Journal of Materials Chemistry B

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/materialsB

Journal Name

RSCPublishing

ARTICLE

Biocompatible D-Penicillamine Conjugated Au Nanoparticles: Targeting Intracellular Free Copper Ions for Detoxification

Cite this: DOI: 10.1039/xoxxoooooox

Received ooth January 2012,
Accepted ooth January 2012

DOI: 10.1039/xoxxoooooox

www.rsc.org/

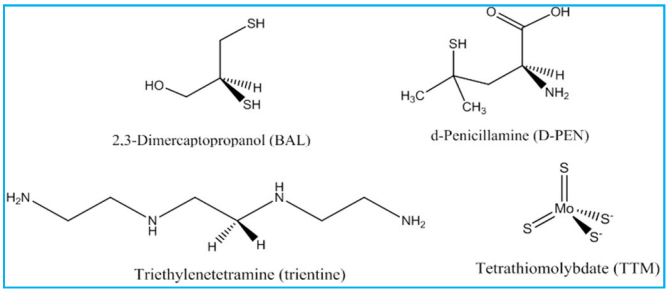
Murthi S. Kandanapitiye, Chamila Gunathilake, Mietek Jaroniec and Songping D. Huang*

High thiophillicicity of the Au-nanoparticle (Au NP) surface leads to covalent attachment of D-penicillamine molecules to Au NPs to form biocompatible D-penicillamine conjugated Au NPs. The latter are highly water-dispersible, exhibit no cytotoxicity, and can readily penetrate the cell membrane to target intracellular free copper ions for selective copper detoxification in the presence of the other divalent essential metal ions including Zn(II), Fe(II), Mn(II), Ca(II), and Mg(II), thus opening up a new avenue for improving the efficacy and pharmacokinetics of D-penicillamine, an important clinical drug currently used to treat the copper overload-related diseases and disorders.

Introduction

Copper is an essential trace element that serves as a catalytic and structural cofactor for many proteins and enzymes in all living organisms.[1-3] However, when it is unbound to proteins or enzymes, the free copper ion can exhibit deleterious effects due to its ability to trigger Fenton-like reactions that produce hydroxyl radicals to cause oxidative damage of proteins, lipids, and nucleic acids.[4-5] Under normal conditions, copper is sequestered and tightly regulated at every stage of its uptake, transport, delivery and excretion in cells to achieve copper homeostasis in order to prevent oxidative stress.[2] Deficiencies in maintaining copper homeostasis are linked to a number of human diseases or disorders including Menkes disease,[6] Wilson's diseases,[7] familial amyotrophic lateral sclerosis,[8] to name but a few. An imbalance of copper metabolism is also implicated in Alzheimer's disease, Parkinson's disease and prion diseases.[9-10] Despite the recent significant progress made in understanding the intracellular trafficking of copper, there is still a limited number of clinical drugs available in the form of chelation therapy to treat the diseases and disorders associated with copper overload.[11-13] This situation is particularly manifesting for Wilson's disease (WD), also known as hepatolenticular degeneration. WD is a recessive genetic disorder characterized by excess copper accumulation in the liver and other vital organs.[14] In 1951, the British anti-Lewisite (BAL) was introduced as the first clinical drug for WD.[15] This chelating agent had been initially developed in World War II (WWII) as an antidote to the chemical warfare agent Lewisite and was later adopted for use in detoxifying heavy metal poisoning by arsenic, gold, antimony, lead or mercury (see **Scheme 1**).[16] Because of some serious side effects including nephrotoxicity and hypertension of BAL, D-Penicillamine (D-PEN; i.e. (2S)-2-amino-3-methyl-3-sulfanyl-butanoic acid; see **Scheme 1**), a metabolite of penicillin was introduced in 1956 as a better clinical drug for WD.[17] In 1982, triethylenetetraamine (trientine; see **Scheme 1**), a less effective copper chelating agent than D-PEN, was introduced as another clinical drug for WD, mainly for the patients who showed intolerance to D-PEN.[18] Currently, the clinical use of triethylenetetramine is limited in the USA because such application has not been approved for the European market. In 1997, the US Food and Drug Administration (FDA) approved the use of zinc acetate as a clinical drug for WD.[19] Unlike other three clinical drugs for WD, this compound is not a chelating agent, but zinc ions from the drug can stimulate the production of metallothionein in gut cells, which in turn binds copper ions to inhibit their absorption and transport to the liver. It has been shown that zinc acetate is only effective as a maintenance therapy for WD.[20] Recently, tetrathiomolybdate (TTM; see **Scheme 1**) was introduced as an investigational drug for WD.[21] Research has shown that TTM forms a non-bioabsorbable form of ternary complexes with copper and food proteins in the gastrointestinal tract to block the intestinal absorption of copper from the diet, thus creating a negative copper balance in the body.[22] Among all these drugs for WD, D-PEN has the highest efficacy, and hence is currently the most widely used drug for WD across the world.[23] However, the side effects of D-PEN are numerous, and several of these are severe. They include bone marrow and immune suppression, skin rash, mouth ulcers, nausea, and deterioration of various neurological functions.[24] The latter side effect is believed to be caused by the ability of D-PEN to mobilize copper ions that are stored in the body tissues and reroute them into circulation, thus increasing the concentrations of copper in the brain.[25] It has been estimated that about half of the WD patients treated with D-PEN would show neurologic deterioration, and a quarter of such patients would suffer irreversible neurologic damage for use of D-PEN.[26] All of these side effects are attributable to the fact that this drug is delivered systemically with no organ-specificity, hence causing a variety of side-effects due to the systemic toxicity of

the drug. Furthermore, all the above-mentioned small molecule-based drugs are unable to penetrate cells to target intracellular free copper ions for detoxification.[7,13]



Scheme 1 Molecular structures of the clinical and investigational drugs based on copper chelation for WD

We[27-28] and the others[29-30] have recently begun to develop cell-membrane permeable chelating agents that have potential to become organ-specific with use of suitable targeting molecules as the next-generation copper detoxifying drugs for treating WD and other copper-overload related diseases and disorders such as angiogenesis in metastatic cancer, fibrosis, inflammation, and autoimmune diseases (e.g. rheumatoid arthritis).[31] In this article, we report our work on the development of a novel delivering system based on gold nanoparticles (Au NPs) for D-PEN that can remove excess free copper ions from the cell. Au NPs were chosen as the carrier for cellular delivery of D-PEN due to their unique properties including surface thiophillicity for covalent conjugation of the chelating agent, the high surface-area to volume ratio for carrying a high payload, and multivalent surface architecture for potential incorporation of multiple therapeutic agents and targeting molecules on the same surfaces.[32-34] We prepared the D-PEN-conjugated Au NPs of the average size at 16±2 nm with superb water dispersability, and examined the kinetics and selectivity of copper binding of such NPs in aqueous solution. We also studied the cellular uptake, cytotoxicity and intracellular copper removal by these NPs to demonstrate their potential as a novel cell-penetrable copper detoxifying agent. Previously D-PEN-capped Au NPs were prepared and temperature-dependent reversible structural changes of such NPs were studied using the infrared spectroscopic method.[35] However, no attempt of using such Au NPs to deliver D-PEN as a drug has been made so far. To the best of our knowledge, the current work is the first example to show that D-PEN Au NPs can be used as a novel biocompatible intracellular copper-detoxifying drug.

Results and discussion

Preparation and Characterization of Core-Shell Au@D-PEN NPs. The preparation of Au@D-PEN NPs was achieved through the double-displacement reaction between the surface-anchored citrate and free D-penicillamine molecules. The citrate-coated Au NPs were synthesized in aqueous solution using sodium citrate as the reducing agent as well as the capping ligand in the modified Turkevich method (for details see the **Experimental**). The as-synthesized citrate-coated Au NPs were first dialyzed in distilled water using regenerated cellulose tubular membrane (MWCO = 12000-14000) for 24 hours to remove the unbound sodium citrate before they were treated with D-penicillamine in aqueous solution for 24 hours. During this time, a small aliquot of NP dispersion was periodically taken out from the dialysis bag, diluted with equal amount of acetone and centrifuged to separate the NPs. Fourier transform infrared (FTIR) spectroscopy was used to analyze the reaction product in order to ensure that the ligand displacement had been completed. Finally, the NPs were dialyzed again for 24 hours to remove the unbound D-PEN molecules. As shown in **Fig. S1**, the FTIR spectra indicate that the final product is D-PEN coated and citrate free. By careful examining the FTIR data, we notice that the peaks around ~ 2586.4 cm^{-1}, attributable to the stretching ν_s(S-H) vibrations of the thiol group found in the free D-PEN ligand, have completely disappeared in the Au@D-PEN NPs, indicating that D-PEN molecules are covalently bound to the Au surfaces *via* the S atom. Additionally, substantial shifts in the vibrational modes and the reduction in the number of peaks for both amine and carboxylate groups were found in the Au@D-PEN NPs as compared to those in the free D-PEN ligand, suggesting that both amine and carboxylate groups also interact with the Au surface (see **Fig. S1** in the ESI). Similar changes of the IR spectroscopic features were previously observed in the D-PEN molecules adsorbed on the bulk Au surface.[36] The observed covalent bonding of D-PEN molecules to the Au surface is fully consistent with the well-established thiophillic nature of Au NP surfaces, and also attests to the existence of the robust coating layers of D-PEN molecules on the Au NPs as the prolonged dialysis against distilled water did not cause any loss of D-PEN molecules from the Au@D-PEN NPs. We have also found that such NPs are highly water-dispersible with the highest amount of D-PEN Au NPs that can be dispersed in distilled water to be 4.5 mg/mL as determined by quantitative disposability measurements.

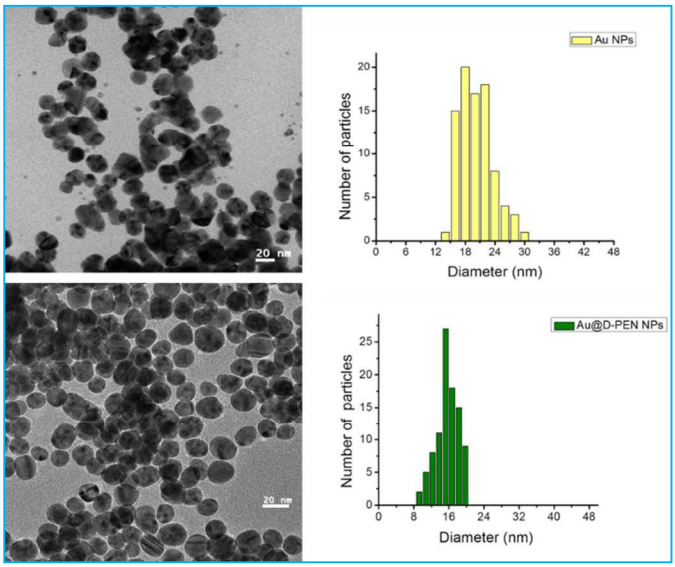


Fig. 1 TEM images of as-prepared Au NPs (upper left) and Au@D-PEN NPs (lower left) and the histograms of the particle size distribution for NPs corresponding to each panel on the right

The transmission electron microscopic (TEM) studies revealed that both the citrate-coated Au NPs and the Au@D-PEN NPs are approximately spherical in shape. However, a careful comparison of their size and particle size distribution shows that there is a statistically significant change in the size and particle size distribution after the ligand displacement reaction. The average size of Au NPs, obtained by measuring and averaging the size of 86 NPs, is 20 ± 4 nm, while the particle size distribution is near Gaussian. The average size of the Au@D-PEN NPs, obtained by measuring and averaging 91 NPs, is 16 ±2 nm, while the particle size

distribution is asymmetric with a negatively skewed tail (see **Fig. 1**). Such changes are caused by a process known as the "digestive ripening", first observed by Klabunde and co-workers in the Au NP-alkanethiol system.[37] The energy-dispersive X-ray spectroscopy (EDS) analysis of individual Au@D-PEN NPs showed the presence of S, C and N in addition to Au, confirming that D-PEN molecules exist as a shell on the Au core rather than being present as a separate phase in the material (see **Fig. S2** of the ESI). Interestingly, we also observed a slight blue shift in the localized surface plasmon resonance (LSPR) of the Au@D-PEN NPs in comparison to the citrate-coated AuNPs, which is consistent with the decrease in the average particle size from Au NPs to Au@D-PEN NPs as shown in **Fig. 2**. Additionally, results from thermogravimetric analysis (TGA) of Au@D-PEN NPs showed a total weight loss of 60%, attributable to the decomposition of D-PEN molecules attached to the Au NPs, when the sample was heated to 600 °C in air (see **Fig. S3** of the ESI).

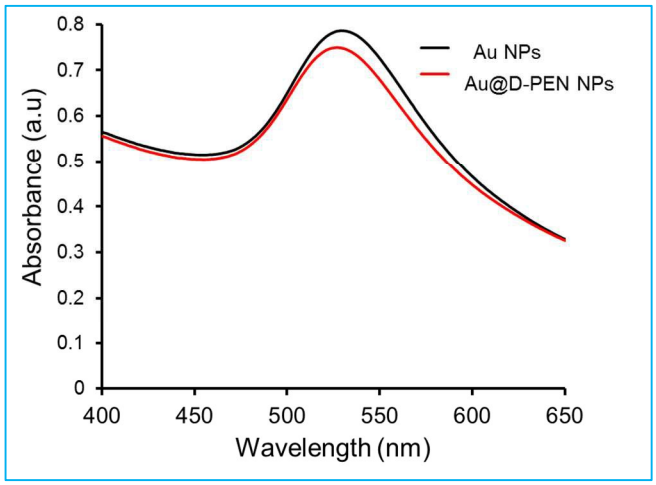


Fig. 2 UV-vis spectra of Au NPs and Au@D-PEN NPs.

Kinetics and Selectivity of Copper Binding by Au@D-PEN NPs in Aqueous Solution. We determined the kinetics and selectivity of copper binding by Au@D-PEN NPs based on the distribution of the metal ions between the solid and liquid phase. First, the kinetics of copper binding was evaluated by monitoring the removal of Cu^{2+} ions from aqueous solution by Au@D-PEN NPs over a time period of 8 hours. The NPs were sealed in a dialysis bag which was then immersed in an aqueous solution containing the Cu^{2+} ions. The decrease of copper concentration in the aqueous solution was measured by elemental analysis using atomic absorption (AA) spectrometry. The results indicate that the copper binding reaction follows a *pseudo* first order law up to the time point of 54 min with a rate constant $k_1 = 7.0\times10^{-5}$ s^{-1}or the half- life of $t_{1/2} = 164$ min as shown in **Fig. 3** (see the ESI for detailed analysis of the kinetic data and thhe presentation of the results in **Fig S4**). These binding measurements suggest that our NPs are kinetically suitable for removing intracellular copper ions. In the meantime, when the content of the dialysis bag was examined by TEM at the end of the copper removal reaction, we found that some D-PEN molecules were detached from the Au surface to form a different type of particulate matter as revealed by the TEM images. The latter is poorly formed and appears as some kind of smudge under the TEM with a much-decreased Au content and an increased Cu content, which suggests that D-PEN molecules are now detached from the Au surface to form separated NPs (see **Figs S5** and **S6** of the ESI). It should be noted that the intracellular copper predominantly exists as Cu(I) because Cu(II) is reduced by various metalloreductases before transported into the cell by the copper transport protein (Ctr1).[38] Since Cu(II) is invariably reduced to Cu(I) upon binding to D-PEN, we could use Cu(II) in place of Cu(I) in these and all of our subsequent studies involving copper in aqueous solution.[39-40]

To evaluate the selectivity of Au@D-PEN NPs toward Cu^{2+} ions in the presence of the other biologically essential divalent metal ions including Mg^{2+}, Ca^{2+}, Fe^{2+}, Mn^{2+} and Zn^{2+}, we investigated the competition of Au@D-PEN NPs for binding different ions in aqueous solution. The competitive binding studies were performed by soaking the dialysis bag with 10 mL Au@D-PEN NPs (3 mM) in a solution containing Cu^{2+}, Mg^{2+}, Ca^{2+}, Fe^{2+}, Zn^{2+} and Mn^{2+} ions each at the 50 ppm level. After 24 hours of incubation, an aliquot of solution was taken out, diluted with 2% HNO_3 acid and analysed by AA to determine the concentration for each metal ion. **Fig. 4** clearly shows that Au@D-PEN NPs are most selective toward the Cu^{2+} ion in the presence of all the other tested divalent metal ions. These results suggest that a proper therapeutic window should exist for selectively removing the Cu^{2+} ion while maintaining the homeostasis of all the other biologically essential divalent metal ions when Au@D-PEN NPs are used as a drug for cellular copper detoxification.

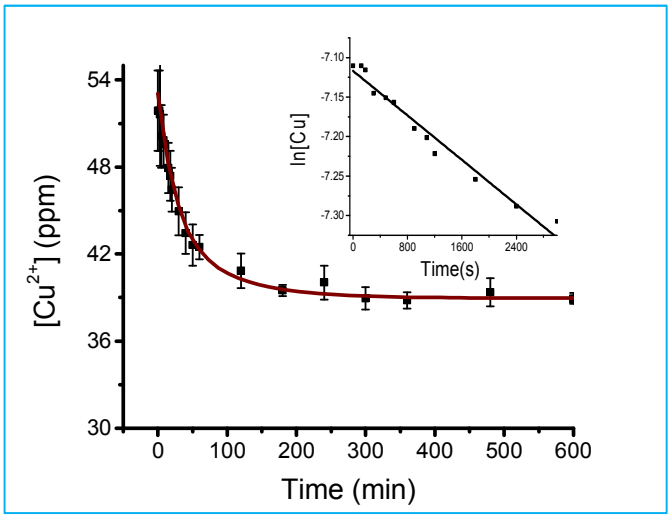


Fig. 3 Kinetics of copper removal from the aqueous solution by Au@D-PEN NPs. The inset shows the curve-fitting plot of a *pseudo* first order law.

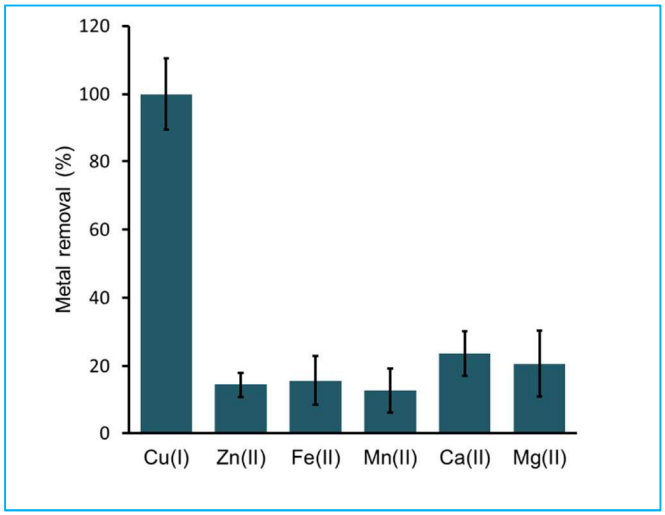


Fig. 4 Selectivity of several biologically essential divalent metal ions by Au@D-PEN NPs in aqueous solution.

Cellular Uptake Studies and Cytotoxicity Assay of Au@D-PEN NPs. We studied cellular uptake of Au@D-PEN NPs in HeLa cells using the fluorescent confocal microscopic imaging technique. As Au@D-PEN NPs themselves are non-fluorescent, 5-carboxyfluorescein dye (CbF) was covalently anchored onto the Au@D-PEN NP surfaces (see the **Experimental** for details and **Fig. S7**). It should be noted that the CbF dye molecule itself is membrane impermeable due to its high anionic negative charge and poor water solubility.[41] For live cell imaging, the cells were first incubated with DMEM media containing the fluorescence dye-conjugated Au@D-PEN NPs for 4 hours and then washed three times with PBS buffer. Finally, Hoechst dye was introduced to stain the nuclei before the imaging studies. The living cells were imaged under a laser scanning confocal microscope without fixation. **Fig. 5** shows the representative confocal fluorescent images of HeLa cells treated with the dye-conjugated Au@D-PEN NPs *vs*. the control cells. As can be seen from the confocal images, strong and uniform green fluorescence signals attributable to the surface-conjugated CbF dye are present in the perinuclear region but not in the nucleus of the cell, indicating an untargeted cytoplasmic distribution of NPs. Furthermore, no specific binding was found to any small organelle in the region, which is consistent with cellular uptake *via* endocytosis. These results suggest that Au@D-PEN NPs possess the ability to penetrate the cell membrane, which provides an important pre-requisite for developing them as a potential cellular copper detoxifying agent (*vide infra*).

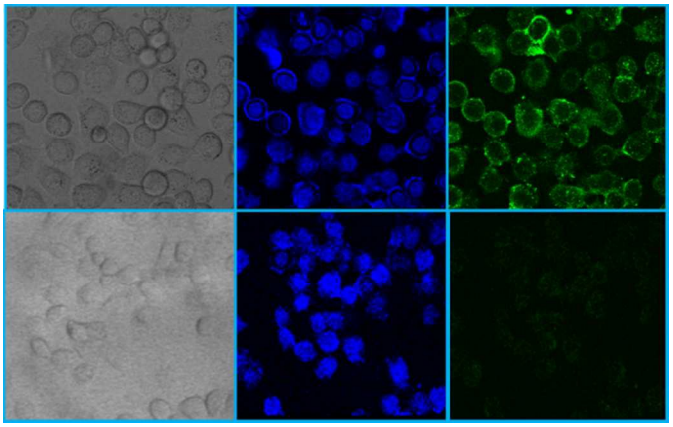


Fig. 5 Confocal microscopic images of HeLa cells: (upper left) bright-field image of cells incubated with dye-conjugated NPs for 4 h; (upper middle) fluorescence image of cells incubated with Hoechst dye (nucleus stain); (upper right) fluorescence image of cells incubated with dye-conjugated NPs for 4 h; (lower left) bright-field image of the untreated cells as the control; (lower middle) fluorescence image of untreated cells incubated with Hoechst dye; and (lower right) fluorescence image of untreated cells.

To assess the cytotoxicity, we performed cell viability assays in HeLa cells using the MTT method. The cells were incubated for 24 h at 37 °C under 5% CO_2 with varying concentrations of Au@D-PEN NPs suspended in Dulbecco's modified eagle medium (DMEM). Each experiment was triplicated and averaged. The percentage cell survival values were calculated in reference to the control cells. **Fig. 6** shows the viability of HeLa cells treated with Au@D-PEN NPs at various concentrations. The results showed that more than 80% of the cells were viable even after incubation with the NPs at the concentration of 88 μM for 24 hrs, and more than 89% of the cells were viable after incubation with the NPs at 52 μM for 24 hrs. If the cell viability curve is converted to a plot using the concentration of D-PEN, the concentration of NPs=88 μM corresponds to the concentration of D-PEN = 1,000 μM, and the concentration of NPs= 52 μM corresponds to the concentration of D-PEN=600 μM (see **Fig. S8**). Therefore, we conclude that these NPs exhibit no cytotoxic effect in the typical therapeutic concentration required for cellular detoxification, i.e. about 500 μM of D-PEN on Au NPs (see the ***Experimental*** for details).

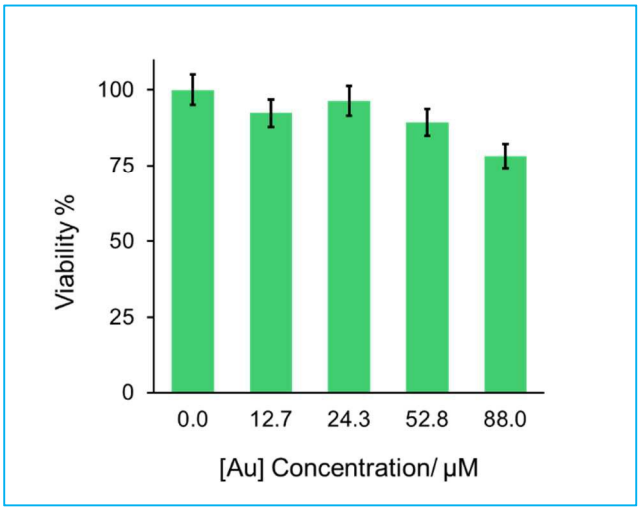


Fig .6 Effect of Au@D-PEN NPs on viability of HeLa cells after 24 hours of incubation

Studies of Intracellular Coper Removal Using Au@D-PEN NPs. To evaluate the ability of Au@D-PEN NPs to remove intracellular copper ions, we deliberately induced the elevated copper level inside the HeLa cells by incubating the cells with the medium supplemented with $CuCl_2$ (250μM) for 12 hrs. After such incubation, the cells remained healthy and thriving. We then incubated the copper-elevated HeLa cells with Au@D-PEN NPs for 4 hrs followed by washing the cells three times with PBS and further incubating them with fresh culture medium. The HeLa cells in each flask were harvested, washed with fresh medium and lysed using concentrated nitric acid at different time intervals. The intracellular copper concentrations were determined for the cell lysates as a function of time by AA. The results were normalized by taking the copper concentration divided by the number of cells to determine the intracellular copper content per cell. As can be seen from the results given in **Fig. 7**, the cells treated with Au@D-PEN NPs showed a substantial decrease in the cellular copper level. Specifically, the intracellular concentration of copper dropped from the highly elevated level of 1040±15 fg/cell to 516±10 fg/per cell after the first 4 hours of incubation, and then to 285±29 fg/cell after 6 hours of incubation in the NP-treated cells, indicating that intracellular copper was restored to a level close to the normal endogenous copper concentration of 263±13fg/cell in the control cells. In contrast, the cellular copper content of the control cells with the elevated copper level that were not treated with Au@D-PEN NPs showed a slight decrease to 870±40 fg/cell after 8 hours of incubation with medium only, probably due to automatic effusion of copper ions from the cells. In a separated study, we incubated the copper-elevated cells with Au@D-PEN NPs in comparison with free D-PEN molecules, and analysed the change of copper concentrations in the culture medium in the intervals of 2, 4, 6 and 8 hour-incubation. At each

time point, there is a statistically significant difference in the percent copper removal from the cells between the two copper-depleting drugs. For instance, after 8 hours of incubation with D-PEN molecules, only 67% of copper is removed from the cells compared to 100% of copper that can be removed by Au@D-PEN NPs. Please note that automatic effusion of copper ions from the cells after 8-hour incubation contributes to high as 20% of copper removal from the cells (see **Fig. 9**). These observations are consistent with the notion that as a free ligand, D-PEN is not cell-permeable because it exists in the zwitterion form with an extremely high hydrophilicity, but the complexation of copper ions by D-PEN in the outside culture medium can create a concentration gradient to facilitate the automatic effusion of copper ions from the cells.[42] We noticed that the cells remained alive and viable during the entire duration of all our copper detoxifying studies (i.e. 8 hours). It is tempting to conjecture that Au@D-PEN NPs show potential to be developed as an organ-specific drug if coupled with a suitable targeting agent. In contrast, the current systemic delivery of D-PEN with poor organ-specificity might be the cause of many undesirable side effects.

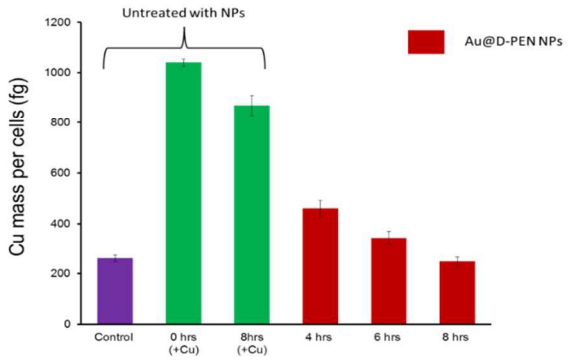


Fig. 7 Kinetics of copper removal from HeLa cells.

Conclusions

D-penicillamine has been in clinical use for treating WD and copper-overload related diseases and disorders for over 5 decades. Currently, it is still the treatment of choice for such illness, and will probably remain so in the foreseeable future. Consequently, D-penicillamine has been designated by the World Health Organization (WHO) as an essential medicine necessary in a basic health system. However, as a non-cell penetrable small molecule, the efficacy of D-penicillamine is far from optimal, while the side effects caused by the use of this drug are numerous and severe. The synthesis and characterization of D-penicillamine NPs reported in this article opens up a new avenue to affect the pharmacokinetics as well as pharmacodynamics of this important clinical drug for improving its efficacy and lowering or even eliminating its undesirable side effects. Work is under way in this lab to test the efficacy of *in vivo* copper removal and safety profile of Au@D-PEN NPs in small animals.

Experimental

Reagents. All chemicals and reagents were purchased from Sigma-Aldrich and used as received without further purification unless otherwise noted.

Synthesis of Au@D-PEN NPs. The citrate-coated Au NPs were synthesized using the modified Turkevich method. In the typical synthesis, an aqueous solution of $HAuCl_4$ (0.25 mM, 100 mL) was first heated to boiling point under rigorous stirring, and a small amount of sodium citrate in distilled water (1%, 5 mL) was added to the $HAuCl_4$ solution. This solution turned from pale yellow to wine red in within a minute to signify the formation of AuNPs. The reaction was boiled with stirring for 30 min before it was cooled to room temperature. The as-synthesized Au NP solution was placed in a dialysis bag made of regenerated cellulose tubular membrane (MWCO=12000-14000) and dialyzed in distilled water for two days to remove the excess of sodium citrate. The purified citrate-coated Au NPs solution (100 mL) was allowed to react with D-penicillamine (0.075 g) for 24 hours. The resulting solution was dialyzed again in distilled water for two days to remove excess D-Penicillamine. The solid product was obtained by lyophilization.

TEM Imaging and EDX Measurements. The samples were first suspended in water, and then placed as droplets onto a carbon-coated copper TEM grid (400-mesh). Specimens were allowed to air-dry and analyzed at 200 KV using a FEI Tecnai F20 transmission electron microscope (TEM) equipped with a field emission gun. The energy dispersive X-ray spectroscopy (EDX) results were obtained with the integrated scanning TEM (STEM) unit and attached EDAX spectrometer. The spatial resolution is <1 nm through the acquisition of high resolution (~0.2 nm) high-angle angular dark field (HAADF) images, which is sensitive to atomic number (Z) contrast.

Thermogravimetric Analysis of Au@D-PEN NPs. The thermogravimetric analysis was conducted on a TA instrument 2950 high-resolution thermogravimetric analyzer from 30° to 800° in air.

Studies of Copper-Binding Kinetics, Selectivity and Capacity of Au@D-PEN NPs. Kinetic studies of copper binding were carried out in aqueous solution. Specifically, 10 mL of NPs (3 mM) were sealed in a dialysis bag (MWCO = 3,500), which was brought in contact with a $CuCl_2$ solution (25 mL) having the initial concentration at ~50 ppm level. The Cu^{2+} concentrations of the solution outside the dialysis bag were periodically analyzed by AA. Selectivity studies were performed by soaking another dialysis bag containing 10 mL of NPs (3 mM) in a solution containing the magnesium, calcium, iron (II), manganese (II), zinc and copper (I) ions. The concentration of each metal ion in the solution was ~50 mg/L. After 24 h, an aliquot of solution was taken out and diluted with 2% HNO_3 acid and analyzed for each metal ion by AA.

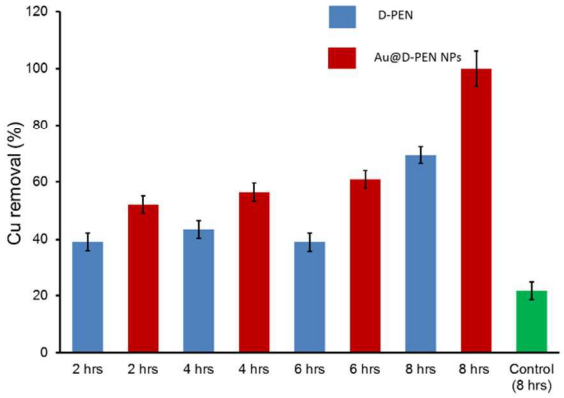


Fig. 8 Comparison of copper removal from HeLa cells by Au@D-PEN NPs vs. free D-PEN.

Cell viability of Au@D-PEN nanoparticles. Cytotoxicity studies were performed using an MTT viability assay. HeLa cells were seeded in a 96-well plate at a density of 2×10^4 cells per well with the DMEM low-glucose medium and incubated for 24 hrs at 37 °C in an atmosphere of 5% CO_2 and 95% air allowing cells to attach to the surface. Cells in each well were then treated with 100 µL of fresh medium containing varying concentrations of the nanoparticles and then incubated for 24 hrs. Control wells contained the same medium without nanoparticles. After 24 hours incubation period, the cells were

incubated with fresh DMEM media containing MTT reagent 10 µL, 1% (w/v) for 4 hours. After the MTT solution was removed, the precipitated violet crystals were dissolved in 100 µL of detergent. The absorbance was measured at 560 and 630 nm using a microplate reader. The assay results were presented as the percentage of viable cells.

Conjugation of fluorescence dye molecules to the surfaces of Au@D-PEN NPs. To prepare dye-conjugated Au@D-PEN NPs, 500 µL of 0.25 mM ethylenediamine solution was added to a 10.0-mL of Au@D-PEN NP solution (~ 250 µM) under vigorous stirring. The resulting mixture was continuously stirred for 12 hours. The product was purified by dialysis to remove unreacted ethylene diamine molecules. Next, 10 mL of nanoparticle solution obtained from previous step was reacted with carboxyfluorescene dye (3 mg) in the presence of the coupling agent 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC; 1.8 mg) for 24 hrs. To remove the unconjugated dye molecules, the resulting product was dialyzed against distilled water for two days and analyzed by fluorescence spectroscopy to confirm that the dye molecules are covalently bound to Au NPs.

Studies of Cellular Uptake of the Dye-Conjugated Au@D-PEN NPs. An 8-well plate was seeded with HeLa cells at a density of approximately 2×10^4 cells per well and incubated for 24 hrs allowing the cells attachment to the surface. The cells were then exposed to fluorescence dye-conjugated Au@D-PEN NPs for 4 hrs of incubation and washed thoroughly several times with PBS buffer. Hoechst nuclear staining dye was then introduced to stain the nuclei before the living cells were imaged under a confocal microscope.

Cellular Copper Detoxification by Au@D-PEN NPs. First, the elevated copper level in HeLa cells was induced by incubating the cells with DMEM medium supplemented with 250 µM of copper (II) solution for 12 hours. The cells were washed three times with PBS and then incubated with the culture medium containing the NPs at 43 µM of Au concentration or 500 µM of D-PEN concentration for 4 hours at 37 °C. After 4 hours incubation period, the cells were washed three times with PBS to remove the non-internalized NPs and further incubated with fresh culture medium for another 4, 6 and 8 hours. The cells grown in separate flasks were then trypsinized, centrifuged, re-suspended in PBS, and counted using a hemocytometer for 4, 6 and 8 hours respectively. The cells were then collected by centrifugation and lysed with 0.50 mL of concentrated nitric acid. The cell lysates were diluted to 5.00 mL with deionized water and filtered though a 200 µm filter. The copper contents in cell lysates were analyzed by AA.

Acknowledgements

We thank NIH-NCI for financial support (1R21CA143408-01A1). The TEM data were obtained using the Cryo TEM Facility at Liquid Crystal Institute, KSU supported by the Ohio Research Scholars Program.

Notes and references

Department of Chemistry and Biochemistry, Kent State University, Kent, OH 44240, USA. E-mail: shuang1@kent.edu

†The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. The authors declare no competing financial interest.

Electronic Supplementary Information (ESI) available: [Fourier transform infrared spectra, energy dispersive X-ray spectra and thermogravimetric (TGA) curves of Au@D-PEN NPs, and fluorescence spectra of carboxyfluorescein dye-labeled Au@D-PEN NPs. Procedures and results of copper-binding kinetic studies]. See DOI: 10.1039/b000000x/

1 R. H. Holm, P. Kennepohl, E. I. Solomon, E. I. *Chem. Rev.,* 1996, **96**, 2239-2314.
2 A. C. Rosenzweig, *Acc. Chem. Res.* 2001, **34**, 119-128.
3 S. Puig, D. J. Thiele, *Curr. Opin. Chem. Biol.* 2002, **6**, 171-180.
4 T. D. Rae, P. J. Schmidt, R. A. Pufahl, V. C. Culotta, T. V. O'Halloran, *Science*, 1999, **284**, 805-808.
5 M. Valko, H. Morris, M. T. Cronin, *Curr. Med. Chem*., 2005, **12**, 1161-1208.
6 C. Vulpe, B. Levinson, S. Whitney, S. Packman, J. Gitschier, *Nature Genet.* 1993, **3**, 7-13.
7 B. Sarkar, *Chem. Rev.* 1999, **99**, 2535-2544.
8 D. R. Rosen, T. Siddique, D. Patterrson, D. A. Figlewicz, P. Sapp, A. Hentati, D. Donaldson, J. Goto, J. P. Oregan, H. X. Deng, Z. Rahmani, A. Krizus, D. McKennayasek, A. Cayabyab, S. M. Gaston, Rr. Berger, R. E. Tanzi, J. J. Halperin, B. Herzfeldt, R. Vandenbergh, W. Y. Hung, T. Bird, G. Deng, D. W. Mulder, C. Smyth, N. G. Laing, E. Soriano, M. A. Pericakvance, J. Haines, G. A. Rouleau, J. S. Gusella, H. R. Horvitz, R. H. Brown, *Nature*, 1993, **362**, 59-62.
9 E. Gaggelli, H. Kozlowski, D. Valensin, G. Valensin, *Chem. Rev*. 2006, **106**, 1995-2044.
10 A. I. Bush, *Trends Neurosci.* 2003, **26**, 207-214.
11 D. M. Taylor, D. R. Williams, *Trace Element Medicine and Chelation Therapy*: Royal Society of Chemistry; Cambridge, 1995.
12 (a) O. Andersen, *Chem. Rev*., 1999, **99**, 2683-2710. (b) O. Andersen, *Mini-Rev. Med. Chem.,* 2004, **4**, 11-21.
13 E. J. Baran, *Curr Med Chem*., 2010, **17**, 3658-3672.
14 M. Frydman, B. Bonné-Tamir, L. A. Farrer, P. M. Conneally, A. Magazanik, S. Ashbel, Z. Goldwitch, *Proc. Natl. Acad. Sci. U.S.A.* 1985, **82**, 1819-1821.
15 R. Peters, L. Stocken, R. Thompson, *Nature,* 1945, **156**, 616-619.
16 D. Denny-Brown, H. Porter, *N. Engl. J. Med.* 1951, **245**, 917-925.
17 J. M. Walshe, *Lancet* 1956, **267**, 25-26.
18 J. M. Walshe, J. M. *Lancet* 1982, **319**, 643-647.
19 G. J. Brewer, R. D. Dick, V. D. Johnson, J. A. Brunberg, K. J. Kluin, J. K. Fink, *J. Lab. Clin. Med.* 1998, **132**, 264-278.
20 K. H. Weiss, D. N. Gotthardt, D. Klemm, U. Merle, D. Ferenci-Foerster, M. Schaefer, P. Ferenci, W. Stremmel, *Gastroenterology* 2011, **140**, 1189-1198.
21 G. J. Brewer, *Transl. Res.,* 2009, **154**, 314-322.
22 H. M. Alvarez, Y. Xue, C. D. Robinson, M. A. Canalizo-Hernández, R. G. Marvin, R. A. Kelly, A. Mondragón, J. E. Penner-Hahn, T. V. O'Halloran, *Science*, 2010, **327**, 331-334.
23 M. N. Schilsky, *Curr. Gastroenterol. Rep.,* 2001, **3**, 54-59.

24 G. J. Brewer, Wilson's Disease: A Clinician's Guide to Recognition, Diagnosis, and Management; Kluwer Academic Publishers: Boston, 2001.
25 G. J. Brewer, C. A. Terry, A. M. Aisen, G. M. Hill, *Arch. Neurol.,* 1987, **44**, 490-493.
26 G. J. Brewer, F. Askari, M. T. Lorincz, *Arch. Neurol.,* 2006, ***63***, 521-527.
27 V. S. Perera, H. Liu, Z.-Q. Wang, S. D. Huang, *Chem. Mater.,* 2013, **25**, 4703-4709.
28 V. S. Perera, N. P. Wickramaratne, M. Jaroniec, S. D. Huang, *J. Mater. Chem. B,* 2014, **2**, 257-261.
29 A. M. Pujol, M. Cuillel, O. Renaudet, C. Lebrun, P. Charbonnier, D. Cassio, C. Gateau, P. Dumy, E. Mintz, P. Delangle, *J. Am. Chem. Soc.,* 2011, **133**, 286-296.
30 A. M. Pujol, M. Cuillel, A.-S. Jullien, C. Lebrun, Cassio, D.; Mintz, E.; Gateau, C.; Delangle, P. *Angew. Chem., Int. Ed.,* 2012, **51**, 7445-7448.
31 G. J. Brewer, *Drug Discovery Today,* 2005, **10**, 1103-1109.
32 P. Ghosh, G. Han, M. De, C. K. Kim, V. M. Rotello, *Adv. Drug Delivery Rev.* 2008, **60**, 1307−1315.
33 D. A. Giljohann, D. S. Seferos, W. L. Daniel, M. D. Massich, P. C. Patel, C. A. Mirkin, *Angew. Chem., Int. Ed.* 2010, **49**, 3280-3294.
34 E. C. Dreaden, A. M. Alkilany, X. Huang, C. J. Murphy, El-M. A. Sayed, *Chem. Soc. Rev.* 2012, **41**, 2740-2779.
35 K.-S. Ock, U. Dembereldorj, J. Park, E.-O. Ganbold, S. Kim, H.-C. Shin, S.-W. Joo, *Spectrochim. Acta A Mol. Biomol. Spectrosc*. 2013, **102**, 419-424.
36 M. Bieri, T. Buürgi, *Langmuir* 2006, **22***,* 8379-8386.
37 X. M. Lin, G. M. Wang, C. M. Sorensen, K. J. Klabunde, *J. Phys. Chem. B* 1999, **103**, 5488-5492.
38 J. H. Freedman, M. R. Ciriolo, J. Peisach, *J. Biol. Chem.* 1989, **264**, 5598-5605.
39 L. E. Scott, C. Orvig, *Chem. Rev.*, 2009, **109**, 4885-4910.
40 A. Gupte, R. J. Mumper, *J. Inorg. Biochem.* 2007, **101**, 594-602.
41 T. O. Pangburn, K. Georgiou, P. S. Bates, E. Kokkoli, *Langmuir* 2012, **28**, 12816-12830.
42 A. Gupte, S. Wadhwa, R. J. Mumper, *Bioconjugate Chem.* 2008, **19***,* 1382-1388.