Electronic Supplementary Material (ESI) for Inorganic Chemistry Frontiers.

Supporting information

A molybdenum-based nanoplatform with multienzymes mimic capacity for oxidative stress-induced acute liver injury treatment

Zhirui Zhang, [a,1] Jiulong Zhao,[b,1] Zheng Chen, [a] Hang Wu,[b] and Shige Wang[a,*]

[a] School of Materials and Chemistry, University of Shanghai for Science and Technology, No. 516 Jungong Road, Shanghai 200093, China.

[b] Department of Infectious Diseases, Changhai Hospital, Naval Military Medical University, Shanghai 200433, China.

[1] These authors contributed equally to this work. * Corresponding email: sgwang@usst.edu.cn (Dr. Wang).

CONTENT

1. Materials and instrumentation

Ammonium tetrathiomolybdate ($(NH_4)_2MoS_4$), bovine serum albumin (BSA)，sodium hydroxide (NaOH), Acetic acid (HAc), sodium acetate (NaAc), N, N-Dimethylformamide (DMF), 2′,7′-Dichlorofluorescin diacetate (DCFH-DA, D6883) from Sigma-Aldrich (Shanghai, China). Polyethylene glycol 400 (PEG-400) was brought from Sinopharm chemical reagent Co. 3,3′,5,5′-Tetra- methylbenzidine (TMB) and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonate) (ABTS) was bought from Macklin (Shanghai, China). Phosphate-buffered saline (PBS, pH 7.4, Na_2HPO_4–NaH_2PO_4, 10 mM) solution was prepared in the laboratory. All chemicals and reagents were of analytical grade and used as received without further purification. Ultrapure water (18.2 MΩ cm^{-1} at 25 °C) purified by a Milli-Q system was used throughout the experiment.

Transmission electron microscope (TEM) imaging was conducted on a Tecnai G220 (Shimadzu, Japan) at 200 keV. The Samples were prepared by redispersing the MPB nanosheets in ethanol. Fourier Transform infrared spectroscopy (FTIR) spectra were measured by an FTIR-8300 series spectrometer (Shimadzu, Japan) in the range of 4,000–400 cm^{-1}. The fluorescence spectra were determined by using an F-2500 spectrofluorometer (Hitachi, Japan). The UV-vis spectra were obtained using a Hitachi U-3010 spectrometer (Hitachi, Japan). A dynamic light scattering (DLS) particle size analyzer (Malvern 2000, USA) was used to determine the hydrophilic diameters of the particles. X-ray photoelectron spectroscopy (XPS) was performed by an ESCALAB 250 Xi Mg X-ray resource (Thermo Scientific, Japan). The X-ray diffraction (XRD) (Rigaku SmartLab SE, Japan) results were obtained with a test range of 2-135° and a

scanning speed of 1-10/min. The concentration of W was detected by inductively coupled plasma (ICP) (Thermo Scientific, iCAP 7400, USA). The electron paramagnetic resonance (EPR) spectroscopy signal was obtained on a Bruker A300 (X-band) spectrometer (Bruker, Germany). All the measurements were performed at room temperature if not specially mentioned.

2. Experimental section

2.1 Preparation of MPB nanosheets

For the synthesis of MPB nanosheets, 150 mg $(NH_4)_2MoS_4$ was firstly dissolved in 30 mL deionized (DI) water. Then, 30 mL PEG-400 was dripped into the mixture solution with a 30-min extensive magnetic stirring in a 100 mL polyphenylene-lined stainless-steel autoclave. After the liquid reacted at 220℃ for 12 h, the nanosheets were produced in the autoclave. The obtained product was centrifuged for 30 minutes (21,000 rpm). The precipitate was thoroughly washed with ethanolamine solution (50%, in water, v/v) 2 times and DI water 2 times. To obtain the MPB nanosheets, previous products were redistributed in DI water, and 0.5 g bovine serum albumin (BSA) was added to the solution. Finally, the above solution was ultrasonically pulverized with a cell pulverizer for 2 h with 520 W power, followed by thoroughly washing with DI water 3 times. The obtained MPB nanosheets were finally stored at 4 ℃.

2.2 H_2O_2 scavenging activity of MPB

Hydrogen peroxide (H_2O_2) detection kit (Nanjing Jiancheng Bioengineering Institute, China) was applied to detect the H_2O_2 scavenging capacity of MPB

nanosheets. The stable yellow complex formed by the reaction of H_2O_2 and ammonium molybdate shows an absorption peak at 405 nm. Then, 163 mM H_2O_2 were incubated with different concentrations of MPB nanosheets (0, 25, 50, 100, 200, and 400 μg/mL) at 37 °C for 2 h, respectively. The concentration of the remaining H_2O_2 was determined based on the absorbance of each sample at 405 nm. Samples containing MPB nanosheets were centrifuged before absorbance detection to determine the absorbance of the supernatant. The H_2O_2-eliminating capacity was calculated as follows:

$$H_2O_{2scavenging}\ (\%) = (A_{blank}\text{-}A_{MPB})/\ A_{blank} \times 100\%$$

(A_{blank} refers to the absorbance value of the blank group, DI water was used instead of the sample; A_{MPB} refers to the absorbance value of the sample group).

2.3 ·OH scavenging activity of MPB nanosheets

The hydroxyl radical (·OH) scavenging activity of MPB nanosheets was tested through two different methods. First, measurement of ·OH scavenging activity through the salicylic acid chromogenic method was applied. After adding 50 μL $FeSO_4$ (9 mM), 50 μL H_2O_2 (8.8 mM), 50 μL salicylic acid (6 mM, dissolved in ethanol) into 0.5 mL MPB nanosheets aqueous solution (0, 25, 50, 100, 200, 400 μg/mL), they were stirred well and incubated at 37 °C for 30 min. Samples containing MPB nanosheets were centrifuged before absorbance detection to determine the absorbance of the supernatant. The characteristic absorbance at 510 nm was measured. The ·OH scavenging rates can be calculated as follows:

$$\cdot OH_{\ scavenging}\ (\%) = (A_{blank}\text{-}A_{MPB})/\ A_{blank} \times 100\%$$

(A_{blank} is the absorbance value of the blank group, DI water was used instead of the sample; A_{MPB} is the absorbance value of the sample group).

The elimination of ·OH by MPB nanosheets was further qualitatively analyzed by electron paramagnetic resonance (EPR). Typically, 20 μL of 10 mM H_2O_2, 1 mM $FeSO_4$, 250 mM DMPO, and 1,000 μg/mL MPB nanosheets were added into the HAc/NaAc buffer (0.5 M, pH 4.5) for reaction. The EPR signal of the sample was immediately detected (Bruker A300 spectrometer, Germany).

2.4 O_2^- scavenging activity of MPB nanosheets

The superoxide anion (O_2^-) scavenging activity was evaluated by a superoxide anion assay kit (Nanjing Jiancheng). The MPB nanosheets with different concentrations (0, 25, 50, 100, 200, and 400 μg/mL) were added to the working solution and stored at room temperature for 10 min. Samples containing MPB nanosheets were centrifuged before absorbance detection to determine the absorbance of the supernatant. The absorbance at 550 nm was measured using a microplate reader (MK3 micro-plate, Thermos Scientific) to detect the O_2^- scavenging rates:

$$O_2^-{}_{scavenging}\ (\%) = (A_{blank}\text{-}A_{MPB})/\ A_{blank} \times 100\%$$

(A_{blank} is the absorbance value of the blank group, DI water was used instead of the sample; A_{MPB} is the absorbance value of the sample group).

The elimination of O_2^- by MPB nanosheets was further qualitatively analyzed by EPR. In details, solution A (containing 1 mM BMPO), solution B (containing 1 mM DTPA and 0.4 mM xanthine) and solution C (containing 0.1 U/mL xanthine oxidase) were prepared with 50 mM phosphate buffer solution (PBS, pH 7.4) separately. Then, 15 μL of solution A, 135 μL of solution B, and 10 μL of solution C were mixed with 50 μL of MPB nanosheets (1,000 μg/mL) and incubated for 5 min at room temperature. The EPR signal of the sample was detected immediately.

2.5 ABTS free radicals ($ABTS^+$) scavenging activity of MPB nanosheets

To produce $ABTS^+$, ABTS stock solution (7 mM) was reacted with potassium persulfate (2.45 mM) in the dark for 16 h. Then, 1 mL of different MPB nanosheet solutions (0, 25, 50, 100, 200, and 400 μg/mL) were mixed with 1 mL ABTS solution and incubated in the dark for 10 min. Samples containing MPB nanosheets were centrifuged before absorbance detection to determine the absorbance of the supernatant. The absorbance at 734 nm was then detected by UV–vis spectroscopy to calculate the ABTS radical scavenging:

$$ABTS^+_{\text{scavenging}}(\%) = ((A_{\text{blank}} - A_{\text{MPB}})/ A_{\text{blank}}) \times 100\ \%$$

(A_{blank} is the absorbance of a standard solution without any MPB nanosheets, and A_{MPB} is the absorbance after the reaction between MPB nanosheets and the radical scavengers, respectively.)

2.6 The SOD-like activity and CAT-like actvity of MPB nanosheets

The SOD-like activity of MPB nanosheets was evaluated using a SOD assay kit (Nanjing Jiancheng, Nanjing, China) according to the manufacturer's instructions. MPB nanosheets (0, 200, 400, 800, and 1,000 μg/mL) were mixed with the working solution. The characteristic absorbance at 450 nm was measured using a multiple plate reader after incubation at 37 °C for 20 min.

The CAT-like activity of the MPB nanosheets was evaluated using a CAT assay kit (Nanjing Jiancheng, Nanjing, China). The remaining H_2O_2 decomposed by CAT can react with ammonium molybdate to produce a pale-yellow complex, which displays an absorbance peak at 405 nm. MPB nanosheets (0, 200, 400, 800, and 1,000 μg/mL) were added to the working solution. The CAT activity of the MPB nanosheets was calculated

by measuring the absorbance at 405 nm after 1 min of exact reaction. Both SOD-like and CAT-like activity can be calculated as follows:

$$\text{SOD/CAT-like activity}\,(\%) = (A_{blank} - A_{MPB})/\,A_{blank}) \times 100\ \%$$

(A_{blank} is the absorbance of a standard solution, DI water was used instead; A_{MPB} is the absorbance after the reaction with MPB nanosheets.)

2.7 The GPx-like activity of MPB nanosheets

The GPx-like activity of MPB nanosheets was calculated using a GPx assay kit (Byotime Biotechnology, Shanghai, China) by detecting the decrease of nicotinamide adenine dinucleotide phosphate (NADPH). The MPB nanosheets (0, 200, 400, 800, and 1,000 μg/mL), the Gpx detection working solution, and the detection buffer were added into a 96-well plate and incubated at room temperature for 15 min to consume GSSG and eliminate interference with subsequent detection. The absorbance at 340 nm of the sample was immediately measured by a microplate reader at a room temperature of 25℃ (recorded as a 0-min reading). The A_{340} value was recorded every 1 min for 15 min consecutively to obtain data for 16 points. Finally, the data before the linear time points were recorded for analysis. The Gpx activity of the whole system was calculated as follows:

$$\text{Gpx-like activity} = [(\ A_{340}(\text{sample}) - A_{340}(\text{blank}))/\text{min}]/\ (\quad \times L\ (\text{cm}))$$

($\boldsymbol{\varepsilon^{\mu M}}$ is the molar extinction coefficient, and the $\boldsymbol{\varepsilon^{\mu M}}$ of NADPH at A_{340} is 0.00622 $\mu M^{-1}cm^{-1}$; L(cm) is the path length when measuring absorbance: 0.276 cm).

2.8 In vitro cytotoxicity measurement

As a typical hemolysis test *in vitro*, KM mice were anesthetized by 4% hydrated aldehyde and punctured the heart to collect blood. All animal experiments in this study

were conducted under the guidance of Changhai Hospital of the Second Military University and in strict accordance with the relevant policies of the National Ministry of Health. The blood was then centrifuged (3000 rpm, 3 min) to harvest mRBCs. The obtained mRBCs were washed three times with PBS and stored in PBS for further use. Then, 1 mL of the mRBCs solution was incubated with 3 mL of different concentrations (25, 50,100, and 200 μg/mL) of MPB nanosheets, 3 mL of PBS (defined as negative control), or 3 mL of water (defined as positive control) in 5 mL centrifugal tubes for 2 h at a 37 °C water bath (n = 3). The treated mRBCs were incubated and centrifuged (5000 rpm, 3 min). The supernatant's absorption at 570 nm was read to calculate the hemolysis ratio.

Using Mouse fibroblast (L929) cells as the model, the cytotoxicity assessment of MPB nanosheets was evaluated in complete Dulbecco's Modified Eagle's Medium (DMEM) in a humidified incubator (37 °C, 5% CO_2). Then, ~ 8 × 103 cells were seeded in each well of a 96-well plate and incubated overnight. The old medium was replaced with fresh DMEM containing MPB nanosheets with a concentration of 200, 150, 100, 50, and 0 μg/mL. After 24 h, these cells were PBS-rinsed 3 times. The viability of treated cells was counted using the CCK-8 kit and Dead/Live staining. The appearance of Dead/Live stained cells was captured using the Leica DM IL LED inverted phase-contrast microscope.

2.9 *In vivo* hemo-compatibility and biodistribution

All animals' feedings and experiments were carried out at the Shanghai Slaughter Laboratory Animal Centre (Animal Center of Changhai Hospital affiliated to the Second Military Medical University). The providing Kunming mice were randomly divided into 4 groups (n = 3). After fasting for 12 h, a group of mice was intravenously injected with 0.1 mL saline as the control group, and each mouse in the experimental group was given an intravenous injection of MPB nanosheets aqueous solution (300 mg/kg). Note that the injected materials will be diluted by mouse blood and the actual concentration of material *in vivo* would not be higher than 200 μg/mL. The body weights of the mice were routinely recorded every 2 days. The mice in the experimental group were euthanized on the 1st day, 7th day, and 28th day after the materials injection, while those in the control group were euthanized on the 28th day. Blood samples were collected for routine and biochemical analysis. Part of the tissues from the main organs (heart, liver, spleen, lung, and kidney) was immersed in 4% paraformaldehyde (Byotime Biotechnology, Shanghai, China) for the next step of hematoxylin-eosin (H&E) staining.

To detect the biodistribution of MPB nanosheets in the major organs, glycerol-induced acute liver injury diagnosed BALB/c mice (n = 3 were intravenously injected with MPB nanosheets at a dose of 300 mg/kg. One day after injection, mice were sacrificed to harvest major organs including the heart, liver, spleen, lung, and kidney. The tissues were weighed, homogenized, and then dissolved in aqua regia to calculate the percentage of injected dose per gram of tissue (%ID/g) by ICP-AES.

2.10 Intracellular ROS detection

At 24 h before the intervention, Raw 264.7 cells (from the Institute of Biochemistry and Cell Biology, the Chinese Academy of Sciences, Shanghai, China) were seeded on a 6-well plate at a density of 100,000 cells/well. Then, the cells were incubated with MPB nanosheets (200 μg/mL) for 4 h, followed by H_2O_2 (500 μM) treatment for 24 h. After a predetermined time, the cells were washed with PBS and collected by trypsin treatment. Raw 264.7 cells were stained with propidium iodide/Annexin V-FITC and analyzed by flow cytometry (CytoFLEX S).

2.11 Assessment of *in vivo* liver injury status

Male BALB/c mice (6-8 weeks old, Shanghai Slaughter Laboratory Animal Centre) were divided into 3 groups (high concentration MPB nanosheets treatment group, low concentration MPB nanosheets treatment group, and control group, n = 5). All animal experiments were carried out in accordance with the protocols approved by the Experimental Animal Center of Changhai Hospital of the Second Military Medical University and the policies of the Ministry of Health. The ACUTE LIVER INJURY model of BALB/c mice was established by intraperitoneal injection of acetaminophen (300 mg/kg), after fasting for 12 h. Note that the injected materials will be diluted by mouse blood and the actual concentration of material in vivo would not be higher than 200 μg/mL. Mice were treated with MPB nanosheets (low concentration group: 20 mg/kg; high concentration group: 40 mg/kg) intravenously, after 30 min of induction. At 12 h after the liver injury induction, the mice were euthanized. Serum and liver samples were collected for liver function test and histological analysis. The status of liver injury in the acetaminophen-induced ACUTE LIVER INJURY model was evaluated through serum biochemical and histological analysis. The concentrations of

serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and tumor necrosis factor-α (TNF-α) levels were determined by the automatic biochemical analyzer.

2.12 Histopathological tests

The livers of the control, treatment, and model groups were first fixed with 4% paraformaldehyde and then treated with ethylenediaminetetraacetic acid for 4 weeks. Before being dehydrated and embedded in paraffin, the samples were then dehydrated with a series of ethanol and fixed in 4% paraformaldehyde for 24 h. Hematoxylin and eosin (H&E) were applied to separate the paraffin sections into 5 μm thick sections. The above-stained sections were detected by using an optical microscope (DM 4000B, Leica Microsystems, Wetzlar, Germany). Suzuki's score was judged within six random fields of view for each segment. During the TdT-mediated dUTP nick end labeling (TUNEL) assays, the de-waxed sections were incubated with protease K for 15 min at 37°C. The One-Step TUNEL Apoptosis Assay Kit (Beyotime Biotechnology, Shanghai, China) was performed according to the manufacturer’s instructions.

2.13 Statistical analysis

The significant difference of data (*, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$) was judged according to the one-way ANOVA method.