundance, number and stoichiometry of heteroatoms associated to the biomolecules and increasingly applied for quantitative proteomics as well. On the other side, ICP-MS analysis suffers from a variety of interferences, mainly arising from polyatomic species and the isotopes of other species having a near mass to charge ratio. Relatively high price and the need of experienced analyst capable of troubleshooting and discriminating the sources of the signal from analyte and other sources are among the limitations of the technique.

If the aim is planar imaging of a solid surface by quantitatively mapping the distribution of elements, micro analytical techniques such as synchrotron radiation X-ray fluorescence (SR-XRF), proton-induced X-ray emission (PIXE), secondary-ion mass spectrometry (SIMS) and laser ablation-inductively coupled plasma mass spectrometry (LA-ICP-MS) are primarily used. With the aid of SIMS, nano synchrotron XRF or LA-ICP-MS techniques very high resolution of the surfaces can be obtained because of excellent focusing preferences of the applied energy (X-ray, ion beam or laser, respectively) in a very narrow area on the target [18].

Synchrotron radiation XAS techniques (comprising XANES and EXAFS) have been applied for structural characterization of metalloproteins [19-22]. These techniques serve valuable information on the oxidation state of metal, molecular symmetry, coordinated atom of ligand, number of ligands and distance between metal and ligands [23] and is especially useful when the kinetically labile complexes are intended to directly analyze without handling which can/may result to the changes in analytes identity [24]. The X-ray absorption phenomena involves the absorption of a photon (synchrotron radiated X-ray) to excite a core electron to an empty or partially filled orbital of the absorbing atom or into the continuum, in what is called an absorption edge [23]. During X-ray absorption phenomena, fluorescent X-ray is also emitted (SR-XRF) because an electron from a higher-energy orbital fills the hole in the core shell. As the use of fluorescent X-ray for detection of elements is more sensitive than X-ray absorbance and the distinct fluorescence energy is characteristic for a given element, simultaneous and multielement mapping of elements in cells and tissues [25] as well as directly on electrophoresis gels [22] is possible.

When the sample was shot by a proton beam instead of X-ray, the method is named PIXE. Similarly, in the principle of SIMS, the material is bombarded with a high energy ion beam (1-30 keV) and the ejected or spattered ions (secondary ions) are analyzed according to mass to charge ratio allowing the information on samples elemental composition. ICP-MS equipped with a laser ablation unit can be directly applied to the analysis of the solid samples such as thin slices of organs [26,27], and plant tissues [28] for elemental mapping and protein spots in GE for spotting heteroatom containing proteins [26]. The comparison of spatially resolved microanalytical techniques for in situ imaging of trace metals have been compared in terms of excitation source (e.g., X-ray, proton, electron, ion or plasma), ability of isotope detection, detection limits, resolution obtained and the analytical depth that excitation beam can penetrate.

Molecular Analysis Techniques

The study of proteins (proteomics) and non-proteins associated to a metal is indispensably carried out by the aid of mass spectrometry techniques. The soft ionizing capability in MALDI and ESI allows the analysis of high molecular weight non-covalent complexes of metals with biomolecules and their identification by MS.

ESI process involves spraying out of a solution including analyte from a capillary with high potential, formation of very small droplets, extraction of solvent through the fly to the vacuum field resulting smaller droplets and positively (single and multiple) charged molecules. Metal complexes by peptides, proteins and carbohydrates can be transferred into gas phase in ionized form without destroying metal coordination by electrospray ionization and analyzed by mass detector. The comparison of mass spectra of the intact metal complex (non-

denaturing form) and ligand only (denaturing form) gives information on binding of metal to bioligand. ESI-MS also can be coupled on-line to the column separation techniques (LC or CE). Main drawback of the technique is low tolerance to non-volatile buffers and other additives that might be used to keep metal complex in native and soluble form or to improve the separation efficiency in chromatographic or electrophoretic run. The identification of molecular ions can be performed by different instrumental techniques providing fragmentation of molecules such as SCID (source collision induced dissociation) and CID (collision induced dissociation). The former is carried out by applying high voltage to the orifice as ionization energy. The latter is performed by tandem mass (MS/MS); the selected molecular ion is filtered by first mass analyzer and fragmented by collisions with a neutral gas molecules and then resulting fragments are analyzed by following mass analyzer.

In MALDI, the sample to be analyzed is mixed by the special molecules called "matrix" in proper rates, deposited on the sample plate and radiated by laser beam to volatilize and ionize the analyte towards mass analyzer. Selection of these matrix molecules, absorbing the excess energy of laser and promoting the ionization, is very critical and depends on the characteristics of analyte and sample. Spectra consist mostly of singly charged molecular ions and fragments. MALDI is more tolerant to the concomitants in the sample than ESI therefore useful and complementary technique for the analysis of species that could not be detected by ESI. Fragmentation techniques through MALDI for structural characterization of molecules include the post source decay (PSD) [29] and o-MALDI [30]. In PSD the fragmentation occurs during flight of the precursor ions after ionization and acceleration away from the source. Since the resulting product ions have less kinetic energy, mass to charge ratio can be resolved in TOF. oMALDI involves cooling the ions produced in MALDI by a system "collisional damping interface", the efficient transport to the quadrupole and TOF respectively. Collision cell fragments the selected precursors in quadrupole and allows the structural analysis by measuring the product ions in TOF. The use of soft ionization sources in proteomics has catalyzed the advancements in the technology of mass analyzers such as ion trap (IT), quadrupole (Q), time-of-flight (TOF), fourier transform-ion cyclotron resonance (FT-ICR) and orbitrap. The performance in terms of resolution, mass accuracy and sensitivity depend upon instrument type, ionization source and scanning properties of instruments. Some instruments consist of mass analyzers in hybride or tandem configuration for multistage analysis [3]. Each configuration, consisting of ionization and analyzer units, has its intrinsic merits and limitations; therefore selection of the technique necessitates the tradeoffs with respect to the type of analysis to be performed. When identification is the primary objective, resolving power and mass accuracy is more prevalent; if quantification is objected, special emphasis is given to the sensitivity, dynamic range and scanning capability [3].

On the other side, the use of molecular spectroscopy techniques such as FT-IRM (Fourier transform-infrared microspectroscopy) and Raman microspectroscopy are complementary tools for structural information and suitable in molecular imaging [23, 31].

Separation Methods prior to MS Analysis of Metal Containing Biomolecules

A huge part of the current research in life sciences depends on mass spectrometry techniques. However, as being in much case metal complexes are not only at minute amounts and labile but also dipped into a complex biological matrix, high resolution separation of the sample components is a prerequisite for a successful MS analysis. Gel electrophoresis (one- and two-dimensional gel electrophoresis), HPLC with different separation principles (i.e., size exclusion, ion exchange, reversed phase or affinity chromatography) in analytical, capillary or nano scale and electrochromatography are employed for the metallobiomolecules separation or purification. An effective separation of components includes sequentially performing two or more separation techniques in a proper series (multidimensional systems). Peak capacity of a system increases with the degree of orthogonality, a system where the properties affecting the separation in one dimension do not affect the separation in other dimension and vice versa [32], and reaches to maximum in a true orthogonal system. As in the

hyphenated systems, aforementioned separation systems are coupled to a species (element) or a molecular specific detector (e.g., HPLC-ICP-MS or CE-ESI-MS) to identify the eluted species. The state-of-the-art hyphenated techniques for bioinorganic speciation analysis has been described [33].

Synergic Use of Atomic and Molecular MS for the Analysis of Metallo-biomolecules

Metallomics and metalloproteomics studies necessitate the investigation of metals present in a covalent or non-covalent arrangement in the biological matrix. The number of separation and/or preconcentration steps applied, on one hand, increases the signal to noise ratio for a reliable determination of analyte, on the other hand decreases the total recovery of analyte. Another difficulty is the necessity of tradeoffs in determining the separation conditions; in most cases an experimental parameter that may be in the favour of a successful separation may not be suitable in identification. When the metal is covalently bound to peptides or proteins, molecular mass data, in theory, allows the identification of metal by isotopic pattern. However, the ionization is frequently suppressed by the more abundant and easily ionizable concomitants in the sample. For many application of protein analysis, the use of ICP-MS has become a synergistic partner to MALDI or ESI-MS. The excellent sensitivity and specificity of ICP-MS is independent from the co-eluting matrix in the search for elemental targets in biomolecules. In addition to the molecular mass spectrometric detection (i.e., ESI-MS) of biomolecules, the use of an element selective detector (ICP-MS) gives valuable information in the analysis of metal bound species. The case of non-covalent binding of metals to the bioligand is more challenging. ICP-MS signal is necessary for proof of the metal but insufficient to be sure that metal bound to biomolecule specifically. Mass spectrum of the complex and auxiliary data such as high throughput-XAS should evidence the complex structure [23].

Complementary use of ICP-MS and ESI-MS has become a frequently chosen tool in the analysis of metal containing biological molecules (metallomics) such as proteins (metalloproteomics) and metabolites (metallometabolomics) [17,34]. Even very recently Hieftje and coworkers developed a TOF mass spectrometer which employs an ICP and an ESI ionization source simultaneously in an instrument allowing atomic, isotopic and molecule structural information from a sample [35,36].

Bioinformatics

Bioinformatics is the study of informatic processes in biotic systems and used primarily in genomics and proteomics [37]. Although the information of metalloproteome is essential for a comprehensive understanding of life processes, the currently available experimental methods or techniques are not capable of achieving such a challenging task. At this point bioinformatics provides a considerable contribution to overcome the limitation of empirical methods by using predictive tools and information technology [38, 39]. The prediction of metal binding domains of proteins at the level of entire proteome can be performed [40] by using the protein sequence data present in continuously updated libraries such as Pfam [41]. Bioinformatics tools allow to prediction of metal binding proteins for a given metal by searching the metal binding sites, metal binding domains, or both. In spite of the limitations of approach and the sources of error, bioinformatics approach to access to the complete information on metalloproteome is crucial and complementary to the currently available experimental methods. Bertini and Cavallaro have very recently reviewed the advances in the design and implementation of bioinformatics resources devoted to the study of metals in biological systems [42].

Areas of Interest and Selected Applications

As a systematic and holistic view of the metal related issues, Metallomics has a wide spectrum of application areas and research interests including environmental chemistry, biogeochemistry, plant and animal biochemistry and physiology, clinical physiology, pharmacology, toxicology, essential element supplementation and nutrition, as well as a potential use in epigenetics. The following section is only intended to give a brief overview of these applications with special emphasis to some recent major applications of Metallomics. For more on practices in the field, reviews by

Haraguchi [15], Szpunar [10], Lobinski et al. [18], Shi and Chance [12], Mounicou et al. [16], Maret [43] and references therein are recommended.

Environment Related Studies

An understanding of how organisms sense, adapt and use metals within the biodiversity characteristic of each specific and dynamic ecosystem or biome is required for the systematic view of transition metal metabolism [44]. Geological factors as well as anthropogenic sources may result in soil or water pollution [45] which necessitates studies on environmental pollution and biogeochemistry. Metallomics may help in understanding of biogeochemical metal cycles [46], metal tolerance and homeostasis in plants [47], and plant proteome analyses involved in metal toxicity [48], as well may contribute to the development of applications for optimized strategies in metal contaminated soils, including microbial-assisted phytoremediation of contaminated land, in situ soil regeneration and identification of biomarkers for ecotoxicological studies [46]. For phytoremediation studies, phytochelatins (PCs) which are enzymatically synthesized peptides involved in heavy metal detoxification and/or accumulation in plants [49] have been traced [50, 51]. A study on PCs in Opuntia ficus in relation to plant and soil levels of Cd, Pb, Cu and Ag has highlighted the complex interplay of environmental conditions in accumulation of heavy metals and PC production [50]. Complexes with bioligands in roots and shoots of two different types of plants (*Brassica juncea* and *Sesuvium portulacastrum*) have been searched following exposure to different amounts of $Pb(NO_3)_2$ [51]. Speciation studies on the extracts using SEC and ion pair reversed-phase chromatography coupled to UV and ICP-MS and identification of the species by MALDI-TOF-MS have revealed presence of the isoform PC3 suggesting that both plants may be useful in studies of phytoremediation with a superiority of S. portulacastrum over B. juncea [51].

The separation of metal-binding proteins has been performed by gel electrophoresis in both denaturing and native (non-denaturing) conditions followed by LA-ICP-MS in detection of metals for samples from plant [52] and animal [53] origins. An integration of metallomics with proteomics and transcriptomics as a "triple -omics approach" has shown promise for environmental biomarker analysis [54,55]. To characterize the metallome of a wildlife species (Mus spretus) and reference species (Mus musculus), SEC-ICP-MS has been applied for trace metal-biomolecule profiling, and reverse-phase and/or ion exchange chromatography with ICP-MS detection on the selected fractions as a second dimension, further purification have been performed. In the proteomic part of the study, MALDI-TOF-MS analysis of tryptic 2-DE spot digest and peptide matching with M. musculus database have been used, while for the transcriptomics part microarrays and absolute quantification using real-time reverse transcription-polymerase chain reaction have been performed [54]. In another research, the response of conventional biomarkers, and changes in protein expression profiles in an aquatic organism (Carcinus maenas) exposed to a complex mixture including heavy metals have been assessed [56]. Briefly, crabs from a contaminated and a reference site have been compared for element load (using ICP-MS), conventional biomarkers (e.g. enzyme activity assays) and altered protein expression profiles (separation using 2-DE, followed by MALDI-TOF and further capLC-μESI-ITMS/MS) and the results have revealed some potential markers [56]. In a very recent study by Zhang et al. [57], to evaluate the aquatic toxicity of lanthanum as a representative of rare earth elements in a test organism (Caenorhabditis elegans), the elemental mapping by microbeam synchrotron radiation X-ray fluorescence (μ-SRXRF) has indicated the dose-dependent pattern of accumulation.

Analysis of Metals Related to Genome

Another area of interest is the search for a correlation of the metal concentration blueprint or speciation with the genome. The correlation may be statistical (presence of a particular gene related to the metal load), structural (sequence of a metalloprotein traceable to a gene) or functional (the presence of a bioligand as a result of a gene-encoded mechanism) [16]. Lahner et al. [58] have shown the utility of elemental profiling as a functional genomics tool to determine the biological significance of connections between a plant's (Arabidopsis thaliana) genome and its elemental profile, and the findings lead to the

conclusion that 2-4% of this species genome is involved in regulating the plant's nutrient and trace element content. Using ICP-MS, 18 elements have been quantified in shoots of 6000 mutagenized M2 A. thaliana plants. Altered elemental profiles have been demonstrated in 51 mutants, among which one mutant contains a deletion in a gene known to control iron-deficiency responses (FRD3) [58]. A genome-wide approach [59] to identify genes that control the yeast ionome has been performed by ICP-AES to simultaneously determine the levels of 13 elements accumulated in a model organism-Saccharomyces cerevisiae. In over 4000 different yeast genes, 212 mutant strains have shown reproducible alterations in ionome profiles when grown on a rich growth medium; few of these mutants (four strains) have been affected for only one element, and only six genes previously shown to be involved in the uptake and utilization of minerals have been identified, indicating that homeostasis is robust under replete conditions [59]. An application, also related to the aforementioned environmental issues, is an analytical approach for the detection and identification of the functional ligand in a metal hyperaccumulating plant, Thlaspi caerulescens [60]. Size exclusion HPLC-capillary electrophoresis with ICP-MS detection has illustrated the presence of a complex produced in response to nickel exposure. For the identification of the gene conferring this resistance, a leaf cDNA library has been expressed in the yeast, and the surviving Ni-resistant clones have been isolated to assess relevancy. One of the genes has been found to correlate with mass spectrometric data, and cDNA sequencing analysis has shown presence of an insert homologous to the nicotianamine synthase gene related to the metabolism and transport of iron. The identity of this complex has been verified by comparison of the electrospray MS/MS spectra obtained from the original plant and Ni-resistant yeast extract with that of chemically synthesized Ni-nicotianamine standard [60].

A promising area of future development is the application of ICP-MS, as a complementary technique, to study the epigenetic events [61]. The rapidly evolving field of epigenetics focuses on the study of heritable alterations in gene expression that occur in the absence of changes in genome sequences [62]. Since carcinogenic metals may perturb DNA-methylation levels as well as global and gene specific histone tail PTM marks, the importance of epigenetics as a possible mechanism underlying the toxicity and cell-transforming ability of these metals has been highlighted [62].

Metallomics in Clinical Studies

Impaired metal homeostasis, especially the defects in metal-transport proteins, has been well-established in diseases such as hereditary hemochromatosis (Fe overload) and Wilson's and Menkes diseases (Cu metabolism) [63]. The possible link between metal accumulation and neurodegeneration with special emphasis to Alzheimer's, Parkinson's and Huntington's diseases as well as amyotrophic lateral sclerosis has recently been reviewed [64]. Metal (Cu, Fe and Zn) containing proteins in Alzheimer-disease brain samples have been detected after separation by 2-DE (i.e. isoelectric focusing followed by SDS-PAGE) and the protein spots in gels have been screened with respect to 67Zn/64Zn, 65Cu/63Cu and 54Fe/56Fe isotope ratios by LA-ICP-SFMS. Identification of singular protein spots following excision from gel, tryptic digestion and MALDI-FTICR-MS has demonstrated existence of proteins which are stable during 2-DE [65]. Application of LA-ICP-MS for investigations of biological tissue imaging with specific emphasis on imaging of metal distributions in brain tissue has recently been published by Becker and Salber [66]. In a comparative study on the metallothionein (MT) isoform (MT-1, MT-2, and MT-3) distributions in brain samples from patients with Alzheimer's disease and controls, by use of CE-ICP-MS has revealed lower levels of MT-1 and MT-3 (determined by metal-specific detection) in the temporal and occipital region samples from patients [67]. Biospecimens used in Metallomics studies are not limited to tissue samples; numerous valuable sources include plasma [68], serum [69], cerebrospinal fluid (CSF) [70,71], and red blood cells [72]. Caruso group has investigated the CSF samples from subarachnoid hemorrhage (SAH) patients sub-grouped according to the presence of vasospasm (vasospastic and nonvasospastic) and controls by their metal containing species [70,71]. A possible Fe-associated protein (RAMP2)

observed only in the SAH vasospastic fraction has been reported after pre-fractionation of samples (using spin concentrators) followed by CapLC-ICPMS and nanoLC-CHIP/ITMS evaluation [70]. More recently, six protein families with possible protein markers to predict cerebral vasospasm have been identified in the SEC fractions using nanoLC-Chip-ESI-ITMS [71].

The "metals in medicine" have been receiving a great deal of attention since the introduction of platinum-based chemotherapy [73], although metals including lithium (bipolar disorder), barium (radiopaque material), and organic bismuth compounds (gastrointestinal disturbances) have long been related to medical therapy. The field of cancer research has gained interest for not only regarding the role of some metal ions in cancer cell formation but also as chemotherapeutic agents [74]. Anticancer drug candidates other than Pt-based agents include gold (III) compounds [75] as well as ruthenium-based agents [76]. The clinical use of Pt-based drugs e.g. cisplatin and analogues has been restricted by side effects as well as inherent or acquired resistance [77]. Approaches to overcome these downsides has given rise to more sophisticated approaches such as the development of photoactivable metallopharmaceuticals in treating localized tumors [78, 79], encapsulation of cisplatin and carboplatin in apoferritin derived from the native protein-ferritin [80], and agents tailored to target biological substrates different from DNA, such as matrix metalloproteinases [81] and cathepsin cysteine proteases [82]. Another strategy has focused on the use of metals as building blocks for three-dimensional constructs, like a scaffold rather than reactive centre [73]. A Ru-based agent (DW1/2) developed according to this principle has been shown to specifically target a protein (glycogen synthase kinase 3β) in human melanoma cells rather than acting on DNA and act as a potent activator of p53, resulting in induction of apoptosis via mitochondrial pathway [83]. Medicinal bioorganometallic chemistry has further expanded the toolbox [73] which can be exemplified by use of the ferrocenyl moiety in the design of ferrocifen, a promising organometallic (pro)drug, a tamoxifen derivative exhibiting antiproliferative effect [84], and ferroquine, an antimalarial agent against chloroquine resistant strains [85]. Target oriented approaches have also emerged. Since a number of proangiogenic factors are Cu-dependent, Cu-lowering therapy with tetrathiomolybdate has been suggested for further evaluation in cancer to inhibit angiogenesis and autoimmune diseases [86].

The analysis of the reactivity of metallodrugs with proteins has become a point of interest in recent years with special emphasis to anticancer agents [87, 88]. In a representative study [87], high-resolution linear trap quadrupole Orbitrap ESI-MS has been used for the analysis of the reactivity of cisplatin, transplatin and Ru-compound RAPTA-C with a mixture containing ubiquitin, cytochrome c and superoxide dismutase. The results have shown that RAPTA-C is more reactive, and can discriminate between the proteins, whereas Pt-based drugs are moderately reactive, without any selectivity [87]. Discrimination between different species of cisplatin and Ru-based drug RAPTA-T and their complexes with human serum albumin, holo- and apo-transferrin has been possible using high resolution SEC-ICP-MS [88]. RAPTA-T exhibited a preferential binding for transferrin, the affinity for holo-transferrin being higher suggesting a cooperative Fe-mediated metal binding mechanism [88]. The analytical techniques and the most common sample treatment procedures currently used in metallomics studies of Pt-containing drugs have been recently reviewed [89]. The hyphenated CE-ICP-MS technique for the in vitro assessment of a novel anticancer gallium compound has demonstrated that the drug remains intact in the simulated intestine juice whereas it undergoes a marked change in speciation in human serum, mostly due to binding to transferrin [90]. These applications are not limited to anticancer therapy. Fe-chelation therapies with special emphasis on the efficacy and adverse health effects [91] as well as the differentiation in terms of metal bound to serum proteins in the bipolar disorder patients either treated with or without lithium [69] have also been investigated.

Quantification

Protein-quantification strategies based on metal labelling and elemental MS (ICP-MS) are still at the development stage; however, they are promising to overcome the known drawbacks of conventional analytical methods, especially in

quantitative proteomics [92]. For an overview of some organometallic derivatizing agents as well as other current labelling strategies including isotope-coded affinity tags (ICAT) please see Bomke et al. [93]. The idea behind labelling with elemental (metal) tags for the improved determination of proteins is the (direct or indirect) chemical introduction of an ICP-MS detectable element (i.e. a heteroatom) [34]. In the direct labelling, either the metal (mostly lanthanides) is introduced in the form of a coordination complex or the heteroelement is bound directly to a specific amino acid via a covalent bond [34]. The compatibility of the metal-coded affinity tags (MeCAT) labelling to analysis workflows such as nanoliquid chromatography/electrospray ionization tandem mass spectrometry (nano-LC/ESI-MSn) for quantitative analysis has been reported [94]. It has been possible to quantify a mixture of differentially Tb-, Ho-, Tm-, and Lu-MeCAT-labelled peptides which are stable under a wide variety of conditions including high salt, low pH, and increased temperature, with a linear dynamic range of 2 orders of magnitude, down to 3.6 fmol. Additionally a representative of a complete proteomic workflow of complex E. coli cell lysates has been shown [94]. The proteins labelled by stable isotopes of Eu, Tb and Ho and separated by SDS-PAGE have been detected by LA-ICP-MS after electroblotting of the target proteins onto nitrocellulose membranes and a range of total protein amounts from 0.015 pmol (BSA) to 105 pmol (lysozyme) has been covered [95].

The second strategy (indirect labelling) follows the principle of immunoassays: Antibodies are labelled with complexes of different lanthanides, and specific antibody-protein reaction is used for the detection [34]. The method characterized by the use of immuno-reaction coupled to an ICP-MS system has been applied to the determination of thyroid-stimulating hormone [96], α-fetoprotein and free β-human chorionic gonadotropin in human serum [97]. Gold nanoparticles labelled antibodies have been utilized in several applications [98, 99]. A sandwich-type immunoassay linked with ICP-MS has been developed for the detection of anti-erythropoietin antibodies (anti-EPO Abs) in human sera and applied to samples from patients with recombinant human erythropoietin (rhEPO) treatment and controls. Recombinant human erythropoietin has been immobilized on the solid phase to capture anti-rhEPO Abs specifically; the captured anti-rhEPO Abs reacted with Au-labelled goat-anti-rabbit IgG antibody followed by detection with ICP-MS after dissociation of the immunocomplex [98]. An immunoassay for the detection of ochratoxin A, a well-known mycotoxin-contaminant, using gold nanoparticle tagged antibodies for ICP-MS and horseradish peroxidase conjugated antibodies for photometric detection has been reported and applied to the quality control of wines, and possibly of some other commodities [99]. An application of LA-ICP-MS which takes advantage of the Western-Blotting-technique to specifically detect proteins by use of antibodies conjugated to gold clusters has been presented and applied to analysis of hamster Mre11-protein in crude lysates of CHO-K1 fibroblasts [100]. Another example has been demonstrated as an alternative route for protein quantification using mercury-containing tags in which proteins are specifically labelled at available sulfhydryl groups of cysteine with p-hydroxymercuribenzoic acid (pHMB) [101]. Two different workflows have been applied to relative and absolute quantification of a model protein (insulin) with the use of complementary MALDI-MS and ICPMS [101]. Multiplexed molecular analysis has also been available using ICP-MS-linked metal-tagged immunophenotyping, to quantitate proteins (intracellular oncogenic kinase BCR/Abl, myeloid cell surface antigen CD33, human stem cell factor receptor c-Kit and integrin receptor VLA-4) on the cell surface as well as intracellularly in permeabilized cells of human leukemia cell lines [102].

Bioinformatics

In a bioinformatics survey, the content of Zn, nonheme Fe and Cu-proteins in 57 representative organisms taken from the three domains of life (12 archaea, 40 bacteria and 5 eukaryotes) have been predicted. It has been shown that the percentage of the number of zinc-proteins in the entire proteome increased from about 5-6% (for prokaryotes) to about 9% (for eukaryotes), indicating a substantial rise in the number of Zn-proteins in higher organisms. In contrast, the number of nonheme proteins is diminished

in the order of archaea (about 7%), bacteria (about 4%), and eukaryotes (about 1%). The number of Cu-proteins represents constantly less than 1% of all proteins in the three groups [40]. Another representative application of bioinformatics deals with the current controversy on the characterization of low molecular weight chromium binding substance (LMWCr) and spots insight on its biological role. Sequence of the peptide (LMWCr) is unknown but the search of database on the basis of reported stoichiometry of amino acids yields the possibility of three decapeptide or a pentameric dimer. Among them pentameric peptide can bind Cr(III) and matches at an acidic region in the α-subunit of the insulin receptor which is an essential constituent of carbohydrate metabolism [103]. A high-throughput-XAS (HT-XAS) method for the analysis of transition metal (Mn, Fe, Co, Cu, Ni, and Zn) content in a large set of selected proteins (654 samples), based on quantitation of X-ray fluorescence signals has been presented [19]. It has been reported that the bioinformatics analysis of the metalloproteins identified by the HT-XAS method in most cases supported the metalloprotein annotation, and identification of the conserved metal binding motif has been shown to be useful in verifying structural models of the proteins. Results indicated that over 10% showed the presence of transition metal atoms in stoichiometric amounts and out of 48 crystal structures, 45 correct predictions have been possible [19]. Bertini and Cavallaro have recently published an outstanding review of bioinformatics in bioinorganic chemistry in the new journal, Metallomics, dedicated to the studies in this field. Review describes the design and implementation of bioinformatics resources and shows the relation between the information of genome and metalloproteome [42].

Miscellaneous Applications

The supplementation of food and feed with mineral elements (e.g. Se, Zn, Fe, Mn, and Cr) has been receiving attention owing to the essential role of many trace elements, their implication in prevention of some diseases and low levels of some of these elements in the diet in many countries [8]. Among the transition metal ions, chromium presents a deep controversy in terms of its biological effects: On the one hand, the highest oxidation state of this element, Cr(VI), has been well-known for its toxic effects [104]. A recent study has demonstrated that the intraperitoneal administration of Cr(VI) to mice causes not only accumulation of Cr but also alterations of Mn, Cu, Fe and Zn levels along with the bioinduction of some Cr, Mn, Fe and Zn-binding proteins in different tissues, especially in liver and kidney [105]. On the other hand, Cr(III) which is regarded as an essential micronutrient still continues to be a matter of ambiguity, especially in terms of efficacy and safety of Cr(III) complexes [106, 107]. Recently, characterization of binding and bioaccessibility of Cr in Cr-enriched yeast has been attempted using a 2D-LC fractionation (size exclusion and reverse phase HPLC) method and ICP-MS for detection of Cr-binding species [108]. On the other hand, selenium-rich yeast metabolome (selenometabolome) has been investigated using normal-phase and hydrophilic interaction liquid chromatography (HILIC) coupled with ICP-MS and ESI Q-TOF MS/MS, which enabled de-novo identification of 12 seleno-compounds out of 15 Se-peaks detected by ICP-MS [109]. In another study, the transport behaviour of copper glycinate complexes through a porcine gastrointestinal membrane has shown the easy penetration of copper glycinate complexes as compared to inorganic copper species indicating the utility of their use in animal nutrition. The complexes have been determined by capillary electrophoresis by UV and ICP-MS detection and characterized by CE-ESI-MS/MS and MALDI-TOF-MS [110]. Apparently, further studies on efficacy and safety of supplements will rely ever more heavily on interdisciplinary collaboration.

Another example is on the effect of arsenic on protein phosphorylation which is an important PTM involved in the regulation of cell signalling [111]. SEC-ICP-MS has been used for detection and isolation of phosphorylated proteins in HeLa cells by monitoring 31P signal, and collected fractions have been separated using nano-LC-CHIP/ITMS system for peptide determination. Spectrum Mill and MASCOT protein database search engines have been used for protein identification and several phosphorylation sites and PTMs have been identified [111]. Studies related to competitive interaction between anta-

gonists may also benefit from Metallomics; for example competitive binding of Zn^{2+} against Cd^{2+} for GSH, has been investigated using CE-ICP-MS, which allowed quantitative determination of the thermodynamic and kinetic parameters of this competitive binding, related to the detoxification of Cd^{2+} in biological system [31].

The field has become so specialized that specific approaches have gained importance and new terms have emerged including toxicometallomics [112] and vascular metallomics [113]. The recent review by Easter et al. [113] has provided detailed prospective on the role of trace metals in vasculature with special emphasis on the role of Cu in the cardiovascular diseases and angiogenesis, as well as a signalling molecule.

Concluding Remarks and Future Perspectives

The research in metallomics has rapidly grown and applied to the different areas in our life. The study of metallomics in line with other –omics on one hand spots the emerging pieces of zigzag puzzle of systems biology; on the other hand contributes to different fields such as molecular biology, pharmacology, toxicology, medicinal chemistry, nutrition, environmental chemistry and physiology of plant and animals. It is expected that the progressive technological advancement in analytical instrumentation and interdisciplinary collaborations will link pure and applied parts for an integrative approach and accelerate the rate of studies in metallomics which will positively reflect to different areas of life.

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