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A Highly Efficient and Extremely Selective Intracellular Copper Detoxifying Agent Based on Nanoparticles of ZnMoS₄

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Abstract

Biocompatible ZnMoS₄ NPs can selectively remove intracellular copper ions *via* ion-exchange rather than chelation. This strategy represents a paradigm shift in designing new-generation intracellular metal detoxifying drugs.

In clinical toxicology, small-molecule chelating agents have been widely used to remove heavy metal ions from the human body in a medical procedure commonly referred to as chelation therapy.¹⁻³ In addition to providing antidotes to heavy-metal intoxications, chelation therapy has been investigated for its potential in treating a variety of metal homeostasis related diseases and disorders.⁴⁻⁹ Despite its medical importance, the number of available chelators used as either the clinical or investigational drugs is, however, surprisingly small.²⁻³ Furthermore, such small-molecule-based drugs seldom possess sufficient selectivity towards a specific metal ion from the body.^{1-3, 10-11} Neither do they have the ability to cross the cell membrane in order to function as intracellular detoxifying agents, unless there is a receptor-targeting moiety conjugated to the drug.^{1-3, 10-12} On the other hand, some chelators may mobilize the metal ions that are deposited in the body tissues and reroute them into circulation, thus increasing the risk of secondary exposure of such metal ions to the brain.¹³⁻¹⁴ In contrast, the use of solid-state compounds as ion-exchangers to selectively remove the target metal ions from the human body by the well-established thermodynamic and kinetic principles of ion-exchange has been largely unexplored.¹⁵⁻¹⁶ We used the Irving-Williams Series as a guide to design a novel nanoparticulate ion-exchanger that exhibits an extremely high selectivity towards copper and can penetrate the cell membrane to act as an intracellular copper detoxifying agent. Specifically, we noticed that the order of relative stability exhibited by the homologous divalent 3d metal complexes shows the trend $Mn^{2+} < Fe^{2+} < Co^{2+} < Ni^{2+} < Cu^{2+} > Zn^{2+}$ with copper and zinc complexes being the most and the second most stable, regardless of the nature of ligand.¹⁷ Thus, if a highly stable solid-state zinc compound is used as an ion-exchanger, it would preferentially undergo ion-exchange with copper, and consequently, act as a detoxifying agent specifically for copper ions while maintaining homeostasis for other biologically essential metal ions such as Mg^{2+} , Ca^{2+} , Mn^{2+} , Fe^{2+} and Zn^{2+} in the body.

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[†]Electronic Supplementary Information (ESI) available online or from the author.

Copper is an essential metal required by all living organisms in several key cellular processes such as mitochondrial respiration, antioxidant defense, neurotransmitter synthesis and connective tissue formation.¹⁸⁻¹⁹ In healthy adults, 85-95% of copper in blood is bound to ceruloplasmin molecules. The remaining 5-15% is loosely bound to albumin or other small molecules. When present as free ions, copper can catalyse Fenton-like reactions in cells to produce reactive oxygen species (ROS) that cause damage to the biological molecules including DNA, proteins and lipids.²⁰ Under normal conditions the concentration of free intracellular copper ions is tightly regulated to prevent oxidative stress from triggering liver and neurodegenerative disorders. However, this is not the case in the Wilson's disease (WD), a rare, yet fatal genetic metabolic disorder characterized by excess copper accumulation in the liver, brain and other vital organs.²¹⁻²² Patients with WD lack the ability to incorporate copper into ceruloplasmin protein due to the mutation in the gene ATP7B.²³ If untreated, WD can lead to severe disability, liver transplantation and death.²⁴ Chelation therapy using D-penicillamine (D-pen or (2S)-2-amino-3-methyl-3-sulfanylbutanoic acid), first introduced by John Walshe in 1956, is the current treatment of choice for WD.^{5-6, 21-22} However, this orally active drug of slow action has a number of serious side effects including bone marrow and immune suppression, skin rash and deterioration of neurological functions.²⁵ The latter is caused by the drug's ability to mobilize copper ions stored in the body tissues and reroute them into circulation, which increases the concentrations of copper in the brain.¹³⁻¹⁴ Triethylenetetraamine (i.e. Trientine®) was later introduced specifically for patients who developed adverse reactions to D-pen, albeit it is a less effective copper chelator.²⁶ Zinc acetate is an FDA-approved drug as a maintenance therapy for WD,²⁷ while the ammonium salt of tetrathiomolybdate (i. e. $[\text{NH}_4]_2\text{MoS}_4$ or TTM) is currently an investigational drug for WD.²⁸ All of these drugs are orally active and can reduce the intestinal absorption of copper, but as small molecules, none of them can penetrate cells to reduce the intracellular copper concentration for the late-stage WD patients who have a significant amount of hepatic copper deposition, which will cause cirrhosis and require liver transplantation. Recently, Pujol and co-workers reported on the synthesis of a cyclopeptide scaffold that contains cysteine-based peptides as the Cu(I)-binding unit and a cluster of carbohydrates as the targeting unit for the asialoglycoprotein receptor (ASGP-R) expressed on the surface of hepatocytes to selectively reduce the intracellular Cu (I) concentration with respect to Zn(II).²⁹⁻³⁰ Herein, we describe the synthesis and characterization of nanoparticles (NPs) of zinc tetrathiomolybdate ZnMoS_4 . We demonstrate that such novel NPs are water-dispersible, biocompatible and able to penetrate cells by receptor-independent endocytosis to selectively remove intracellular copper *via* ion-exchange rather than chelation. The reaction of $(\text{NH}_4)_2\text{MoS}_4$ in formamide (FM) with $\text{Zn}(\text{O}_2\text{CCH}_3)_2$ in an aqueous solution containing 3-mercaptopropionic acid, NH_4OH and polyvinylpyrrolidone (PVP) led to the formation of PVP-coated ZnMoS_4 NPs. (see Supporting Information). PVP is a polymer commonly used as a surface-coating agent to enhance biocompatibility and water dispersibility in a variety of inorganic nanoparticle preparations.³¹ Transmission electron microscopic (TEM) studies revealed that the PVP-coated ZnMoS_4 NPs are near spherical in shape and have an average size of 16 ± 2 nm, as determined by counting and averaging the size of 23 particles in this TEM picture frame (Figure 1). The energy dispersive X-ray spectroscopic (EDX) measurements showed distinctive signals of Zn, Mo and S from several individual NPs randomly selected from the

TEM sample (Figure S1). The Fourier transform infrared (FT-IR) spectra of these NPs showed the presence of the surface-coated PVP. The bands at approximately 1660 cm^{-1} , 1420 cm^{-1} and 1285 cm^{-1} are the characteristic IR bands of PVP (Figure S2). The X-ray powder diffraction (XRD) patterns of both the NPs and bulk materials of ZnMoS_4 match those of the ternary solid-state phase of the same composition previously reported in the literature (Figure S3).³² Although the structure determination for this compound has not been carried out yet, it is tempting to conjecture that both the Mo and Zn centres have tetrahedral coordination while the S atoms are engaged in a double-bridging mode of bonding, which is reminiscent of the coordination environment in the GeS_2 structure.³³

The reaction between $(\text{NH}_4)_2\text{MoS}_4$ and a copper(II) salt failed to give the anticipated CuMoS_4 . Instead, a solid-state compound containing the copper(I) center was formed, presumably *via* a redox reaction between the Cu^{2+} ion and the MoS_4^{2-} ligand. The direct reaction of $(\text{NH}_4)_2\text{MoS}_4$ with a copper(I) salt gave the same product. The XRD pattern of this compound matches that of Cu_2MoS_4 whose crystal structure was determined from the X-ray powder data to be a layered compound with both the Mo and Cu centers in tetrahedral coordination environment with each S atom coordinating to two Cu atoms and one Mo atom within a layer.³⁴⁻³⁵ The K_{sp} values for ZnMoS_4 and Cu_2MoS_4 are 2.7×10^{-11} and 9.1×10^{-19} , respectively, as determined through a static method in which the bulk materials of each compound were allowed to equilibrate with deionized water at room temperature, and the metal concentrations were then analyzed by chemical analysis (see Supporting Information). We expected the difference in the equilibrium concentrations of $[\text{Zn}^{2+}] = 5.218 \times 10^{-6}\text{ M}$ and $[\text{Cu}^+] = 6.102 \times 10^{-7}\text{ M}$ to provide a driving force for the ion-exchange between ZnMoS_4 NPs and $\text{Cu}^{2+}/\text{Cu}^+$ ions to occur. This hypothesis was quickly confirmed by several semi-quantitative studies in aqueous solution. The kinetics of the *in vitro* ion-exchange of ZnMoS_4 NPs with Cu^+ ions was determined only because the intracellular free copper is most likely to exist in this oxidation state.³⁶⁻³⁷ Since Cu(I) ions are prone to the disproportionation reaction in aqueous solution to form Cu(II) ions and the Cu(0) metal, acetonitrile was used to stabilize this oxidation state in water by forming a Cu(I)-acetonitrile complex. The results showed that the ion-exchange reaction between PVP-coated ZnMoS_4 NPs and Cu^+ ions followed a *pseudo* first-order reaction up to the reaction time point of ~60 min with a rate constant $k_1 = 3.0 \times 10^{-4}\text{ s}^{-1}$ or a half-life $t_{1/2} = 38.5\text{ min}$. There is a change of reaction rate after this point to a slower and second-order reaction with a rate constant $k_2 = 1.8 \times 10^{-1}\text{ mM}^{-1}\text{ s}^{-1}$. The shift to the slower and second-order reaction is presumably attributable to the need for Cu^+ ions to penetrate into the NPs to react with the inner Zn^{2+} ions after the surface layer is consumed (Figure 2 and Figure S4).

The copper removal capacity of the NPs in aqueous solution was also determined. The capacity of a solid-state material to remove metal ions from a solution is often expressed as the maximum amount of metal in milligrams that can be removed by one gram of the given material. The metal removal capacity (q , mg/g) at different time intervals can be calculated using the mass balance equation $q = (C_i - C)(V/W)$, where C_i (mg/L) is the initial metal concentration, C (mg/L) is the metal concentration remaining in solution after adsorption at given time t (min), V (L) is the volume of metal solution, and W (g) is the weight of NPs. The copper removal capacity for the PVP-coated ZnMoS_4 NPs was found to be 130 mg/g

(Figure 3), indicating that these NPs can effectively remove copper from the aqueous solution. On the other hand, good selectivity towards the intended metal ion is probably a more important criterion for evaluating the performance of copper detoxifying drugs because other endogenous metal ions, being both extracellular and intracellular, will compete for coordination sites in the same agent. Such a competition not only lowers the efficiency, but also disturbs the homeostasis of other essential metals. The typical approach towards designing a selective therapeutic metal detoxifying ligand relies mostly on the hard-soft acid-base theory³⁸ with other considerations such as the chelating effect, biomimetic tailoring of coordination environment³⁹ and pro-ligand approaches⁴⁰. Thus far the design and synthesis of novel small-molecule chelating agents with high metal binding selectivity has remained a challenge.¹⁻⁴ In contrast, our PVP-coated ZnMoS₄ NPs should possess intrinsically high selectivity towards copper as dictated by the the Irving-Williams Series. To evaluate such selectivity, the NPs sealed in a dialysis bag were submerged in an aqueous solution containing Cu⁺, Fe²⁺, Mn²⁺, Ca²⁺ and K⁺ ions, each at the ~100-pm level for 48 hours. The solution concentration of each ion was analyzed before and after the competition reaction. The results were normalized against the removal of the Cu⁺ ion from the solution, and expressed as the percent removal.

As can be seen in such a high selectivity is most likely unachievable by use of small chelating agents. Besides the high selectivity, the use of ZnMoS₄ NPs as the ion-exchanger for copper removal offers an additional benefit, i.e. the Zn²⁺ ions released from this ion-exchange reaction may stimulate the production of zinc metallothionein in the body. Zinc metallothionein has the ability to inhibit the intestinal absorption of copper by the metal displacement reaction with copper in the protein. To visualize the cellular uptake of the PVP-coated ZnMoS₄ NPs, a fluorophore was conjugated to the surface of NPs by the EDC coupling reaction to permit the optical detection of the fluorescently labeled NPs inside cells (see Supporting Information). The cellular uptake of the dye-label NPs in HepG2 cells was visualized using the confocal fluorescence microscopic technique. HepG2 cells were used as an *in vitro* model of drug development target for WD due to their morphological and functional differentiation that are comparable to human hepatocytes. The fluorescent images of the live HepG2 cells treated with dye-labeled NPs showed strong fluorescent signals that are uniformly distributed in the entire cytoplasm without specific binding to any small organelles, while the untreated HepG2 cells gave almost no fluorescent signal (Figure 5). This observation seems to suggest that the cellular uptake of these NPs is *via* endocytosis. Additionally, continued monitoring of the fluorescence signals in these living cells showed that the residence time of the NPs within the cell is about 5 hours before exocytosis takes place (see Figure S6 in ESI). The cytotoxicity of the NPs was evaluated using an MTT viability assay. The 96-well plates were seeded at 1×10^4 HepG2 cells per well and incubated for 5 hours to allow cells to attach to the surface. Cells were then exposed to varying concentrations of NPs, followed by incubation for 24 or 48 hours. After 24-h incubation with a concentration of 50 μ M, the cell viability was found to be *ca.* 95 $\pm$ 4% while the cell viability became 81 $\pm$ 8% after 48-h incubation with the same concentration (Figure 6).

To study the intracellular copper removal by these NPs, we first induced an elevated copper level in HepG2 cells by incubating the cells with a medium supplemented with CuSO₄ to reach the saturation of copper uptake in these cells (see Supporting Information). The copper-saturated HepG2 cells were then incubated with PVP-coated ZnMoS₄ NPs at 37 °C for different times. As can be seen in Figure 7, such copper-saturated cells treated with NPs showed a significant decrease in the cellular copper level after 1.5 hour of incubation. An overall substantial decrease in copper concentration lasted as long as 6 hours of incubation. Longer incubation than 6 hours did not cause the intracellular copper level to go down any further, presumably because the remaining PVP-coated ZnMoS₄ NPs along with the absorbed copper ions had exited the cells by now via exocytosis.

Conclusions

In summary, our approach to developing the ZnMoS₄ NPs as an efficient and selective cellular copper detoxifying agent represents a new strategy for designing the next-generation therapy for WD. This shift of paradigm from chelation to ion-exchange should also be applicable to the search of novel treatments for other metabolic disorders caused by metal overload and for heavy metal intoxications. Copper is a cofactor for more than a dozen key angiogenic promoters in cancer angiogenesis.⁴¹⁻⁴² Preliminary studies showed that our ZnMoS₄ NPs can inhibit basic fibroblast growth factor, i.e. FGF2-induced tube formation by HuVEC cells in an *in vitro* 3D cell-culture model (see Supporting Information). Furthermore, these NPs were well-tolerated by nude mice as an injectable. Depletion of copper in normal as well as cancerous nude mice as the model for treating WD or solid tumors is currently underway. The results will be published in due course.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
TEM image of PVP-coated ZnMoS₄ NPs

Figure 2.
Kinetics of copper removal by PVP-coated ZnMoS₄ NPs

Figure 3.
Copper removal capacity of PVP-coated ZnMoS₄ NPs

Figure 4.
Selectivity of metal removal by PVP-coated ZnMoS₄ NPs

Figure 5.
Confocal microscopic images of HepG2 cells: fluorescence image (upper left) and bright field image (upper right) of cells incubated with dye-conjugated NPs for 3 hours; fluorescence image (lower left) and bright field image (lower right) of the untreated cells

Figure 6. Viability curve of HepG2 cells incubated with PVP-coated ZnMoS₄ NPs for 24 hours and 48 hours (MTT assay)

Figure 7.
Kinetics of copper removal from HepG2 cells