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Metalloproteomics for Biomedical Research: Methodology and Applications

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Abstract

Metals are essential components in life processes and participate in many important biological processes. Dysregulation of metal homeostasis is correlated with many diseases. Metals are also frequently incorporated into diagnosis and therapeutics. Understanding of metal homeostasis under (patho)physiological conditions and the molecular mechanisms of action of metallodrugs in biological systems has positive impacts on human health. As an emerging interdisciplinary area of research, metalloproteomics involves investigating metal-protein interactions in biological systems at a proteome-wide scale, has received growing attention, and has been implemented into metal-related research. In this review, we summarize the recent advances in metalloproteomics methodologies and applications. We also highlight emerging single-cell metalloproteomics, including time-resolved inductively coupled plasma mass spectrometry, mass cytometry, and secondary ion mass spectrometry. Finally, we discuss future perspectives in metalloproteomics, aiming to attract more original research to develop more advanced methodologies, which could be utilized rapidly by biochemists or biologists to expand our knowledge of how metal functions in biology and medicine.

Contents

INTRODUCTION

Metals are involved in many cellular and subcellular functions and play critical roles in life processes (1). For example, intra- and intercellular communications rely largely on the concentration gradients of potassium and sodium. Calcium (Ca) ions play essential roles in blood clotting and muscle contraction. Iron (Fe) is essential for oxygen transport, while zinc (Zn), as the most abundant transition metal ion, plays a vital role in processes such as the regulation of DNA transcription. Metal ions have been utilized in biological processes to the extent that we cannot imagine a life without metals. Through evolution, metal ions have been coopted in living organisms with numerous roles.

Dysregulation of metals results in various diseases. Increasing evidence shows that the disruption of metal homeostasis is associated with a number of neurodegenerative diseases. For instance, Alzheimer's disease (AD) and Parkinson's disease (PD) have been characterized by elevated tissue Fe and by miscompartmentalization of copper (Cu) and Zn, e.g., accumulation in amyloid (2). Metals are also frequently incorporated into pharmaceuticals as diagnostic and therapeutic agents. The clinical success of cisplatin as an anticancer drug has accelerated the research of metals in medicinal chemistry (3). Subsequently, several other platinum (Pt)-based drugs, including carboplatin and oxaliplatin, have been approved by the US Food and Drug Administration, and more Pt-based compounds with certain functionalizations are being developed to improve therapeutic efficacy and reduce systematic toxicity (4, 5). In addition, many other metal-based anticancer agents, such as those containing ruthenium (Ru), gold (Au), arsenic (As), and osmium (Os), are being developed (6). Moreover, bismuth (Bi) drugs, e.g., colloidal bismuth subcitrate (CBS, or De-Nol®) and ranitidine bismuth citrate (Tritec®), are currently used to treat *Helicobacter pylori* infection when combined with antibiotics as either triple or quadruple therapies, even for antibiotic-resistant strains (7).

Although important in life processes, metals can be toxic at excessive concentrations. Thus, organisms have developed elaborate systems for metal homeostasis, including uptake, transport,

storage, and secretion. Such events are often accomplished through proteins. Metals serve as cofactors for approximately one-quarter to one-third of human enzymes/proteins (8), namely metalloproteins, with the intrinsic metal ions providing catalytic, regulatory, and structural roles critical for protein function. Metalloproteins are found in almost all life forms to facilitate biological processes such as respiration, photosynthesis, hydrogen, nitrogen fixation, and molecular oxygen reduction. However, metalloproteins are not well characterized, owing to the lack of well-established experimental methods to analyze the complete set of metalloproteins encoded by an organism; for instance, many microbial metalloproteomes remain uncharacterized (9). Moreover, due to diverse and poorly recognized metal-binding-site geometry and composition, it is impossible to predict the numbers and types of metals that an organism assimilates from its environment or uses in its metalloproteins solely on the basis of genome sequence information (10).

How metals participate in life processes is an extremely challenging issue to address, particularly at a systems level, owing to the diverse coordination geometries and multiple oxidation states of metals as well as the complexity of metal-biomolecule interactions in living systems. Recently, new research areas, namely metallomics and metalloproteomics, have emerged to study the metals and metalloids within a cell or tissue/organ. The term metallomes was initially coined by Williams to refer to the soluble (mobile) metals in a cell (11) but was subsequently extended to encompass all metal and metalloid species present in a cell or tissue (12). Metallomics, an integrated biometal science in symbiosis with genomics and proteomics (13), is the study of a metallome and the interactions and functional connection of metals with proteins and other biomolecules (12). Metalloproteomics, a subdiscipline of metallomics, aims to systematically identify large sets of proteins associated with metals (metalloproteins and metal-binding proteins) and to analyze their regulation, modification, interaction, structural assembly, and function, as well as their involvement in disease states and physiological processes (14). A focus of metalloproteomics is to identify novel proteins contributing to metal homeostasis.

In the past decade, there has been an increasing interest in the use of metalloproteomics to understand metal homeostasis and the roles that metals play in biology and medicine. Many comprehensive reviews can be found, including metalloproteomics for clinical research (15), microbial metalloproteomics (16), metalloproteomics in neurodegeneration (17), metalloproteomics in disease processes (14, 18), and the use of metalloproteomics approaches to unveil the mechanism of action of metallodrugs (19–22). In this review, we highlight major developments in metalloproteomics methodology and application. We focus on the important research findings derived from metalloproteomics, including the mechanisms of action of therapeutic metals, the roles of metals in disease processes, and metal homeostasis in microbes. We also discuss emerging single-cell metalloproteomics. Finally, we discuss challenges and the outlook for metalloproteomics in human health.

METHODOLOGIES FOR METALLOPROTEOMICS

Modern atomic and molecular spectroscopy–based hyphenated techniques consist of diversified methods for separation, detection, and characterization of metals in life processes and metalloproteomics. In fact, many well-established proteomics approaches are applicable for metalloproteomics, particularly the platform for protein separation and identification (23). The continued development of techniques in protein separation and metal detection in biological systems has led to new potential for this research field. Here, we briefly summarize up-to-date important metalloproteomics approaches; detailed information on individual methodology, including instrumental developments, applications, and limitations, can be found elsewhere (12, 16, 24).

Methods Based on Inductively Coupled Plasma Mass Spectrometry

Inductively coupled plasma mass spectrometry (ICP-MS) is a robust and highly sensitive technique capable of quantitative detection of metals in biological systems and has the advantage of allowing for simultaneous multiplex quantification of elements (25). ICP-MS is one of the most frequently used techniques in metallomics for the detection of metals in different biological samples. This technique can be easily adopted to detect metals from protein samples. Prior to this technique, metalloproteins or metal-binding proteins had to be separated from other biological species via different separation techniques (**Figure 1*a***) such as liquid chromatography (LC) or high-performance liquid chromatography (HPLC), capillary electrophoresis (CE), and gel electrophoresis (GE). Although CE exhibits high efficiency in protein separation, CE–ICP-MS (26) is of limited use in analysis of metalloproteins or metal-binding proteins in real biological samples owing to insufficient detection limits. In contrast, LC–ICP-MS has been widely used for metalloproteomics study. However, the relatively low separating resolution of LC requires multidimensional chromatographic steps. The combination of multidimensional LC with ICP-MS has been successfully utilized for analysis of microbial metalloproteomes (9).

GE is the most frequently used separation technique in the field of genomics and proteomics. Through using 2D nondenaturing polyacrylamide gel electrophoresis (PAGE), the proteomes of Fe-rich *Ferroplasma acidiphilum* were well separated, and Fe-binding proteins were then confirmed by ICP-MS (27). Column-type continuous-flow GE coupled with ICP-MS, namely GE–ICP-MS, was recently reported (28). A column-type gel, either a native or a denaturing gel, can be prepared according to the protein targets of interest. A T-connection is used to split the elutes from the column gel system into two parts, with one part for online metal measurement by ICP-MS and the other for protein identification through biological MS analysis. The method has been successfully employed to identify Bi-binding proteins from *H. pylori* (28, 29) and metallo-anticancer drug-binding proteins in cancer cells (30). This method has also been utilized to determine the stoichiometry of metal binding to metalloproteins in vivo by using sulfur (S) signals to quantify protein concentrations (31). To further enhance the resolution of protein separation, weak anion exchange LC was combined with GE–ICP-MS, namely 2D GE–ICP-MS or LC–GE–ICP-MS, as such proteins are separated according to differences in protein isoelectric points as well as molecular weights (32). 2D GE–ICP-MS significantly enhances separation efficiency relative to 1D GE–ICP-MS (33), which has been successfully utilized to profile silver (Ag)-associated proteomes in *Escherichia coli* (32) and *Staphylococcus aureus*, as discussed below (34).

Laser ablation (LA) coupled with ICP-MS (LA–ICP-MS) is a powerful technique for in situ analysis and imaging of metal or metallodrug distribution in biological samples, since it offers high spatial resolution, excellent sensitivity, and feasibility for quantitative imaging (12, 35). As the most frequently used method for protein separation, PAGE or 2D PAGE, either denaturing or nondenaturing, is often introduced for direct in situ imaging of metals on protein bands or spots by LA–ICP-MS analysis. This method has been successfully employed to detect Bi- and Zn-binding proteins of *H. pylori* in gel (35).

Fluorescence-Based Approaches

Given that some metal-protein interactions are weak and even transient, small-molecule-based fluorescence approaches (**Figure 1*b***) have emerged for real-time visualization and mapping of different metalloproteins in live cells (36, 37). As(III)-based probes, e.g., FlAsH-EDT2, are well-known representatives of fluorescence probes based on the interaction between As and two pairs of vicinal thiols; FlAsH-EDT2 has been successfully employed to image proteins with genetically fused tetracysteine tags (38), as well as to study protein-protein interactions and associated

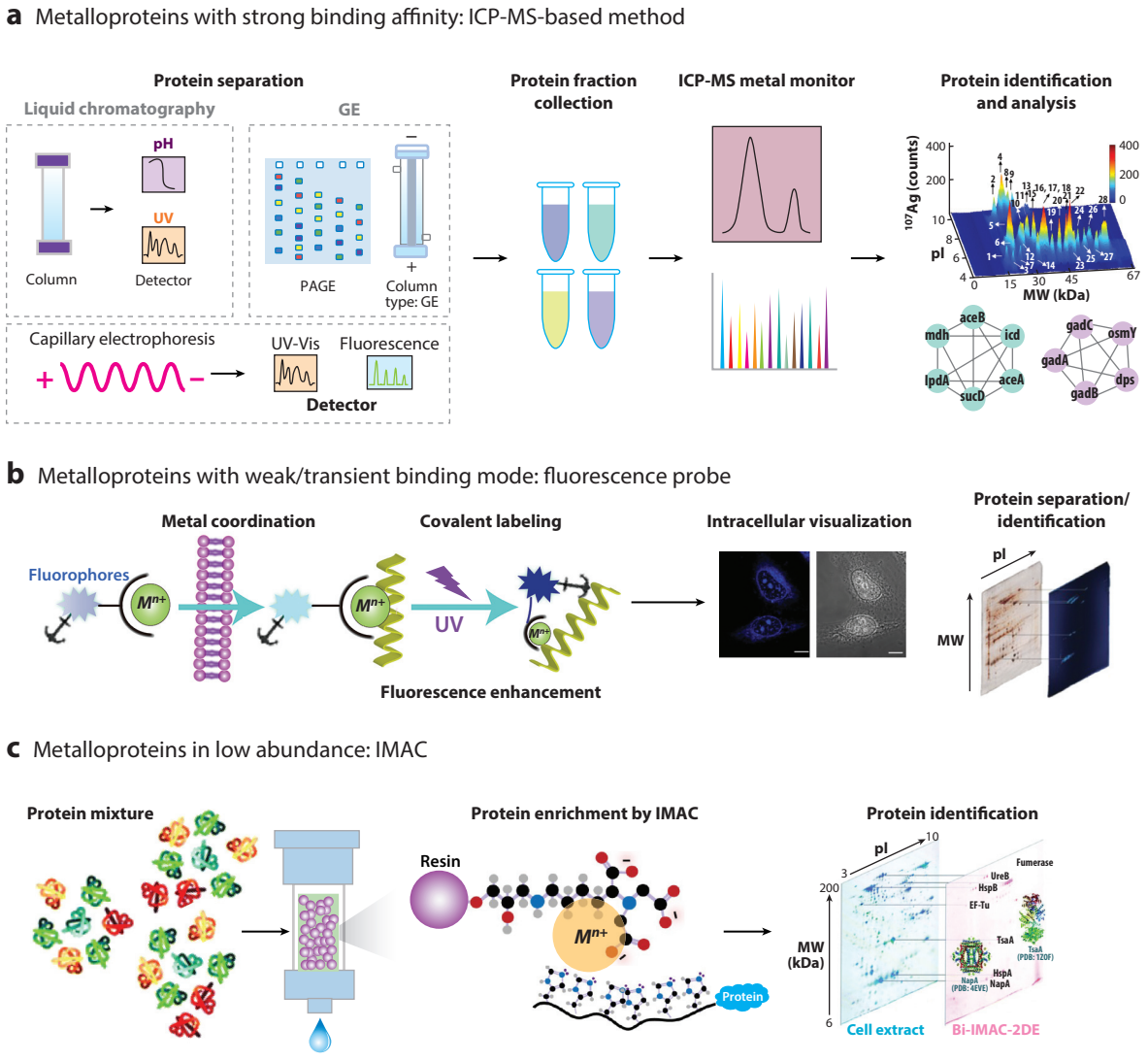


Figure 1

Representative techniques for metalloproteomics study. (*a*) General scheme for ICP-MS-based approaches for tracking metalloproteins. (*b*) Fluorescence-based approach for visualization and identification of metal-binding proteins. (*c*) IMAC for separation, enrichment, and identification of metal-binding proteins in low abundance. Abbreviations: 2DE, two-dimensional gel electrophoresis; GE, gel electrophoresis; ICP-MS, inductively coupled plasma mass spectrometry; IMAC, immobilized metal affinity chromatography; MW, molecular weight; PAGE, polyacrylamide gel electrophoresis; pI, isoelectric point.

protein conformational changes (39). Additionally, various nitrilotriacetic acid (NTA)-based fluorescence probes have been developed via conjugation of fluorophores with *mono*-NTA or *di*-, *tri*-, and *tetra*-NTA derivatives to mimic the concept of FlAsH (37). Among these probes, nickel (Ni)-NTA-AC—a probe consisting of a *mono*-Ni-NTA moiety, a small-membrane-permeable fluorophore (a coumarin derivative), and an aryl azide moiety—is the only one that can rapidly enter cells to label and visualize hexahistidine (His6)-tagged proteins (40). The introduction of the aryl azide moiety further facilitates the efficient protein labeling of the probe. By changing

the conjugated fluorophores (coumarin, BODIPY, fluorescein), probes with different colors (blue, red, green) were obtained, indicating great potential for multiplex imaging in live cells. These probes have been successfully utilized to track His-tagged proteins in mammalian and bacterial cells as well as plant tissues (40–42). Importantly, these probes (termed M^{n+}-TRACER) are metal tunable and could be further extended to mine different metal-binding proteins, e.g., Bi(III) (29, 43), Ni(II)/Cu(II) (44), Fe(III) (45), and gallium(III) [Ga(III)] (46). The M^{n+}-TRACER targets the metal-binding proteins guided by metal ions to achieve specific labeling, and the aryl azide group provides an additional covalent bond between the probe and its target protein upon photoactivation to enable downstream protein separation and identification (**Figure 1*b***). In combination with the proteomics approach [2D GE, matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS),] a large set of putative metalloproteins have been tracked on a proteome-wide scale in different biological systems (29, 44–46).

Immobilized Metal Affinity Chromatography

Immobilized metal affinity chromatography (IMAC) is a powerful technique for separation and enrichment of metalloproteins on the basis of differential binding affinities for immobilized metals [e.g., Cu(II), Zn(II), As(III), Co(II), Ni(II), and Bi(III)] on a solid-phase support (**Figure 1*c***) (47–49). When IMAC is combined with other protein-separating strategies (e.g., 2D GE, 2D LC) and protein identification techniques (e.g., MALDI-MS), extensive metalloproteins, even those in low abundance, can be profiled and identified. IMAC has great potential in metalloproteomics research due to its low cost, high recovery yields, easy operation, superior protein separation, and enrichment efficiency (50, 51).

Synchrotron Radiation–Based Methods

Advanced nuclear analytical techniques have been extensively applied in metalloproteomics to monitor metals or metallodrugs in biological samples (52). With the advances in instrumentation for third-generation synchrotron microprobe beamlines, the utilization of synchrotron radiation X-ray fluorescence (XRF) for high-resolution elemental mapping in tissues, cellular compartments, or even individual cells has received growing interest (53). X-ray absorption spectroscopy (XAS) is a valuable tool for probing the changes in metal centers' chemical environment, such as metal oxidation states in cells, the coordination motif of the probed metal, and the identity and number of adjacent atoms. As a noninvasive technique, XAS has been successfully used to monitor the biotransformation of metallodrugs in biological fluids (54). High-throughput XAS was introduced to directly measure metal contents in 654 proteins, with more than 10% of proteins being found to contain stoichiometric amounts of transition metals (55). Particle-induced X-ray emission is another superior nondestructive X-ray spectrographic technique and has potentially wide applications for metalloprotein structural analysis owing to its high throughput and multiplex capability (56). Recently, a new 3D cryo correlative and quantitative methodology was developed through combining cryo–soft X-ray tomography and cryo-XRF tomography; this technique has been implemented to map the intracellular distribution of iridium-based compounds with super high resolution (57). This original correlative and quantitative method can be readily extended to track different elements involved in biochemical processes in different organelles of human cells.

APPLICATIONS OF METALLOPROTEOMICS

Given that metals or metallodrugs often bind and functionally perturb the biological functions of metalloproteins and/or metalloenzymes, systemic identification of metal- or metallodrug-binding

proteins is particularly important in understanding their roles in biology and medicine, including their roles in disease processes or in regulation of normal biological events and their modes of actions as therapeutics. In the past decades, we have witnessed extensive advances in metal-based research driven by metalloproteomics study (58). Here, we highlight some important discoveries generated by metalloproteomics studies.

Mechanism of Action of Metals as Therapeutics

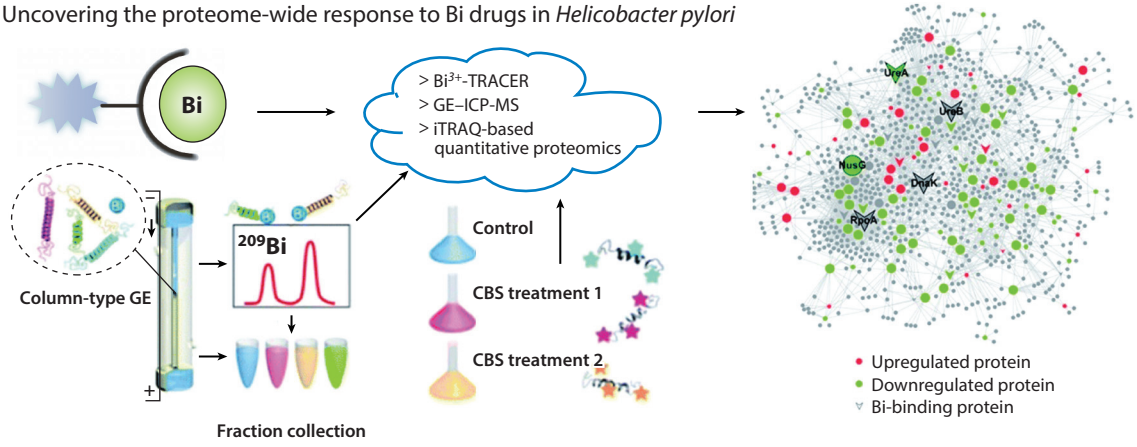
In addition to Pt-based drugs (cisplatin, oxaliplatin, carboplatin), numerous metal-based anticancer (e.g., As, Ru, Au, Os) and antibacterial (e.g., Bi, Ag, Ga) agents/drugs have been developed (59). Systemic identification of putative protein targets of a metallodrug is necessary to generate a holistic view of the mode of action of a metallodrug, in turn guiding rational design of better drugs. In recent years, metalloproteomics approaches have resulted in tremendous progress in unveiling the molecular mechanism of action of metallodrugs (21, 22, 24, 25).

Antimicrobial agents. In the past few decades, enormous efforts have been made in the development of different metalloproteomics strategies to uncover the molecular mechanisms underlying different antibacterial metallodrugs (24). Bi drugs in combination with antibiotics, e.g., tetracycline and metronidazole, are clinically used as the first-line therapy to treat *H. pylori* infection (60). However, the molecular mechanism of action of Bi drugs remains largely unknown. By using Bi-IMAC combined with LC-MS, more than 300 Bi-binding peptides from 166 proteins in *H. pylori* were identified, and the preferential binding of Bi for cysteine and His was revealed (49). GE–ICP-MS was used to successfully identify seven Bi-targeted proteins in *H. pylori* (28), including the urease subdomains UreA and UreB, the GTP-binding elongation factor EF-Tu, the Fe(III) ABC transporter periplasmic Fe-binding protein CeuE, the key ROS/RNS defense enzyme alkyl hydroperoxide reductase AhpC, cell binding factor 2, and the previously hypothetical protein HP1286. Interestingly, although Bi^{3+} binds to urease via the UreA and UreB subunits, the enzyme's activity is hardly inhibited at enzyme levels. Instead, Bi drugs inhibit urease activity through binding and functional disruption of UreG, thus perturbing the maturation of urease; UreG is therefore a more efficient drug target for the design of urease inhibitor (43). The Bi^{3+}-TRACER-based fluorescence approach was used to identify more than 40 Bi-binding proteins from *H. pylori* (29); 9 of these proteins, e.g., UreA/B, HspA, tuf, and tsaA, were also previously identified by GE–ICP-MS. The rest of the 31 proteins were tracked only by the fluorescence-based approach, possibly due to weak binding or the low resolution of 1D GE–ICP-MS in protein separation.

Through the integration of different metalloproteomics approaches, including GE–ICP-MS and a fluorescence-based approach with iTRAQ-based quantitative proteomics, a total of 63 Bi-binding and 119 Bi-regulated proteins were identified from *H. pylori* (**Figure 2*a***) (29). Further bioinformatics analysis combined with bioassays showed that Bi drugs disrupt multiple essential pathways (such as ROS defense and pH buffering) of the pathogen by binding and functional perturbation of key enzymes. Mapping Bi-influenced proteins to protein-protein interaction networks resulted in a large Bi-influenced protein interaction network, in which a large quantity of Bi-targeting proteins were grouped together not only as hub proteins but also as bottleneck proteins (29). This multitarget mode of action of Bi may contribute to the sustainable antimicrobial activity of Bi drugs against *H. pylori* and the low likelihood of *H. pylori* developing resistance to Bi drugs. Further integration of metalloproteomics with metabolomics and transcriptomics enabled a thorough examination of the cellular responses of *H. pylori* under CBS administration. Bi drugs may inhibit *H. pylori* through perturbing initially the TCA cycle and then urease activity, resulting in increased oxidative stress, suppressed energy production, and extensive downregulation of

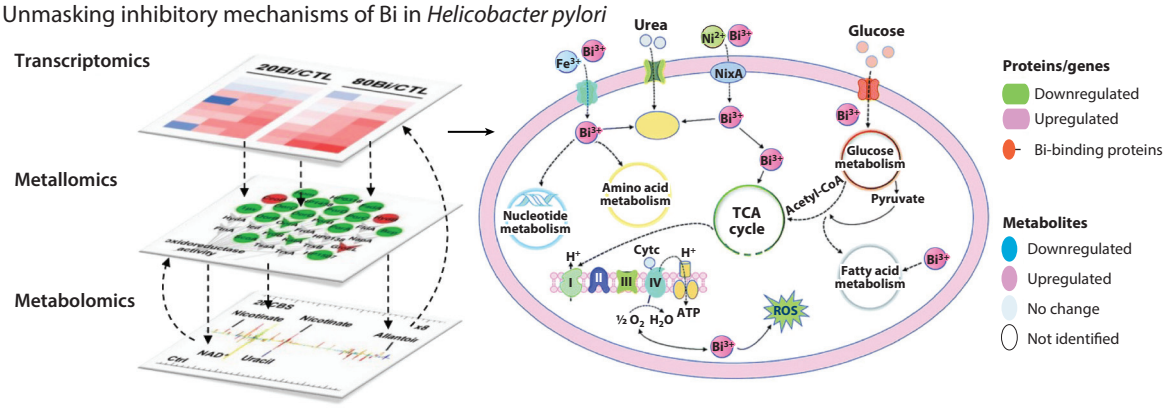
INTEGRATION OF DIFFERENT METHODS

a Uncovering the proteome-wide response to Bi drugs in *Helicobacter pylori*

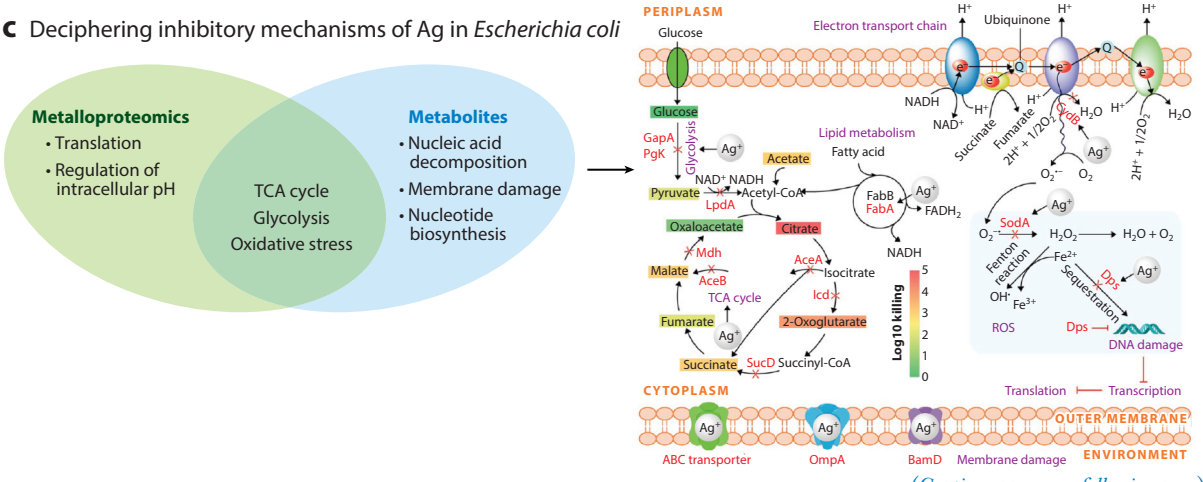


INTEGRATION OF MULTI-OMICS

b Unmasking inhibitory mechanisms of Bi in *Helicobacter pylori*



c Deciphering inhibitory mechanisms of Ag in *Escherichia coli*



(*Caption appears on following page*)

Figure 2 (*Figure appears on preceding page*)

Integrative approaches for uncovering the mode of action of antibacterial metallodrugs. (*a*) Bi-associated proteomes of *Helicobacter pylori* uncovered by a combination of Bi^{3+}-TRACER, GE–ICP-MS, and iTRAQ-based quantitative proteomics. (*b*) The influence of Bi on a diverse array of intracellular pathways of *H. pylori*, as revealed by integration of transcriptomics, metallomics, and metabolomics. (*c*) The molecular mechanism of Ag antimicrobial activity, as deciphered by integration of metalloproteomics and metabolomics. Abbreviations: CBS, colloidal bismuth subcitrate; GE, gel electrophoresis; ICP-MS, inductively coupled plasma mass spectrometry. Adapted with permission from References 29, 32, and 61.

the *H. pylori* metabolome (**Figure 2*b***) (61). The antimicrobial mechanisms of Ga(III)-based drugs were also revealed; i.e., Ga(III) suppresses the metabolism and energy utilization of *Pseudomonas aeruginosa* through targeting the RNA polymerases RpoB and RpoC (46).

Ag [and Ag nanoparticles (NPs)] exhibits broad-spectrum antimicrobial activity, boosts antibiotic efficacy against gram-negative bacteria, and restores susceptibility of a bacterial strain resistant to antibiotics in animal models (62). However, the molecular mechanism of action of Ag at a systems level is not well studied. LC–GE–ICP-MS was used to identify a total of 34 proteins from *E. coli* (32). By integration with metalloproteomics, metabolomics, bioinformatics, and systems biology, the antimicrobial actions of Ag^+ were decoded at a systems level for the first time. Ag induced systematic damage and death of the bacterium through primarily disrupting glycolysis and the TCA cycle, followed by further destroying the adaptive glyoxylate pathway and suppressing cellular oxidative stress responses (**Figure 2*c***) (32). Key enzymes, including glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and malate dehydrogenase, were further validated as vital targets of Ag (63, 64). By using a similar approach, the molecular mechanism underlying the antimicrobial activity of Ag against *S. aureus*, one of the five most common causes of hospital-acquired infections, was unveiled at a systems level (34). Different from the mode of action of Ag against *E. coli*, Ag eradicates *S. aureus* through primarily targeting glycolysis via inhibition of multiple essential enzymes and inducing ROS elevation through functional disruption of the redox homeostasis system at a late stage, leading to the upregulation of oxidative pentose phosphate pathway (oxPPP) enzymes to alleviate Ag(I) stress. However, the activation of oxPPP is ultimately futile due to the key oxPPP enzymes, e.g., 6-phosphogluconolactonase and 6-phosphogluconate dehydrogenase, being inhibited by Ag^+. The multitarget mode of action of Ag contributes to its suppression of antibiotic selection effects on pathogens as well as the sustainable susceptibility of *S. aureus* to Ag. This shotgun mode of action of Ag allows it to enhance the efficacy of a broad range of conventional antibiotics and to resensitize methicillin-resistant *S. aureus* to antibiotics.

Anticancer agents. Metalloproteomics are widely utilized for molecular target identification of protein-binding anticancer metallodrugs (65). Photoaffinity labeling with click chemistry, together with chemical proteomics, has been widely used to track drug putative targets. Such a strategy is also applicable for profiling metallodrug-binding proteomes. To mine the cellular protein targets of Pt compounds, researchers used chemical proteomics to selectively label and isolate platinated proteins in yeast treated with azidoplatin, a Pt compound with azide coordination (66). The azide-modified Pt(II) reagent enables the labeling of extracted proteins with fluorophores for in-gel visualization, as well as the modification of biotin for subsequent streptavidin enrichment and analysis by LC-MS/MS. Through this Pt(II) pull-down method, a total of 152 Pt-binding proteins were isolated and identified, with 7 proteins being discovered to be involved in the endoplasmic reticulum (ER) stress response.

Drug resistance is frequently observed in cisplatin-treated patients. To understand the resistance mechanisms, a quantitative proteomics approach was used to quantify proteome alteration in cisplatin-resistant cell lines (67). A total of 374 proteins showed significant variation between cisplatin-resistant and cisplatin-sensitive cells, providing new potential markers and therapeutic

targets to combat cancer recurrence. Given the frequently observed mitochondrial dysregulation in most malignancies, alterations of mitochondrial protein abundance in cisplatin-sensitive and cisplatin-resistant cells were quantified by comparative organellar proteomics (68). Multiple proteins (e.g., ALCAM and AKAP) involved in evasion of apoptosis, tumor invasiveness, and metastasis showed elevated levels in resistant cell lines. Recently, the proteomes of tumors from Pt-resistant and Pt-sensitive patients with high-grade serous ovarian cancer (HGSOC) were quantitatively analyzed using multilevel proteomics (69). Cancer/testis antigen 45 (CT45) was revealed as an independent prognostic factor associated with disease-free survival from HGSOC. Further phosphoproteomics and interaction proteomics analyses demonstrate the relevance of CT45 and DNA damage through direct interaction with the protein phosphatase 4 complex, providing a new chemosensitivity mediator and immunotherapy target of ovarian cancer.

Arsenic trioxide (ATO; also known as Trisenox®) is a clinically used drug to treat acute promyelocytic leukemia. Enormous efforts have been made toward understanding its mechanism of action through tracking As-binding proteins via various techniques (70, 71). For example, by using a human proteome microarray consisting of 16,368 affinity-purified N-terminally GST (glutathione *S*-transferase)-tagged proteins with a biotinylated As molecule (**Figure 3*a***) (72), a total of 360 proteins were identified, and the proteins involved in the glycolysis pathway, including the rate-limiting enzyme in glycolysis, hexokinase 1, were found to be highly enriched. Moreover, the homologous enzyme, hexokinase 2, was validated as one of the key anticancer targets of As, representing a general mechanism underlying the suppressive effects of As on cancer cells. By using an arsenic-biotin conjugate combined with proteomics techniques, the As-binding proteins in MCF-7 cells, including metabolic enzymes, structural proteins, and stress response proteins, were enriched and identified (73). In addition, As was found to bind to β-tubulin, inhibiting tubulin polymerization. Recently, an efficient pull-down strategy was developed using an azide-labeled arsenical, *p*-azidophenylarsenoxide (PAzPAO), to capture As-binding proteins in living cells, followed by enrichment and identification by shotgun proteomics (**Figure 3*b***) (74). This strategy has enabled 48 As-binding proteins to be captured and identified in A549 cells, among which thioredoxin and peroxiredoxin-1 are the two most abundant proteins. Additionally, GAPDH was identified to interact with PAzPAO through binding to Cys152 and Cys156 at its active site, possibly inhibiting the hyperactive glycolytic pathway typical of cancer cells.

As an emerging As-based anticancer drug candidate, *S*-dimethylarsino-glutathione (ZIO-101) exhibits superior anticancer properties against a variety of solid tumors and hematologic malignancies. However, the molecule targets of ZIO-101 remain elusive. By using GE–ICP-MS, histone H3.3 was identified as the only protein in leukemia cells that binds to ZIO-101 (30). Binding of ZIO-101 to histone H3.3 via cysteine residues disrupts the formation of histone H3.3 dimers, facilitates the denaturation of nucleosomes, and consequently results in TRAIL-induced apoptosis and inhibition of tumor growth. Further complementary dynamic transcriptomic analysis and biochemical characterizations reveal that ZIO-101 suppresses the proliferation of leukemia cells via activating the ferroptosis pathway and that *HMOX1*, a gene dramatically upregulated by ZIO-101, is critical for ZIO-101-induced ferroptotic cell death (75). Importantly, ZIO-101 and the kinase inhibitors dasatinib and dactolisib were found to synergistically kill leukemia cells, providing a novel therapeutic strategy to fight against drug-resistant leukemia.

Ru compounds can overcome Pt-based drug resistance, making them effective anticancer drug candidates. To unveil cellular targets of RAPTA, a class of experimental Ru anticancer drugs, a pull-down assay that combined an affinity probe with a protein identification technique (nano-electrospray ionization quadrupole time-of-flight MS) (76) enabled the identification of 15 cancer-associated Ru-binding proteins involved in metastasis, angiogenesis, proliferation, and cell cycle regulation, providing a general strategy for direct mining of intracellular metallodrug-binding

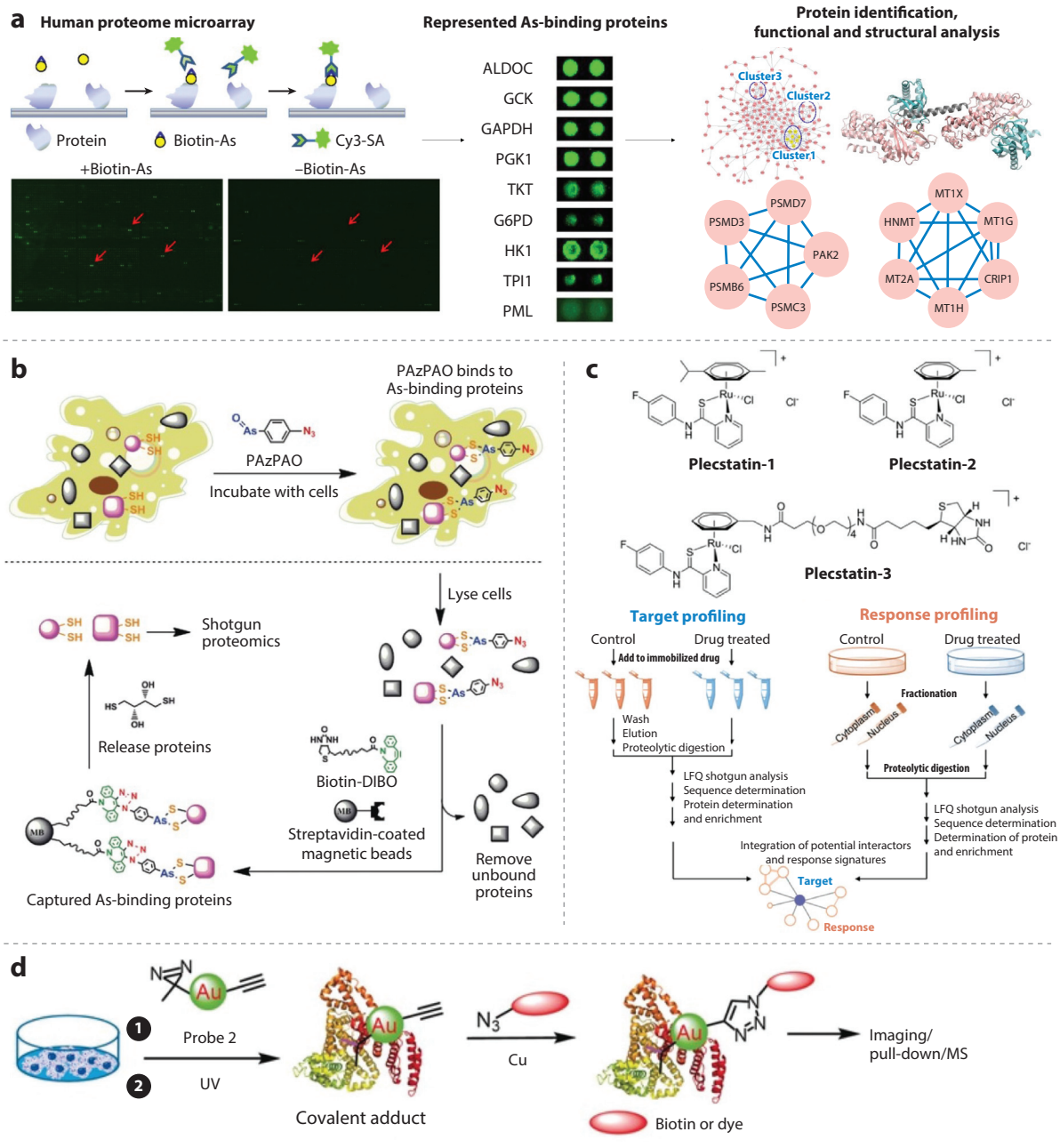


Figure 3

Integrative approaches for uncovering the mode of action of anticancer metallodrugs. (*a*) Profiling of As-binding proteins by proteome microarray. (*b*) Exploration of protein targets of As-based compounds by photoaffinity probes. (*c*) Identification of binding proteins of Ru(arene) compounds by integrating metallodrug pull-down and target-response profiling approaches. (*d*) Uncovering protein targets of anticancer Au compounds by photoaffinity probes. Abbreviations: LFQ, label-free quantification; MS, mass spectrometry. Adapted with permission from References 72, 74, 77, and 80.

proteins. Using an integrated proteomics-based target-response profiling approach (**Figure 3*c***), Gerner and coworkers (77) provided a comprehensive view of the Ru-binding proteomes in cancer cells. In this study, derivatives of Ru(arene) compounds with a hydrophilic biotin linker were immobilized on streptavidin-modified beads to capture the Ru-binding proteins from cancer cell lysates. Combined with the subsequent pull-down and target-response profiling approaches, this approach resulted in the identification of the Ru-binding proteomes. Unexpectedly, plecstatin, a Ru(arene) pyridine carbothioamide, was found to selectively bind to plectin, a scaffold protein and cytolinker that is involved in the organization of nonmitotic microtubules of colon carcinoma cells. In addition, several plectin partner proteins (e.g., receptors, keratins) were also identified in the target-response profiling network, suggesting that the Ru-based compound can interfere in the signaling pathways of cancer cells. Very recently, by integrating proteomics, transcriptomics, transmission electron microscopy (TEM), and nanoscale secondary ion MS (NanoSIMS) imaging, a Ru-based complex (KP1339) was demonstrated to directly interact with the ribosomal proteins RPL10 and RPL24, subsequently inducing ER stress and modulating GRP78 in cancer cells (78).

Au compounds exhibit various bioactivity, including antimicrobial and anticancer properties. The mechanism of action of various Au compounds has also been actively investigated by metalloproteomics approaches. Che and coworkers (79) synthesized a new clickable photoaffinity probe of gold(III) tetra-phenyl-porphyrin chloride ([Au(TPP)]Cl) through appending a linker (hexaethylene glycol), a clickable tag (alkyne), and a photoaffinity tag (benzophenone) onto one of the *meso*-phenyl rings of the porphyrin ligand of [Au(TPP)]Cl. By utilizing this probe, the chaperone protein heat shock protein 60 (Hsp60) was identified as a direct target of [Au(TPP)]Cl, which disrupts the chaperone activity of Hsp60. To further explore the molecular targets of Au(III) *N*-heterocyclic carbene (NHC) complexes, Che and colleagues (80) employed a clickable photoaffinity probe containing a small photoaffinity diazirine group and a clickable alkyne moiety onto NHC (**Figure 3*d***). Through this approach, multiple proteins, including Hsp60, vimentin, nucleophosmin, and YB-1, were identified as putative cellular targets of Au(III) NHC complexes. These proteins were also found to interact with Pt(II) and Pd(II) analogs that have the same structural geometry, suggesting the structural significance of pincer-type NHC-containing metal complexes in anticancer activity (80).

Despite recent enormous efforts, a systemic understanding of the mode of action and identification of the authentic protein targets of metallodrugs represent the major challenges in the field of metals in medicine owing to the intrinsic complexity of drug-target interactions in cells. Integration of multi-omics approaches may facilitate mechanistic studies of metallodrugs. Subsequent validation by bioassays, gene knockout, molecular biology, and structural biology is necessary to differentiate authentic from fake targets.

Metals in Neurodegenerative Disease Processes

It is well recognized that metals (e.g., Cu, Zn, Fe) are closely associated with neurodegenerative disorders, including AD, PD, and Huntington's disease (81). However, the roles of metals in the pathophysiology of disease remain elusive due to the complexity of the brain and the large numbers of metalloproteins involved in neurodegenerative diseases. Systemic investigation of metal-associated proteomes in the neurological system may provide more information to elucidate how metals modulate neurological disease processes.

A systems biological approach that integrated data mining of metal-binding proteins, the interactome, and meta-analysis was employed to explore the metalloproteomes involved in the pathogenesis of PD (82). Significant metal-associated protein hubs in PD were revealed, with an interdependency between metal concentration and gene expression being uncovered. Significant

changes in cerebrospinal fluid and serum levels of metals (Al, Ca, Cu, and Mg) suggest an ideal and feasible diagnosis method for PD. A systematically comparative analysis of metals (Fe, Cu) and their major molecular forms [ferritins, ceruloplasmin, neuromelanin, manganese superoxide dismutase (SOD), and Cu/Zn-SOD] in the locus coeruleus and substantia nigra (SN) showed that neuromelanin buffers free metals and their toxicity in both the locus coeruleus and SN and that there are higher Fe mobilization and toxicity in the SN, which may contribute to the higher vulnerability of the SN relative to the locus coeruleus in Parkinsonian syndromes (83).

Given the tight association between altered Fe homeostasis and different diseases (e.g., cancer, neurodegeneration), a first-generation bioluminescence probe, iron-caged luciferin-1 (ICL-1), was developed to image labile Fe stored in living animals (84). By using this labile Fe probe, alterations in Fe status (Fe deficiency or overload) were studied in a systemic bacterial infection model. Through combining ICL-1, ICP-MS/LA–ICP-MS, and transcriptional assays, the increased Fe accumulation in infected tissues accompanied by transcriptional changes was found to be consistent with increases in both Fe acquisition and retention. The ability to track Fe status in living animals provides a powerful technology for the investigation of Fe dysregulation in neurological disease pathogenesis. Tetrathiomolybdate (TM) is an active agent for the treatment of Cu disorders; however, how TM inhibits Cu-regulated proteins is unclear. The interaction of TM with the Cu metallochaperone Atx1, as studied by LA–ICP-MS, electrospray ionization (ESI)-MS, and crystallography, revealed that such an interaction inhibits metal transfer functions between Cu-trafficking proteins; this finding indicates that the proteins involved in metal regulation are viable drug targets (85).

Metal Homeostasis in Microbes

Although essential for living organisms, metal ions can be toxic at elevated levels. Thus, organisms have developed elaborate systems to regulate metal acquisition, storage, transport, and secretion through biosynthesis of a series of metalloproteins/chaperones (86, 87). Comprehensive characterization of cytoplasmic metalloproteins from microorganisms by metal-based identification and purification via LC, high-throughput tandem MS (HT-MS/MS), and ICP-MS revealed that metalloproteomes are much more extensive and diverse than previously recognized and are largely uncharacterized (9). Proteome-wide identification of metalloproteins may be facilitated with metalloproteomics approaches, and subsequent functional assignment of the identified metalloproteins on the basis of, for instance, changes in the structure and abundance of the involved proteins in response to metal overload and deficiency will accelerate progress toward an understanding of metal homeostasis.

Multicomponent efflux complexes constitute a primary mechanism for bacteria to expel toxic metals for survival (88). Single-molecule super-resolution imaging together with genetic engineering approaches in living *E. coli* cells revealed structural alterations in CusCBA, a tripartite RND (resistance-nodulation-division) family efflux complex, that were induced by metal stress [Cu(I) and Ag(I)] (89). The periplasmic adaptor protein CusB appears to be a key metal-sensing protein that mediates complex assembly and disassembly under metal depletion and overloading conditions, providing a new mechanism of metal homeostasis in bacteria. The factors driving Cu trafficking between protein partners were investigated by an ESI-MS-based strategy to determine the Cu(I) binding affinities of a representative set of intracellular Cu proteins involved in enzymatic redox catalysis, trafficking to and within various cellular compartments, and storage. This investigation found that affinity gradients are likely to drive Cu to cellular destinations (90). Trafficking processes of metal ions (Cu and Ag) were also studied by XAS through inserting a selenium probe (SeM) sequentially into the metal-binding sites of Cus proteins (91).

Metal-bound CusB is required for activation of the Cus efflux pump through regulation of metal ion transfer from CusF to CusA in the periplasm. Moreover, yersiniabactin (Ybt), a type of virulence-associated siderophore in uropathogenic *E. coli*, binds to Cu(II) in human urine, as revealed with the aid of a novel MS-based screening technique, LC-CNL (LC–constant neutral loss) (92). Competitive binding of Cu(II) and Fe(III) to Ybt was verified to protect uropathogens, which prevents catecholate-mediated reduction of Ybt and helps bacteria resist Cu toxicity by sequestering host-derived Cu(II). Subsequent tracking of the Cu route in Ybt by quantitative MS combined with ^{64}Cu radiolabeling revealed the transportation and conversion of Cu(II)/Cu(II)-Ybt by the FyuA-YbtPQ import system (93), which may be attributable to the minimized toxicity and nutritional prevalence of Cu in *E. coli*.

Ni participates in important biochemical processes in many microbes, in particular *H. pylori*, a pathogen that is responsible for chronic gastritis, peptic ulcers, and even stomach cancer. Ni homeostasis in microbes has been extensively studied and summarized in a number of reviews (87, 94, 95). Several approaches have been developed to identify Ni proteomes in bacterial cells (96).

Ni-based IMAC combined with 2D GE and MS enabled the identification of several Ni-interacting proteins from *H. pylori*, including proteins with cellular processes (HspA, HspB, TsaA, and NapA), enzymes (urease, fumarase, GuaB, Cad, PPase, and DmpI), membrane-associated proteins, and storage proteins (97), all of which may play important roles in Ni homeostasis. Similarly, Ni-SOD, phycoerythrin, and cysteine synthase were identified as Ni-binding proteins and other metalloproteins in the marine cyanobacterium *Synechococcus* sp. WH8102 (98). Two novel Ni-containing enzymes, alanyl-tRNA-editing hydrolase and Cupin (a putative sugar-binding protein), were also identified from *Pyrococcus furiosus* by a robust strategy using LC, HT-MS/MS, and ICP-MS (9). Recently, with the aid of a novel fluorescence-based approach using a membrane-permeable Ni^{2+}-TRACER, a total of 44 Ni-binding proteins were tracked from *H. pylori* cells, among which 11 proteins had been previously identified by IMAC (40, 44, 97, 99). Some of the identified proteins—including *Hp*UreB, *Hp*HspA, and *Hp*UreG, which play critical roles in Ni homeostasis—had been previously demonstrated to be Ni^{2+}-binding proteins on the basis of in vitro characterization (100–102). For example, *Hp*UreG, a specific Ni-dependent GTPase, plays an important role in urease maturation by interacting with other metallochaperones such as UreE (103), which also interacts with HypA to facilitate Ni transfer between these two proteins. Such Ni translocation mediated by protein-protein interactions was observed by a Ni^{2+}-TRACER fluorescence probe (104). *Hp*HspA, a GroES homolog as a cochaperonin in *H. pylori*, plays an essential role in Ni^{2+} transport as a specialized Ni chaperone protein and may also be a drug target (101, 102, 105). UreA and UreB—which are two subunits of urease, an enzyme with a critical role in *H. pylori* survival and pathogenesis—were also identified as Ni-binding proteins. Urease maturation, the process of insertion of Ni into urease, requires the cooperation of a battery of accessory proteins, including UreE, UreF, and UreG. In addition, two [NiFe] hydrogenase accessory proteins, HypA and HypB, participate in this process only in *H. pylori*, possibly owing to the extremely important role of Ni in this pathogen (94). Moreover, two Ni-binding proteins, Hpn and Hpnl, have been discovered, via different biochemical/molecular biological approaches, to be Ni storage proteins in *H. pylori* (106–108). However, these proteins were not identified by the abovementioned methodologies, possibly because these proteins are associated with inner membranes. Similarly, the Ni-responsive regulator NikR, a Ni-dependent regulatory protein that controls the activation and repression of gene transcription in *H. pylori* (109), was also not identified by metalloproteomics approaches. Therefore, the metalloproteomics approach should be employed in conjunction with other biochemical/biophysical and molecular biology approaches to elucidate metal homeostasis in organisms.

EMERGING SINGLE-CELL METALLOPROTEOMICS

The cell is a highly heterogeneous and dynamic system whose components change over time and space. Bulk analysis, which uses stochastic average results to demonstrate biological phenomena, fails to illustrate cell-to-cell variation and events that occur in different cell subpopulations. A recent focus is studying individual cells. Although metalloproteomics study at the single-cell level is in its infancy, recent advances in techniques for single-cell analysis will facilitate metalloproteomics study in heterogeneous multicellular tissues and organisms.

Time-Resolved ICP-MS

Despite the wide application of ICP-MS for elemental quantification in bulk solution, ICP-MS for single-cell analysis has become increasingly appreciated. Time-resolved ICP-MS (TR-ICP-MS) measures elements on a cell-by-cell basis through modulating the sampling system, which has been successfully utilized to track the uptake of metallodrugs at the single-cell level in different cell lines (110), in cells at different states (111), and in cells with varied drug sensitivity (112).

TR-ICP-MS (113) was used to monitor the uptake of Bi-based drugs in single *H. pylori* cells, and intracellular Fe uptake was disrupted by Bi; this result was consistent with the binding of Bi(III) to transferrin/lactoferrin (114) inducing Fe deprivation in the pathogen. In addition to being used in direct tracking of metallodrug uptake, TR-ICP-MS has been used to study the intrinsic mechanism of action of ATO through quantification of specific cell viability indicators, namely (*a*) cisplatin levels and (*b*) apoptosis- and differentiation-related marker proteins conjugated with lanthanide tags (**Figure 4*a***) (110). By monitoring intracellular drug levels and cellular status, a relationship between intracellular As levels and ratios of death, apoptosis, and differentiation of cells was revealed, suggesting a positive correlation between intracellular As content and cytotoxicity. Given the critical role of the cell cycle in cancer development and therapy, the uptake of As-based drugs (ATO and ZIO-101) in single leukemia cells across the cell cycle was further evaluated by using double thymidine block together with TR-ICP-MS to synchronize cells (**Figure 4*a***) (111). The observed cell cycle–dependent uptake and cytotoxicity of As-based drugs suggest that the cell cycle may need to be taken into account to precisely evaluate the uptake and cytotoxicity of anticancer drugs. Thus, the efficiency of anticancer drugs may be enhanced in combination with cell cycle perturbation agents.

Mass Cytometry

As a superior emerging technique for single-cell analysis, mass cytometry, also referred to as CyTOF, addresses the challenge of multiparametric analysis in TR-ICP-MS. Mass cytometry is a next-generation flow cytometry technique in which antibodies are labeled with heavy metal ion tags, usually lanthanides, allowing for the combination of many more antibody specificities (theoretically >100) in a single sample (e.g., a single cell) (115). The advent of mass cytometry dramatically broadens the application of ICP-MS and the utilization of metals for single-cell bioassays (116). Mass cytometry has been used to examine the cellular response of metallodrugs. For example, through direct quantification of cisplatin accumulation by mass cytometry, a set of compounds that inhibit cisplatin efflux by the ABCC2 pump were investigated regarding their ability to ameliorate cisplatin resistance (117). Elevated cisplatin uptake and sensitizing of cisplatin-resistant cells were observed for certain tested compounds, providing a promising method for drug screening to combat drug resistance. Furthermore, through quantification of cisplatin in individual tumor cells, in combination with the cell cycle and cell proliferation biomarker IdU, dose- and time-dependent cell cycle arrest induced by cisplatin was observed in cisplatin-treated BxPC-3 and ME-180 xenografts (118).

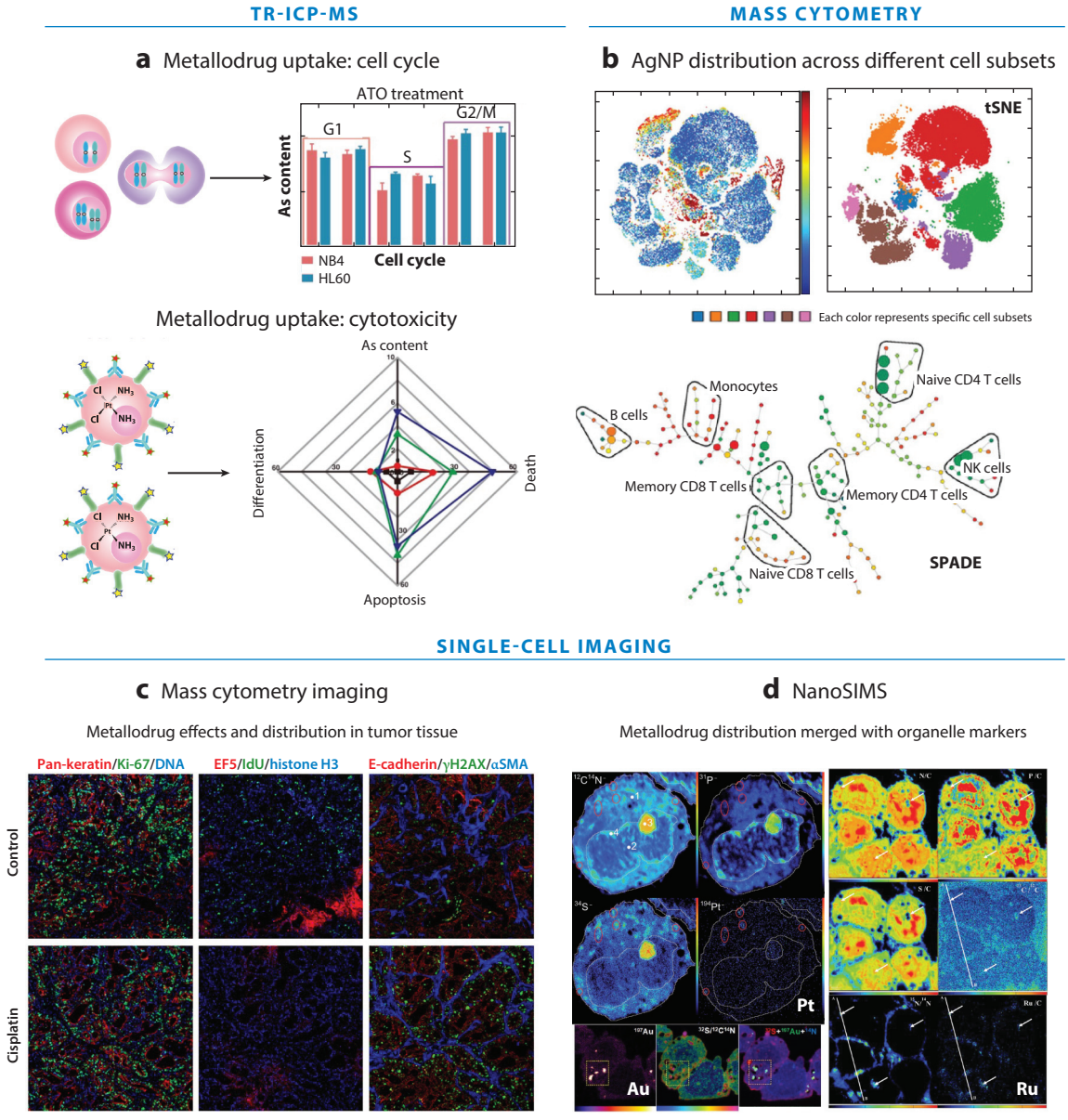


Figure 4

Emerging single-cell techniques for metalloproteomics study. (*a*) Tracking uptake of metallodrugs (here, As) in cells to decipher the correlation of metallodrug uptake and cell cycle stage/metallodrug cytotoxicity. (*b*) Multiplex assay by mass cytometry for identification of intracellular metal distribution in different cell subsets. (*c*) Uncovering the distribution and effects (proliferation, DNA damage, target proteins) of metallodrugs (here, cisplatin) in a tumor. (*d*) Cellular distribution of different metallodrug candidates (Pt-, Ru-, and Au-based therapeutic agents) merged with organelle markers by NanoSIMS. Abbreviations: AgNP, silver nanoparticle; ATO, arsenic trioxide; NanoSIMS, nanoscale secondary ion MS; SPADE, spanning-tree progression analysis of density-normalized events; TR-ICP-MS, time-resolved inductively coupled plasma mass spectrometry; tSNE, t-distributed stochastic neighbor embedding. Adapted with permission from References 110, 111, 122, 125, 127, 128, and 133.

In addition, mass cytometry has been used in NP-related research. As measured by mass cytometry, Ag in single bacterial cells at different physiological states treated with AgNPs or $AgNO_3$ has a microenvironment-dependent heterogeneous distribution among cells in a population (119). Mass cytometry was also applied to investigate the interaction of NPs with immune cells, justifying their potential utilization in biomedical applications (120), as well as to track inorganic NPs (e.g., AuNPs) in tandem with highly multivariate cellular phenotyping to evaluate the in vivo fate of inorganic nanomedicines (121). The cell type–dependent distribution of NPs provides a guideline for the design of novel NP-based therapeutics. In another study, AgNPs were demonstrated to preferentially accumulate in monocytes and B cells relative to other populations (**Figure 4*b***) (122). In combination with single-cell RNA sequencing, the distinct responses of these two cell types to AgNPs—i.e., NRF2-mediated oxidative stress in B cells and NFAT-regulated immune response and Fcγ-mediated phagocytosis in monocytes—were elucidated, providing deep insight into how AgNPs perturb the immune system at a molecular level.

Through the integration of TR-ICP-MS, mass cytometry, and synchrotron XAS, the uptake, toxicity, and detoxification mechanisms of AgNPs were recently investigated in human T lymphocytes (123). TR-ICP-MS enabled cell-internalized AgNPs to be identified and characterized, mass cytometry quantified cell-associated Ag, and XAS revealed the corresponding chemical speciation and distribution of cell surface–bound and intracellular Ag, revealing the interactions and biotransformation of AgNPs in and around human T lymphocytes. Cell-internalized Ag is the main driving force of AgNP toxicity, while Ag_2S and Ag–cysteine are the major transformed forms, with the extent of transformation being dependent on size and surface functionality.

Although the application of mass cytometry to single-cell metalloproteomics study is in its infancy, the powerful multiplex capability of mass cytometry endows it with great potential for the simultaneous examination of (*a*) metal distribution in cells of different types or statuses and (*b*) multifaceted effects of metals on cells based on proteomics information, which will facilitate the elucidation of the molecular mechanisms of action of metals in life processes in the near future.

Single-Cell Multiplex Imaging

Organs and tissues are made up of multiple cell types in a unique microenvironment consisting of matrices, fluids, and other cells. A comprehensive understanding of biological events, especially for the immune response, cannot be achieved without the exploration of cellular behavior in its natural state. The importance of in situ quantification of different biomolecules at the single-cell level is becoming increasingly recognized, given that analysis in a native environment will result in more accurate information.

Mass cytometry imaging. Imaging mass cytometry (IMC) enables highly multiplexed imaging at the single-cell level through combination of a LA system with mass cytometry (124). Multiplex imaging by IMC provides a comprehensive, spatially resolved view of cell types and their states in tissues or organs and shows enormous potential to guide therapeutic design and clinical outcomes through evaluation of therapeutic and immune responses. IMC in metalloproteomics study is currently focused on elucidating the fate of metallodrugs in cells.

Mass cytometry was used to directly visualize Pt localization in malignant or normal tissues of cisplatin-treated mice (**Figure 4*c***). In combination with multiple markers to define cell lineage, the DNA damage response, cell proliferation, and functional status, a holistic view of the cellular response to cisplatin was revealed, demonstrating extensive binding of Pt to collagen fibers and the slow release of stroma-bound Pt (125). The preferential binding of cisplatin to collagen-rich structures was also observed in gastric cancer tissues (126).

Nanoscale secondary ion mass spectrometry. NanoSIMS is an emerging technique for in situ elemental and isotopic analysis, with extremely high resolution at a single-cell level. This technique's extremely high resolution, sensitivity, and multiplex capability endow it with great potential to track the cellular uptake and subcellular distribution of different metallodrugs, e.g., Pt (127) and Ru (128).

A major advantage of NanoSIMS in metal-based study is simultaneous visualization of ligands labeled with metals and isotopes (e.g., ^{13}C, ^{15}N). This approach allows the dynamic transformation of metallodrugs in cells to be unveiled. Furthermore, different organelles can be simultaneously visualized with metallodrugs through isotope (e.g., ^{13}C, ^{15}N) labeling, facilitating the visualization of interactions between metallodrugs and cellular components (**Figure 4*d***). As revealed by NanoSIMS, Pt in cisplatin predominantly accumulates in S-rich structures in the nucleus and cytoplasm of human colon cancer cells (127). The colocalization of Pt with phosphorus-rich chromatin regions provides further evidence of cisplatin as a DNA-targeting agent (129). Confocal laser scanning microscopy and NanoSIMS analysis showed that Pt–N bonds partially dissociate during the accumulation process, and this finding may shed light on the mechanism of action of cisplatin. The different processing of Pt drugs in acidic organelles may be related to their detoxification and modes of action. The distribution of Pt in kidney and tumor samples of mice treated with two investigational Pt(IV) anticancer compounds was imaged by the combination of LA–ICP-MS, TEM, and NanoSIMS, showing the accumulation of Pt in cytoplasmic S-rich organelles in both kidney and malignant cells (130). The preferential aggregation of oxaliplatin (131) and other new Pt(II) anticancer compounds in cytoplasmic organelles was also validated by NanoSIMS (132). By integration of NanoSIMS and electron microscopy (EM), a Au-based compound (AuMesoIX) was recently found to target cellular thiol proteins in the cytosol of cancer cells (133).

Clearly, these single-cell imaging techniques are very powerful tools not only for elucidating metallodrug accumulation in cells or organs but also for understanding the mode of action of metallodrugs, and such techniques should find more application in future metal-based research.

CONCLUSION AND PERSPECTIVES

Given the fundamental roles that metals play in biological and (patho)physiological processes, comprehensive elucidation of the mode of action of metals will improve our understanding of metal-involved life processes, thus positively impacting human health and well-being. The intrinsic complexity of metal-related activities requires multiple techniques to comprehensively characterize the biomolecules that interact with metals. As an emerging field of research, metalloproteomics integrates the advantages of metallomics and proteomics and has become an increasingly important strategy to decipher the molecular mechanisms of metal-based life processes.

Although substantial progress has recently been made in deciphering how metals function, extensive metalloproteomes remain largely uncharacterized in many organisms. The integration of multi-omics will be an efficient strategy to provide multifaceted knowledge of metal-involved events. Additionally, a combination of techniques from multiple disciplines (e.g., molecular biology, genetics, computer sciences, biophysics/biochemistry) will facilitate the comprehensive characterization of metalloproteomes to better understand the roles of metals in biology and medicine. Furthermore, the complex data derived from integrated techniques, multi-omic approaches, and single-cell metalloproteomics pose a challenge for subsequent data clarification. As an indispensable tool in the current big data era, artificial intelligence has been incorporated into metalloproteomics study as a potent tool for target screening, identification, and prediction (134) and will stimulate exciting new findings in these fields. More importantly, continued

developments in proteomics (135, 136), single-cell analysis (137, 138), and computer science (139, 140)—as well as the emergence of new advanced technologies such as multiplexed ion beam imaging (124, 141, 142), cryo-EM (143), and single-cell RNA sequencing (144)—will boost metalloproteomics-related research, facilitating unprecedented discovery of the roles that metals play in life processes and providing a solid basis for systems biology and precision medicine.

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