— **Review** —

Research Tools and Techniques for Copper Metabolism in Mammals

Yasumitsu Ogra*

Laboratory of Chemical Toxicology and Environmental Health and High Technology Research Center, Showa Pharmaceutical University, 3–3165 Higashi-Tamagawagakuen, Machida, Tokyo 194–8543, Japan

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Copper (Cu) is an essential component of biological redox reactions and its deficiency is fatal to the body. At the same time, Cu is extremely toxic when present in excess. In this regard, several groups of Cu-regulating proteins in the body act to regulate the concentration of Cu within a certain range. However, the overall mechanism underlying the maintenance of Cu homeostasis in the body and cells remains poorly understood. In this review, recent research tools, such as animal models and gene-modified animals, and techniques, such as speciation and imaging of Cu, are highlighted.

Key words —— copper, chaperone, speciation, imaging

INTRODUCTION

Copper (Cu) is an essential metal for organisms that utilize oxygen for respiration, and is required as a cofactor of redox-regulating enzymes, such as Cu,Zn-superoxide dismutase (Sod1), ceruloplasmin, lysyl oxidase, tyrosinase, and dopamine β-hydroxylase.[1, 2] However, the redox-active property of this metal may have toxic effects on cells due to the generation of harmful reactive oxygen species (ROS).[3, 4] Cu in the body is present in the mono (cuprous, Cu^{+}) or divalent (cupric, Cu^{2+}) form. Cuprous ions are readily oxidized to cupric ions and Cu cannot exist as cuprous ions without being coordinated by appropriate ligands. In other words, Cu in the monovalent form readily reduces chemicals, as in the case of the production of ROS. Given these circumstances, it is said that cells have a dependable system for Cu homeostasis that efficiently distributes this essential metal to cuproenzymes, thereby avoiding damage to proteins, DNA, sugars, and lipids. In particular, the influx, efflux, and intracellular distribution with fixation of the oxidation state of Cu are strictly regulated.

As mentioned above, Cu is an essential component of biological redox reactions and its deficiency is fatal to the body. At the same time, Cu is extremely toxic when present in excess. In this regard, the body has to regulate the concentration of Cu within a certain range. The body utilizes Cu efficiently in a Cu-deficient state and detoxifies it when present in excess. Several groups of Cu-regulating proteins have been identified in mammalian cells as mentioned above (Fig. 1).

The first group consists of Cu transporters that transport Cu across the plasma membrane. Copper transporter 1 (Ctr1) is an integral membrane protein that is structurally and functionally conserved from yeast to human, and is a high-affinity

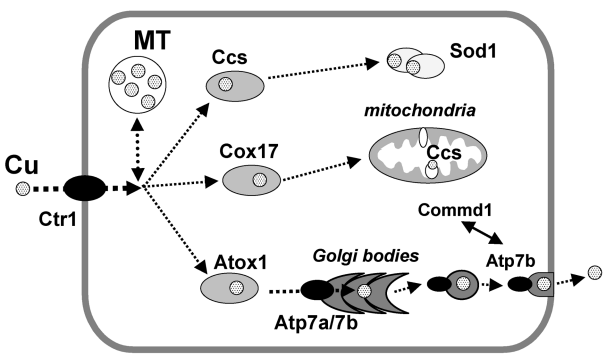

Fig. 1. Proposed Scheme of Mechanisms Underlying Cu Metabolism in a Mammalian Cell

*To whom correspondence should be addressed: Laboratory of Chemical Toxicology and Environmental Health and High Technology Research Center, Showa Pharmaceutical University, 3–3165 Higashi-Tamagawagakuen, Machida, Tokyo 194–8543, Japan. Tel. & Fax: +81-42-721-1563; E-mail: ogra@ac.shoyaku.ac.jp

Cu importer into eukaryotic cells.[5–7] Ctr2, which has high homology to Ctr1, has been identified in mouse and human.[8] Initially, Ctr2 was recognized as a low-affinity Cu importer. However, it was suggested that Ctr2 has different functions from Ctr1; namely, Ctr2 is localized not only in the plasma membrane but also in the surface of late endosome and lysosome, although its function is still unclear.[9, 10] The expression of Ctr1 was increased under the Cu-depleted state, whereas that of Ctr2 was markedly suppressed by the Cu depletion.[11, 12] Thus, the putative function of Ctr2 may be different from that of Ctr1. Cu-transporting P-type ATPases, *i.e.*, Atp7a and Atp7b, are expressed on the Golgi apparatus and participate in the efflux of Cu from cells.[13] Atp7a is expressed in all tissues except liver, whereas Atp7b is expressed primarily in the liver. As discussed in greater detail in the later section, disorders of ATP7A and ATP7B in human result in Cu deficiency [Menkes disease and occipital horn syndrome (OHS)] and Cu toxicosis (Wilson disease), respectively.[14–19] In addition to the efflux of Cu, this pathway is also involved in the secretion of cuproenzymes, including lysyl oxidase, extracellular Sod, tyrosinase, and ceruloplasmin. These cuproenzymes receive Cu in the Golgi apparatus and traverse the secretory pathway after protein maturation, *i.e.*, post-translational modification. Thus, the dysfunction of this pathway results in the loss of activity of secretory cuproenzymes.

The second group consists of intracellular Cu delivery proteins, or the so-called "Cu chaperones." Cu transported by Ctr1 associates with one of three Cu chaperones, Atox1, Cox17, or copper chaperone for SOD1 (Ccs), to be escorted to organelles or cuproenzymes. First, Atox1 hands over Cu to Atp7a and Atp7b expressed on the surface of the Golgi apparatus.[20–22] Atox1 coordinates Cu^+ to transfer Cu^+ to a Cu-binding domain repeat with the consensus GMTCXXC in the N-terminus of Atp7a and Atp7b.[23] Second, Cox17 is required to load Cu to cytochrome *c* oxidase (Cco) via SCO1 and Cox11, which are recipient proteins of Cu on the mitochondrial inner membrane.[24, 25] The molecular mechanisms underlying Cu delivery in the mitochondrial intermembrane space have been demonstrated.[26, 27] Third, Ccs delivers Cu to Sod1 in cytosol by forming a heterodimer between itself and Sod1.[28–30] Cu is transferred from Ccs to Sod1 by a series of ligand exchange reactions between Ccs and Sod1.[31, 32] Alternative pathways for Cu loading onto Sod1 are speculated because tissues from $Ccs^{-/-}$ mice and yeast ccsΔ strain have significant levels of Sod1.[33]

The third group of Cu-regulating proteins is metallothioneins (MTs), in particular, MT-I and MT-II, or the so-called classical MTs. MTs actually bind excess intracellular Cu via Cu-thiolate clusters to mask the toxicity.[34] It has been also suggested that MTs alleviate Cu deficiency by maintaining intracellular Cu concentration. Thus, MTs may play a dual role in Cu homeostasis in mammalian cells.[35]

The fourth group includes a novel type of Cu-regulating protein that was recently characterized, *i.e.*, Cu metabolism gene Murr1 (mouse U2af1-rs1 region 1) domain 1 (Commd1).[36] Although it does not have any apparent Cu^+-binding motifs in its molecule unlike the Cu-regulating proteins in the first three groups, it is reported that Commd1 binds Cu^{2+} [37, 38] and is implicated in the Cu efflux pathway by cooperating with Atp7b.[39] The dysfunction of Commd1 causes Cu toxicosis that results from abnormal Cu accumulation in the liver of Bedlington terriers.[40–45] Whether Commd1 is directly or indirectly involved in Cu homeostasis is still a controversy.

As mentioned above, Cu is an essential element and yet a harmful metal in the body and cells. Thus, it needs to be strictly controlled by several Cu-regulating factors. However, the overall mechanisms underlying Cu homeostasis in the body and cells are poorly understood and many researchers are groping for ways to unravel the mechanisms at the molecular level. Molecular-biological techniques to analyze genes and proteins have provided many new insights in this research field. In addition, techniques for the direct detection of Cu are expected to pave the way toward further insights. In this review, recent research tools and techniques for Cu metabolism are highlighted.

RESEARCH TOOLS

Spontaneous Mutants and Gene-modified Animals

Atp7a: The Atp7a gene maps to X chromosome and human Atp7a gene (ATP7A) encodes a protein that is 1500 amino acids in length and has a molecular weight of 165 kDa.[15, 16, 19] The protein is expressed in all tissues except the liver. Menkes disease is a severe neurodegenerative disorder arising from a defect in ATP7A. OHS is also caused by the same genetic defect as Menkes disease but shows less severe clinical manifestations than Menkes dis-

ease. Mutations in the Atp7a gene, the mouse homologue of the ATP7A gene, have been suggested to be responsible for the mottled, blotchy, and brindled phenotypes.[46–48] As the defect in Atp7a causes systemic Cu deficiency, these phenotypes are a result of the dysfunction of the cuproenzyme tyrosinase, which is involved in the biosynthesis of the pigment, melanin. Macular mouse is another strain that has a defect in Atp7a.[49]

Atp7b: The Atp7b gene maps to autosome and human Atp7b gene (ATP7B) encodes a protein that is 1411 amino acids in length.[14, 17, 18] The Atp7b protein is predominantly expressed in the liver, kidney, and placenta. Wilson disease arises from a defect in ATP7B and shows Cu toxicity primarily in the liver.[50] It was reported that the long Evans rat with a cinnamon coat color (LEC rat) showed spontaneous development of fulminant hepatitis and liver cancer. The LEC rat also presented with abnormal Cu accumulation in the liver and a remarkable decrease in ceruloplasmin activity.[51, 52] A partial deletion at the 3′ end of the Atp7b gene was found in the LEC rat.[53] The toxic milk mouse is also known to spontaneously present with Cu accumulation in the liver and decreased ceruloplasmin activity.[54, 55] Identification of the causative mutation in murine Atp7b gene has proved that toxic milk mouse is also a true model of Wilson disease.[56]

Commd1: Bedlington terrier is a canine breed that presents with spontaneous Cu toxicosis characterized by massive Cu accumulation in the liver to result in chronic hepatitis and cirrhosis.[57, 58] Unlike Wilson disease and it animal models, plasma ceruloplasmin level in Bedlington terrier is normal. This indicates that Cu transport to the Golgi apparatus is not defective. However, Cu excretion from the trans Golgi network to the plasma membrane may be defective. Commd1 was identified as the responsible gene for Cu toxicosis in Bedlington terrier.[36, 59] Dogs show different Cu metabolism from other mammals: although non-ceruloplasmin-bound Cu is mainly bound to albumin in most mammals, it is bound to amino acids in dogs as canine albumin lacks a specific Cu binding site because of a mutation of histidine to tyrosine in the site, and the physiological hepatic Cu level in dogs is high compared to other mammals.[60–62] Thus, the clinical manifestations of the defect in Commd1 may be canine-specific. Indeed, Commd1-null mice showed different symptoms from Bedlington terrier. Commd1-null embryos died *in utero* between 9.5 and 10.5 days postcoitum.[63]

MT: Mice deficient in two major MT isoforms (MT-1 and MT-2) were established.[64] However, fibroblasts established from the MT-null mice did not show higher sensitivity to Cu exposure than wild-type cells.[65] On the other hand, to determine the function of MT in the presence of Atp7a deficiency, Atp7a-deficient (Mo-brJ) females were crossed with MT-null males. Most Mo-brJ males as well as heterozygous Mo-brJ females with an MT-null background died before embryonic day (E) 11.[66] This suggests that MT plays a significant role in Cu homeostasis. Thus, the role of MT in Cu metabolism continues to be an enigma.

Others: Ctr1 mediates Cu influx via high-affinity uptake on the plasma membrane.[67] Ctr1-null mouse showed embryonic lethality due to severe Cu deficiency. Because of this, a mouse bearing conditional knockout in the intestinal epithelium was developed.[68–70] The mouse presented with systemic Cu deficiency because of a defect in Cu uptake from feeds by the intestinal epithelium.[71]

Atox1-null mice failed to thrive immediately after birth, with 45% of pups dying before weaning. Surviving animals exhibited growth failure, skin laxity, hypopigmentation, and seizures because of perinatal Cu deficiency.[72] These clinical features resembled or were more severe than those of mice having defective Atp7a because Atox1 functions at the upstream of Atp7a, *i.e.*, the Cu chaperone for Atp7a. Furthermore, Atox1-deficient cells accumulated high levels of intracellular Cu and metabolic studies have indicated that this defect was because of impaired cellular Cu efflux.[73]

Although mouse embryos homozygous for Cox17 disruption developed normally up to E6.5, they died between E8.5 and E10.[74] Cox17-null embryos exhibited severe reductions in Cco activity at E6.5. Cco is one of the key enzymes in the respiratory chain in mitochondria. Thus, the dysfunction of Cco is critical for the embryos.

Ccs-null mice are viable and possess normal levels of Sod1 protein. However, they exhibited marked reductions of Sod1 activity compared with control littermates.[28] Metabolic labeling with ^{64}Cu has demonstrated that the reduction of Sod1 activity in Ccs-null mice is the direct result of impaired Cu incorporation into Sod1 and that this effect was specific because no abnormalities were noted in Cu uptake, distribution, or incorporation into other cuproenzymes. Consistent with the loss of Sod1 activity, Ccs-null mice showed increased sensitivity to paraquat and reduced female fertility, phenotypes

that are characteristic of Sod1-null mice.[33)]

Antibodies

Antibodies against all Cu-regulating factors, *i.e.*, Atp7a, Atp7b, Ctr1, Commd1, MT, Atox1, Cox17, and Ccs, are commercially available. In addition, antibodies against Ctr1,[5,75)] Atp7a,[76)] Atp7b,[77)] Atox1,[21)] Cox17,[78)] and Ccs[79)] were produced by researches.

Cu Chelators

Penicillamine/trientine: Penicillamine[80–82)] and trientine[83–85)] are used to treat Wilson disease in the clinical setting (Fig. 2). These chelating agents should be taken on an empty stomach because they interfere with the absorption of Cu from foods. Urinary Cu excretion is enhanced by the treatment with these agents.[86)] Trientine shows less toxicity and effectiveness than penicillamine.[85)] Penicillamine is also used to treat rheumatoid arthritis and kidney stones (cystinuria).

Tetrathiomolybdate: Tetrathiomolybdate (TTM) is a molybdenum-containing molecule that has strong and specific affinity for Cu. It is also used in the clinical setting to treat Wilson disease with neurological manifestations.[87–91)] Cu that accumulates in the liver of Wilson disease patients and their animal models, such as LEC rats, is predominantly bound to MT. The affinity of Cu for MT is known to be too high, rendering both penicillamine and trientine ineffective. However, TTM is able to remove Cu from MT.[92–96)] The mechanism of Cu removal from MT by TTM has been shown.[94,97,98)] Recently, the effects of TTM as a therapeutic agent for Huntington disease were evaluated.[99)]

Bathocuproine Sulfonate (BCS): BCS is a water-soluble and Cu(I)-specific chelator.[100,101)] Although BCS is not used for Cu toxicosis in the clinical setting, it can induce to the Cu deficiency in cultured cells.[35)] The effect of BCS on Alzheimer's disease was evaluated.[102)]

RESEARCH TECHNIQUES

Speciation

According to the recommendations of the International Union of Pure and Applied Chemistry (IUPAC) interdivisional working party, "speciation" is described as the distribution of an element amongst defined chemical species in a system.[103)] A species is defined as a form of an element specified on the basis of its isotopic composition, electronic or oxidation state, and/or complex or molecular structure. The term "speciation analysis" denotes the identification and/or measurement of the quantities of one or more chemical species in a sample.[103)] To achieve speciation analysis, hyphenated techniques are used.[104,105)] The term of "hyphenated technique" was introduced by Hirschfeld[106)]

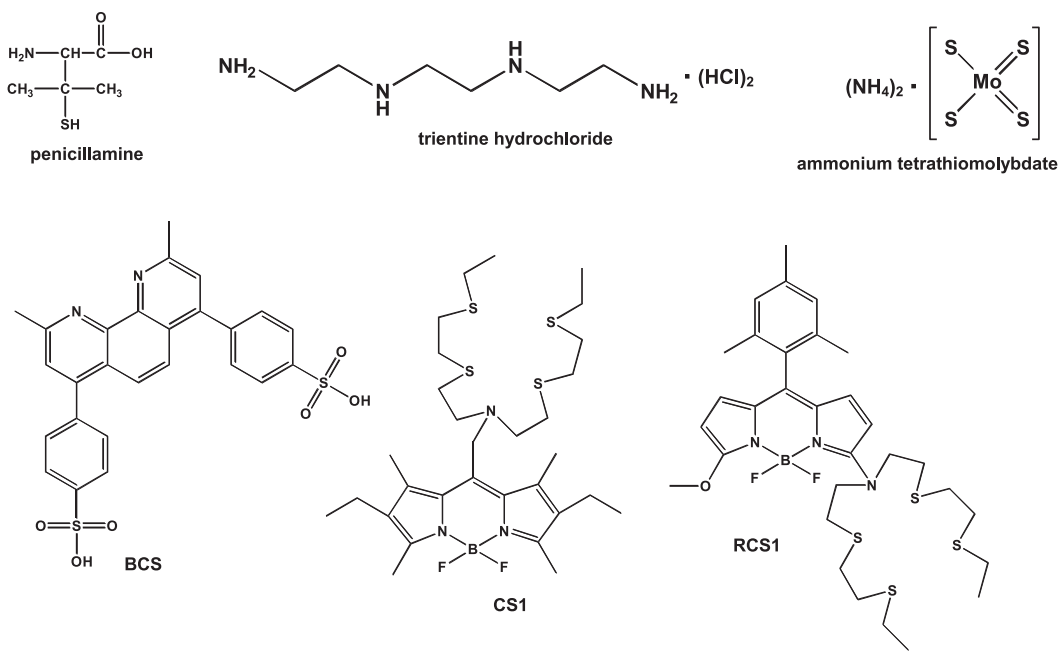

Fig. 2. Structures of Cu Chelators and Fluorescent Probe

and refers to an on-line combination of a chromatographic or an electrophoretic separation technique with a sensitive and element-specific detector, such as an atomic absorption photometer, an atomic emission photometer, or a mass spectrometer. For Cu speciation, an inductively coupled plasma mass spectrometer (ICP-MS) presents several advantages over other detectors in terms of sensitivity for the discrimination of Cu isotopes, *i.e.*, ^{63}Cu and ^{65}Cu. Indeed, Cu speciation in biological samples by an HPLC-ICP-MS (an HPLC hyphenated with an ICP-MS) was reported.[107–110] In particular, abnormal Cu metabolism in the liver of LEC rats was shown by HPLC-ICP-MS.[111–114] The mechanism underlying the removal of Cu from MT by TTM was also revealed by HPLC-ICP-MS.[94]

Although HPLC-ICP-MS is a unique, sensitive, and robust technique for biological samples, it has two inevitable disadvantages. First, it requires a substantial volume of sample of μl order when a conventional HPLC column is adopted. When we used conventional HPLC-ICP-MS in our previous experiments, the injection volume and the flow rate were 20–200 μl and 0.6–1.0 ml/min, respectively. As the flow rate of conventional HPLC is comparable to the flow rate for sample introduction into an ICP-MS, the eluate can be directly introduced into an ICP-MS without splitting or addition of sheath flow. This is considered to be both a strong point and a weak point. The direct introduction does not reduce the sensitivity of ICP-MS; however, the requirement for such a large volume of sample limits the applicability of conventional HPLC-ICP-MS. Indeed, samples that can be acquired in large amounts, such as blood plasma, tissue extract, and urine, have been analyzed by conventional HPLC-ICP-MS. Second, HPLC-ICP-MS can be used to analyze soluble samples, such as tissue supernatant. However, because Cu-containing biomolecules exist not only in the soluble fraction but also in plasma membrane and organelles, the determination of Cu distributed to the insoluble fraction is necessary. To overcome the disadvantages, other techniques have been recently developed.

Micro HPLC-ICP-MS (*Nano-speciation*): As pointed out above, the speciation analyses of such biological samples as blood plasma, tissue extract, and urine using conventional HPLC require relatively large volumes of sample of μl order. However, samples with limited volumes, including extracts of gene-modified cells, digested spots from two-dimensional (2D) electrophoresis, tissue biopsy extracts, and fetus/neonate, are not applicable to conventional HPLC-ICP-MS. Thus, an analytical technique for samples having ultra-small volumes, *i.e.*, micro/capillary HPLC-ICP-MS, is needed in place of conventional HPLC. The flow rate of micro/capillary HPLC is of the order of several μl/min, which is too low to allow direct connection to a conventional nebulizer, and the large dead volume (40–100 cm^3) of the most commonly used double-pass Scott spray chamber results in long washout times and peak broadening. To this end, a specialized interface between a micro/capillary HPLC and an ICP-MS consisting of a total consumption micronebulizer operating at flow rates in the range 0.5–7.5 μl/min and a small dead volume spray chamber were developed.[115, 116]

Using the interface mentioned above, it was reported that MT-isoform-specific knockdown was observed by reverse transcription (RT)-PCR and 2D micro HPLC-ICP-MS. 2D micro HPLC-ICP-MS, which consists of a gel filtration column and an anion-exchange column, was an effective tool to separate MT isoforms prepared from cultured cells.[117] Indeed, a 100 nl aliquot of a cell supernatant was sufficient for injection into the column (thus, the technique was called nano-speciation), suggesting that the minimum cell number required for our 2D micro HPLC-ICP-MS system was 2.0×10^3. This is the first report of the combination of nano-speciation with the RNAi technique. The techniques shown in this study are expected to contribute to clarifying the physiological and/or biological roles of MT isoforms. Moreover, as the RNAi technique has wide-ranging applications, the combination of nano-speciation with the RNAi technique may open new doors in the study of metallomics. However, one drawback of this system is that it focuses on the separation of two MT isoforms. On the other hand, one-dimensional (1D) separation with a gel filtration column is robust and suitable for screening Cu distribution in tissue supernatants. In this sense, to improve separation on 1D gel filtration HPLC, HPLC-ICP-MS equipped with a narrow bore gel filtration column was developed to analyze a minute amount of tissue extract, and the relationship between the amount of Cu in the MT-bound form (Cu-MT) and MT mRNA expression was evaluated to reveal Cu metabolism in a mutant animal model. A hemizygote bearing a mutation in Atp7a located on the X chromosome, *i.e.*, the male blotchy mouse, showed typical symptoms of Cu deficiency. Due to the Cu de-

ficiency, the mouse showed severe growth retardation and died before weaning. As the organs of this neonatal mouse were too small to be analyzed by conventional HPLC-ICP-MS, narrow bore HPLC-ICP-MS was used and the injection volume and the flow rate were set at 1.0–5.0 μl and 40 μl/min, respectively. Narrow bore HPLC-ICP-MS revealed that all the examined organs of the male blotchy mouse presented with systemic Cu deficiency except the kidneys.[118] The kidney accumulated Cu in the form bound to MT. The nano-speciation of Cu with capillary/micro/narrow bore HPLC is a useful technique that enables observation of unique Cu metabolic processes.

Cu Imaging

The bioimaging of metals/metalloids, which involves mapping of the distribution of metals/metalloids in tissue and cell specimens, is an emerging technique that is expected to overcome the disadvantages of speciation. Metal bioimaging techniques are divided into two categories. One category includes techniques that use specific analytical instruments. For instance, laser ablation coupled with an ICP-MS (LA-ICP-MS), scanning X-ray fluorescence microscopy (SXFM), and secondary ion mass spectrometry (SIMS) belong to this category. Several excellent reviews for LA-ICP-MS,[119–121] SXFM,[122,123] and SIMS[124] have appeared. Some studies of Cu imaging are likewise available: Cu distribution in the brains of aged mice was analyzed by LA-ICP-MS,[125] and Cu, Zn, and Fe distributions in fibroblasts established from Atox1-deficient mice were visualized by SXFM.[126] However, these techniques require special instrumentation, *e.g.*, a synchrotron source for SXFM and a laser ablation system with an interface to ICP-MS for LA-ICP-MS. Thus, these techniques do not seem to be viable for general use.

Cu(I)-specific Fluorescent Probe: Contrary to the imaging techniques mentioned above, fluorescent probes and fluorescence microscopes are familiar to many biological and biochemical researchers. The imaging of living cells with a Cu-specific fluorescent probe offers a potentially powerful method for studying Cu metabolism. Yang *et al.* reported 4-3-phenyl-5-[4-(1,4,7,10-tetrathia-13-aza-cyclopentadec-13-yl)-phenyl]-4,5-dihydro-pyrazol-1-yl-benzoic acid (CTAP-1), the first Cu(I)-responsive probe for cellular use.[127] CTAP-1 uses a pyrazoline dye platform appended to an azatetrathiacrown receptor for Cu(I) coordination. Upon UV excitation at 365 nm, the probe produces up to 4.6-fold fluorescence turn-on response in the presence of Cu(I). Fixed NIH 3T3 fibroblasts grown in the presence of elevated levels of Cu showed greater fluorescence in the presence of CTAP-1 than cells grown in basal medium.[127] In parallel with the study mentioned above, another new type of Cu(I)-specific fluorescent probe with visible excitation and emission was reported for cellular Cu imaging.[128,129] 8-[*N*,*N*-Bis(3′,6′-dithiaoctyl)-aminomethyl]-2,6-diethyl-4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3*a*,4*a*-diaza-*s*-indacene [Coppersensor-1 (CS1)] combines a 4,4-difluoro-4-bora-3*a*,4*a*-diaza-*s*-indacene (boron-dipyrromethene, BODIPY) reporter and a thioether-rich receptor to achieve selectivity and sensitivity for Cu(I) over other biologically relevant metal ions. CS1 also exhibits ten-fold turn-on response and picomolar affinity for labile Cu(I) (Kd = 3.6 pM) in aqueous solution. Confocal microscopy experiments in human embryonic kidney (HEK) 293T cells have established that CS1 can respond to changes in labile intracellular Cu concentrations in living samples.[128] Cu(I) distribution in Atox1 or Commd1 knockdown cells was also determined by CS1.[130,131] An improved CS1, rational CS1 (RCS1), is also reported, which may be applicable to the ratiometric imaging of Cu(I).[132]

CONCLUSION

The techniques and tools for metallomics, including Cu biology, are undergoing continuous development. Nano-speciation has been evolving into single-cell speciation, a technique that enables analysis of the entire metal/metalloid-containing species in a single cell. The development of more selective and sensitive fluorescent probes for individual metals/metalloids is one of the exciting topics highly awaited by chemical biologists. These techniques that directly monitor metals/metalloids in biological samples are complementary to molecular biological, cell biological, and biochemical techniques, and the complementary use of these techniques is anticipated to provide novel insights into the research of metallomics.

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