Supplementary Information

A Highly Efficient and Extremely Selective Intracellular Copper Detoxifying Agent Based on Nanoparticles of $ZnMoS_4$

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Materials.

Human hepatocellular carcinoma cell line (HepG2) was obtained from the American Type Culture Collection (Rockville MD, USA) and cryopreserved at -200 ºC prior to use. Dulbecco's Modified Eagle's Medium (DMEM, M0643, Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum, 2.2 g/L $NaHCO_3$, 1 mM sodium pyruvate, 100 U/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL amphotericin and 1% penicillin-streptomycin was used at 37 °C in an atmosphere of 5% CO_2 for culture and other cellular experiments with HepG2 cells.

Synthesis and characterization of $ZnMoS_4$ nanoparticles. $ZnMoS_4$ nanoparticles were synthesized according to the following procedure: 111 mg of $Zn(O_2CCH_3)_2$ in 6 mL of deionized water was added to a mixture of 3-mercaptopropionic acid (25 mL) and 1N NH_4OH (10 mL) solution dropwise. Next 130 mg of $(NH4)_2MoS_4$ in 20 mL of formamide (FM) solution was added into the above mixture under stirring. Finally, polyvinylpyrrolidone (PVP; 3 g, MW= 8000) was added as a surface-coating agent. The sample was dialyzed using regenerated cellulose tubular membrane (MWCO is 3500) against distilled water for two days. The solid product was collected by lyophilization.

TEM imaging and EDX measurements. $ZnMoS_4$ nanoparticles were first dispersed in water by by sonication. Next, one drop of the suspension was placed onto a carbon-coated copper TEM grid grid (400-mesh) and specimens were then allowed to air-dry prior to analysis. TEM measurements measurements were made using a FEI Tecnai F20 transmission electron microscope (TEM) equipped with a field emission gun and analyzed at accelerated voltage of 200 kV and an emission emission current of 30 mA. The energy dispersive X-ray spectroscopy (EDX) results were obtained for a selected area of the sample 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.

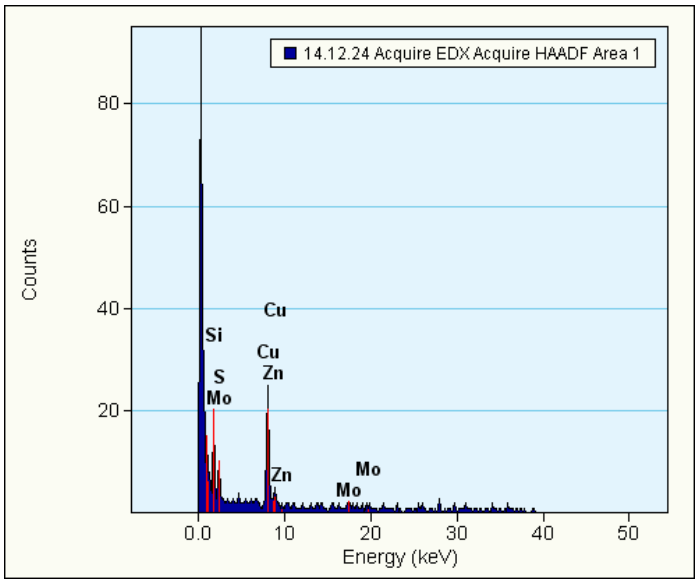


Figure S1. EDX spectrum of a representative $ZnMoS_4$ nanoparticle (Note the copper peak is due to the use of copper TEM grid).

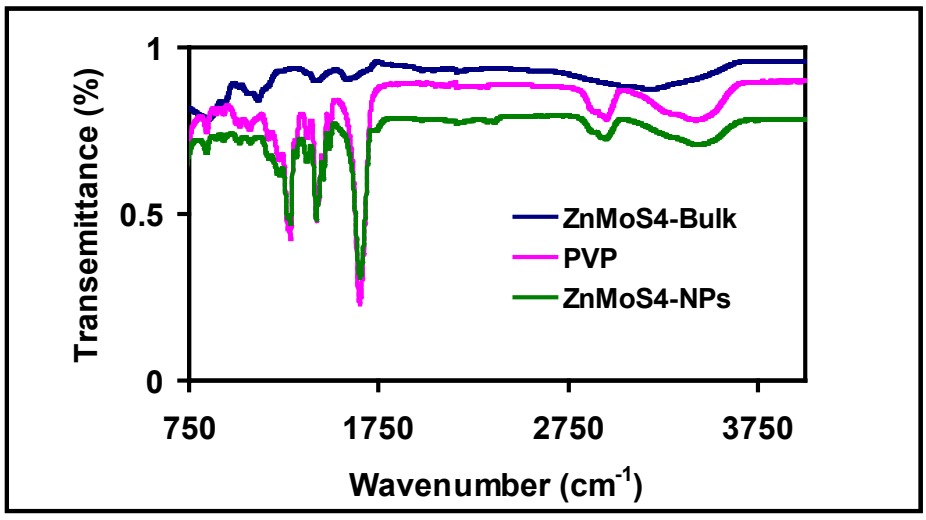


Figure S2. IR spectra of the bulk (blue), nanoparticles (green) of $ZnMoS_4$ and pure PVP (purple).

Powder X-ray Diffraction Analyses. The X-ray diffraction (XRD) measurements were recorded for the samples using a PANanalytical, Inc. X'Pert Pro (MPD) Multi-Purpose Diffractometer with Cu Kα radiation (1.5406 A) at an operating voltage of 45 kV. The $ZnMoS_4$ nanoparticles prepared at room temperature using the above described procedure gave a product with the elemental composition of Zn:Mo:S=1:1:4, but with poor crystallinity. In order to increase the crystallinity, the reaction mixture was charged into a Teflon-lined autoclave and heated at 120 ºC for 1 hour. After dialysis and lyapholization, the isolated $ZnMoS_4$ nanoparticles were found to be crystalline (Figure 3SA), although the size of nanoparticles had grown to larger than 100 nm. Similarly, the bulk $ZnMoS_4$ material was prepared under solvothermal conditions at temperature of 150 ºC. In a typical reaction, 87 mg of $(NH_4)_2MoS_4$ and 85 mg of zinc acetate were reacted in 1 : 1 mixture of ethanol and formamide solution (2 mL) in a sealed Teflon-lined hydrothermal autoclave for 12 hrs. The final dark brown powder was separated by centrifugation with acetone and water mixture (1:1 volume ratio). The X-ray diffraction (XRD) patterns of both the bulk $ZnMoS_4$ (Figure 3SB) and nanoparticle samples showed three main diffraction lines at 28.70°, 47.65°, 56.51°, with d values of 3.10781, 1.90663, 1.62704 respectively, which matched the previously reported values for $ZnMoS_4$.[1]

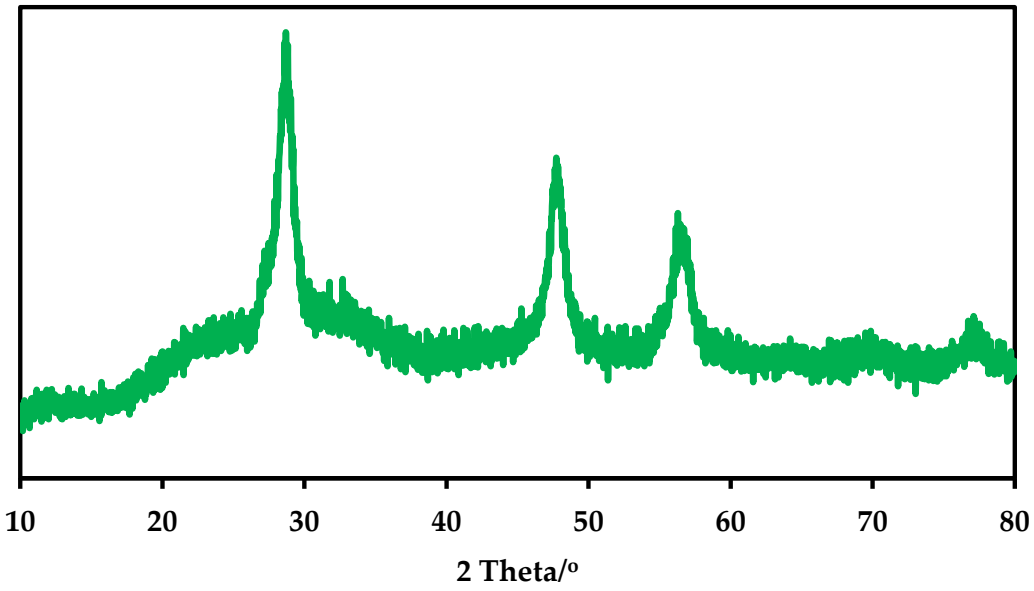


Figure S3A. PXRD pattern of PVP-coated $ZnMoS_4$ NPs.

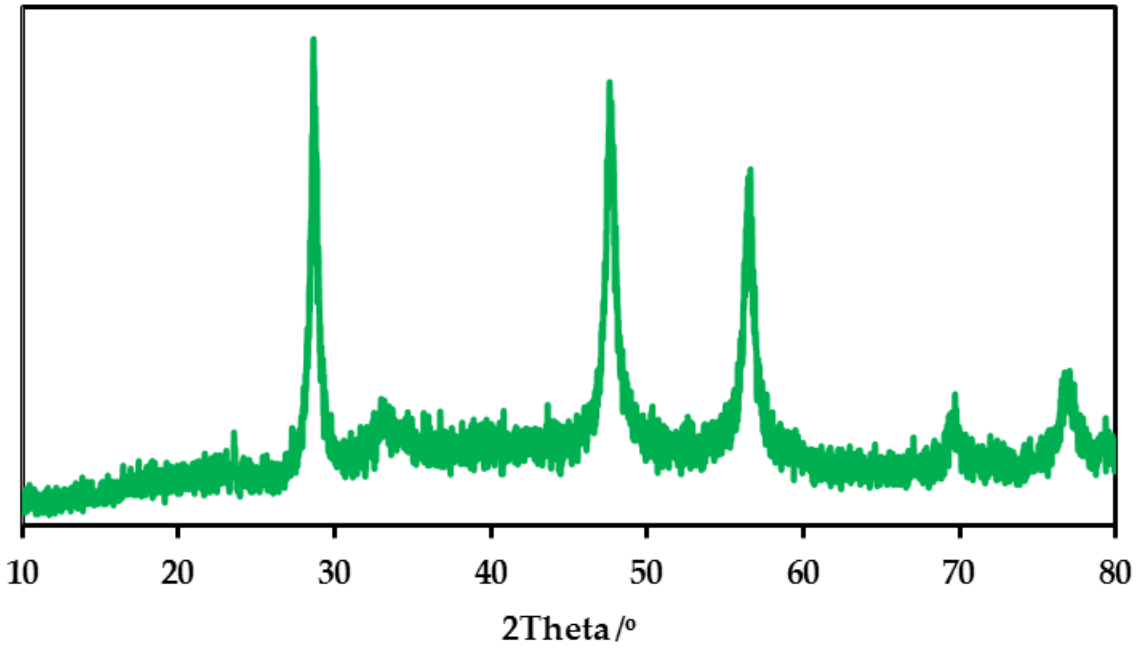


Figure S3B. PXRD pattern of bulk $ZnMoS_4$ material.

Determination of the K_{sp} values (solubility products) for $ZnMoS_4$ and Cu_2MoS_4

$ZnMoS_{4(s)} \longrightarrow Zn^{2+}{}_{(aq)} + MoS4^{2-}{}_{(aq)}$ [1]

$K_{sp} = [Zn^{2+}]\,[MoS_4{}^{2-}]$

$Cu_2MoS_{4(s)} \longrightarrow 2\,Cu^{+}{}_{(aq)} + MoS_4{}^{2-}{}_{(aq)}$ [2]

$K_{sp} = [Cu^{+}]^2\,[MoS_4{}^{2-}]$

The K_{sp} values for the above compounds were determined according to the following procedure: about 300 mg of each compound was weighed into separate 1 L beakers. 500 mL of deionized water was then added to each beaker, and the solutions were stirred at 25 ^{o}C for 48 hrs. The solutions were kept undisturbed for another 24 hrs to allow any suspended particles to settle.

25 mL aliquot from each supernatant solution was pipetted into 50 mL beakers and heated to dryness. Next 1 mL of concentrated nitric acid was added to each beaker and diluted the solutions by adding 2 mL of deionized water. The metal ion concentration was determined using AA for each of the solutions.

Studies of Zn-Cu ion-exchange kinetics, capacity and selectivity in aqueous soltuion. The kinetics of copper removal was done by using 50 mL of 500 µM copper(I) solution. Because Cu(I) disproportionates in water, but can be stabilized by the addition of acetonitrile. Furthermore, Cu(I) is more soluble in acetonitrile than in water.[2] Therefore, copper(I) chloride was dissolved in 80% acetonitrile solution to prepare Cu(I) solution. 100 mg of $ZnMoS_4$ nanoparticles was placed in a dialysis bag followed by adding copper solution while stirring. Then aliquots of outside solution were taken out at different time intervals. The aliquots were then evaporated to dryness and re-dissolved in 2% HNO_3 acid and diluted with deionized water before analyzing the copper content with AA. The kinetics data of the ion-exchange reaction can be fitted into two separate rate laws. A *pseudo* first-order up to the reaction time point of ~60 min with a rate constant of $k_1=3.0\times10^{-4}$ s^{-1} with a half-life of $t_{1/2}$ = 38.5 min (Figure S4A). The second part can be fitted to the second order rate law with a rate constant of $k_2= 1.8 \times 10^{-1}$ $mM^{-1}s^{-1}$ (Figure S4B).

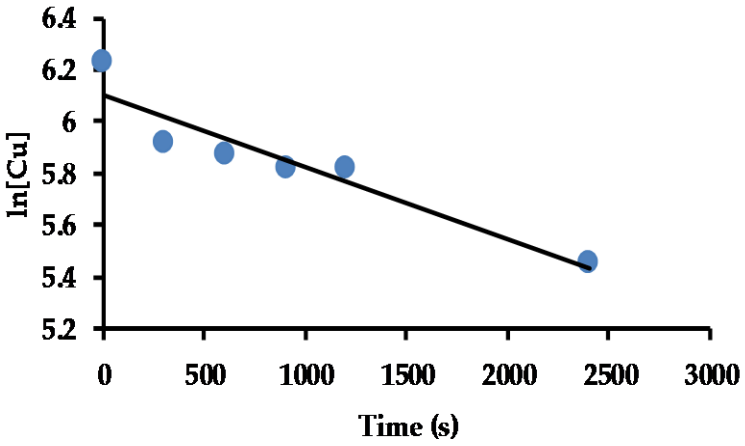


Figure S4A. PXRD pattern of $ZnMoS_4$.

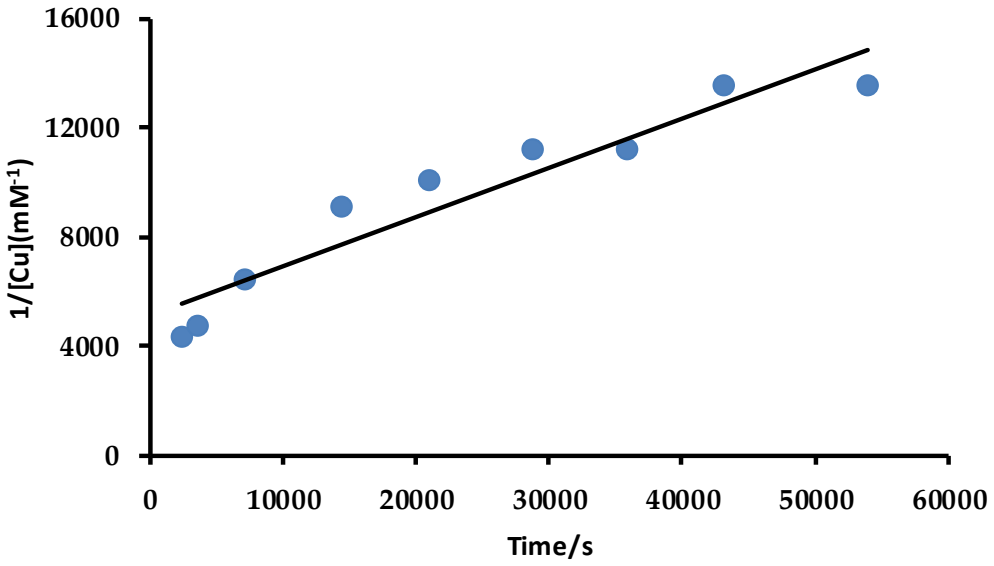


Figure S4B. PXRD pattern of $ZnMoS_4$.

Competitive removal studies were performed by soaking the dialysis bag containing 5 mL nanoparticles (10 mM) in a solution of copper(I), manganese(II), iron(II), zinc(II), calcium(II) and potassium(I). The concentration of each metal in the competitive solution was ~100 mg/L. After 48 hrs, an aliquot of solution was taken out and diluted with 2 % HNO_3 acid and analyzed for each metal ion using AA.

Nanoparticle-surface conjugation of fluorescence dye molecules and cell uptake studies. Confocal scanning microscopy was used to investigate the cellular uptake studies. Nanoparticles were resuspended in 2 mL of PVP solution (100 mg of PVP in 1 mL). 1.5 equivalents of ethylenediamine were then added to it under vigorous stirring. The resultant mixture was stirred for another 24 hrs and excess ethylenediamine was removed by dialysis against distilled water for two days. Then 5 mL of carboxyfluorescein dye (0.35 mg/mL) was reacted with 1.2 eq of EDC (≈1.5 mg) for 24 hrs. The ethylenediamine coated nanoparticles were then added to the 2 mL of above reaction mixture and stirred for Ca. 24 hrs. Finally the product was dialyzed to remove excess dye. The fluorescence spectra of the carboxyfluorescein dye and the nanoparticles after attaching the dye were obtained to confirm the conjugation of the dye to the nanoparticle

surface. The fluorescence spectra shown in Figure S5 clearly show that the presence of dye on the surface of the nanoparticles. The fluorescent dye-labeled nanoparticles were then incubated with HepG2 cells to visualize the cellular uptake of these nanoparticles by confocal microscopy. Briefly, HepG2 cells were seeded in an 8 well chamber at a density of approximately 1.3×10^5 cells per well in complete medium in the absence of antibiotics and incubated for 24 hrs at 37 ^{o}C. The cells were then incubated with dye-labeled nanoparticles for 3 hrs at 37 ^{o}C. The cells were washed with PBS three times and then imaged using a confocal microscope with 488 nm excitation wavelength. Furthermore, continued monitoring of the fluorescence signals in these living cells showed that the residence time of the NPs within the cell is about 5 hurs before exocytosis takes place (see **Figure S6**)

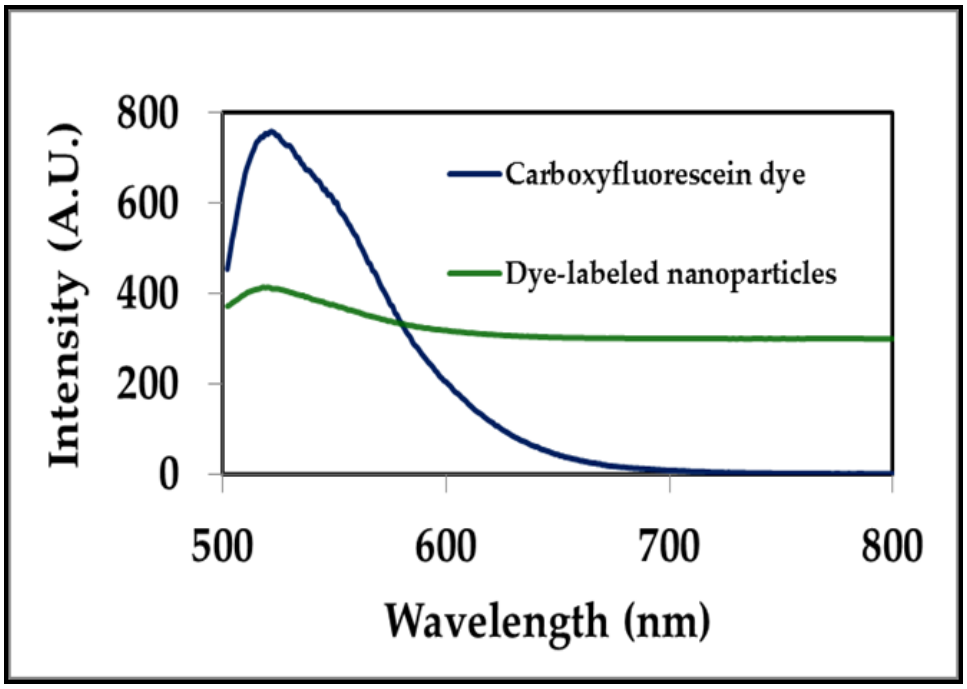
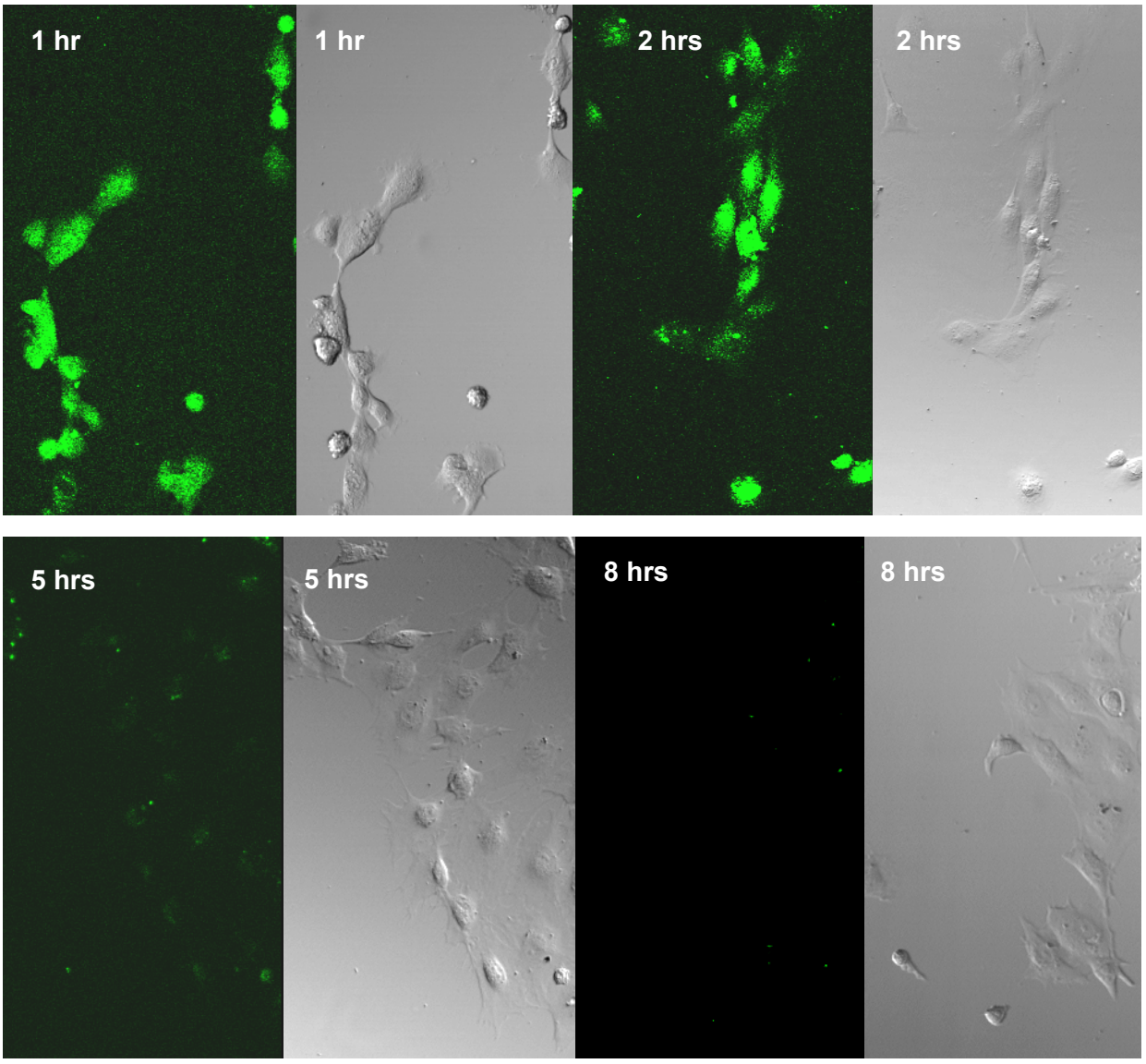


Figure S5. Fluorescence spectra of carboxyfluorescein dye and dye labeled nanoparticles.



Cell Viability Assays. Cytotoxicity studies were performed using the MTT viability assay. HepG2 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 5 hrs at 37 °C in an atmosphere of 5% CO_2 and 95% air to allow 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 or 48 hrs. Control wells contained the same medium without nanoparticles. The cells were then trypsinized and re-suspended in 100 μL medium without serum, then added to 100 μL of 0.4% trypan blue solution. Viable and non-viable cells were counted using a hemocytometer. Each

concentration was tested in replicates of three. The assay results were presented as percent viable cells.

Evaluation of cellular copper detoxification by $ZnMoS_4$ nanoparticles. Copper ions were introduced into HepG2 cells by incubating the cells with the DMEM medium supplemented with 300 μM of copper sulfate solution. Copper uptake kinetics by HepG2 cells were determined by measuring the copper levels of the cells at different time intervals. For that, the cells were washed three times with PBS and resuspended in serum free medium and counted using hemocytometer. Atomic absorption measurements were used to determine the final copper concentration of the HepG2 cells after incubation for different time periods with copper sulfate solution. The cells were lysed by concentrated nitric acid and diluted with deionized water before taking the atomic absorption measurements. These studies showed that the copper concentration in the cells became saturated after 12 hr-incubation. The copper-saturated cells were then washed three times with PBS and further incubated with the culture medium containing nanoparticles (200 μg/ mL) at 37 ^{o}C for different time periods. The cells were washed three times with PBS to remove the un-internalized nanoparticles and further incubated with the fresh culture medium. To quantify the concentration of copper in cells from each cell culture flask (T25 flask), the cells were washed three times with PBS at different time intervals and lysed using concentrated nitric acid. After dilution with deionized water, the concentration of each lysate was determined by AA. Additionally, the ICP measurements showed that the concentration of molybdenum in the cells treated with NPs is 371 fg per cell. After 2 hrs of incubation, the concentration of molybdenum is decreased to 111 fg per cell, and after 6 hrs of incubation, the molybdenum can no long be detected. This seems to suggest that TTM can exit cells via exocytosis. We have now included a paragraph in ESI to describe this experiment.

Endothelial cell tube formation assay. 10^4 HuVEC cells in 100 uL suspension of basal media with or without FGF-2 (50ng/mL) were seeded into a 96 well plate coated with 50 uL BME gel. Tube formation was assessed after 12 hours of PVP-$ZnMoS_4$ NPs treatment (20uM, 50 uM, 100 uM, 150 uM) by staining cells with Calcein AM. Effects of NPs were quantitated by counting the number of branches/tubular structures under 200X fluorescent microscope. Inhibition of tube formation was observed in those wells that were treated with all the four nanoparticle concentrations.

References:

1. G. H. Guo, Y. T. Tao, Z. P. Song, K. L. Zhang, *J Solid State Electrochem.,* 2007, **11**, 90-92
2. J. Irangu, R. B. Jordan, *Inorganic Chemistry,* 2007**, 46**, 1451-1458.