Supporting Information

Ag@*S*-nitrosothiols core-shell nanoparticles for chemo and photothermal synergistic tumor target therapy

Tuanwei Liu,[#,*,a] Xiao Li,[#,a] Jilan Wang,[#,b] Ping Zhang,[a] Xiaoying Huang,[a] Zhide Zhang,[*, a] Dian-shun Guo,[*, a] and Xinlin Yang[*, c]

a) College of Chemistry, Chemical Engineering and Materials Science, Key Laboratory of Molecular and Nano Probes, Ministry of Education, Collaborative Innovation Center of Functionalized Probes for Chemical Imaging in Universities of Shandong, Shandong Normal University, Jinan 250014, PR China.

b) Department of Anesthesiology, Shandong Provincial Hospital Affiliated to Shandong University, Jinan 250021, P. R. China.

c) Key Laboratory of Functional Polymer Materials, Ministry of Education, Institute of Polymer Chemistry, College of Chemistry, Nankai University, Tianjin 300071, PR China.

# These authors equally contributed to this work.

* Corresponding authors.

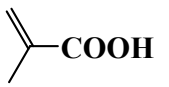
E-mail addresses: twliu2010@163.com (Tuanwei Liu), zdzhang@sdnu.edu.cn (Zhide Zhang), chdsguo@sdnu.edu.cn (Dian-shun Guo), xlyang88@nankai.edu.cn (Xinlin Yang).

Materials and chemical structures

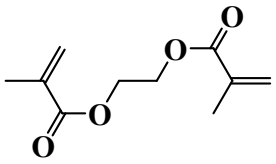
Materials

Silver nitrate solution, citric acid trisodium salt, ammonium hydroxide, (methacryloxy)-propyltrimethoxysilane (MPS), ethanediamine, hydrofluoric acid, benzoyl peroxide (BPO) were purchased from Tianjin Fuyu Fine Chemical Co. Ltd. Methacrylic acid (MAA), ethyleneglycol dimethacrylate (EGDMA), hyaluronic acid (HA), *N*-hydroxysulfosuccinimide sodium salt (NHS), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI), 4-dimethylaminopyridine (DMAP), and tetraethyl orthosilicate (TEOS) were purchased from Alfa Aesar and used without purification. Griess reagent (S0021) was purchased from Shanghai Beyotime Institute of Biotechnology. Acetyl-*D*-penicillamine were purchased from Shanghai Macklin Biochemical Technology Co. Ltd, China.

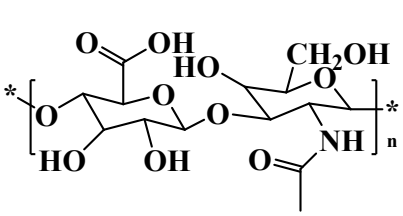
Chemical structures



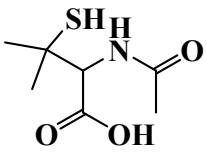
(MAA)



(EGDMA)



(HA)



Acetyl-D-penicillamine

Preparation of $Ag@SiO_2$ core-shell nanoparticles

60 mL of sodium citrate solution (1 %) was added into silver nitrate solution (1.5 mmol/L, 150 mL) under stirring. After heating at 100 ^{o}C for one hour, the mixture continued to stir at room temperature and formed Ag nanoparticles suspension.

50 mL of the Ag nanoparticles suspension was added into 150 mL of ethyl alcohol and stirred at room temperature for 5 mins. 5 mL of ammonium hydroxide was added into the mixture and stirred for 15 mins. After that, twice amount of 20 μL TEOS were added into and continued to stir 8 hours at room temperature, getting the Ag@SiO_2 core-shell nanoparticles suspension. The Ag@SiO_2 nanoparticles were afforded by centrifugation-decantation-resuspension cycle for three times, and dried in a vacuum oven till constant weight

Preparation of Ag@SiO_2-P(MAA-*co*-EGDMA) NPs core-shell nanoparticles

100 mg of the Ag@SiO_2 nanoparticles were dispersed in 20 mL of ethyl alcohol. Under stirring, 2 mL of ammonium hydroxide and 200 mg of MPS were added into the mixture with continuous stirring for 48 hours. The MPS modified Ag@SiO_2 nanoparticles were afforded by centrifugation-decantation-resuspension cycle for three times.

100 mg of MPS-Ag@SiO_2 nanoparticles were ultrasonic dispersed in 40 mL of acetonitrile. 0.42 mL of MAA, 0.96 mL of EGDMA and 10 mg of AIBN were then added into the mixture. The reaction system began to be heated and distilled 20 mL of acetonitrile. The resultant Ag@SiO_2-P(MAA-*co*-EGDMA) core-shell nanoparticles were purified by centrifugation-decantation-resuspension cycle for three times.

Preparation of the Ag@P(MAA-*co*-EGDMA)-HA core-shell nanoparticles

100 mg of the Ag@SiO_2-P(MAA-*co*-EGDMA) core-shell nanoparticles were dispersed in 30 mL of acetonitrile. Under stirring, 35 mg of EDCI and 3 mg of DMAP was added into the mixture, following with addition of ethanediamine (10 ml). After stirring at room temperature for 48 hours, the Ag@SiO_2-P(MAA-*co*-EGDMA)-NH_2 nanoparticles were purified by centrifugation-decantation-resuspension cycle for three times.

In order to selectively etch the SiO_2 core nanoparticles and afford the rattle-type Ag@P(MAA-*co*-EGDMA)-NH_2 nanoparticles, we added 100 mg of Ag@SiO_2-P(MAA-*co*-EGDMA)-NH_2 nanoparticles into 10 mL of ethyl alcohol. Then, 1.0 mL of hydrofluoric acid was added into the mixture and stirred overnight, following the addition of saturated sodium hydroxide solution for neutralization. The Ag@P(MAA-*co*-EGDMA)-NH_2 nanoparticles were purified by centrifugation-decantation-resuspension cycle for three times.

In the next step, 100 mg of Ag@P(MAA-*co*-EGDMA)-NH_2 nanoparticles, 10 mg of NHS, and 6 mg of EDCI were added into 30 mL of acetonitrile, following addition of HA solution (0.2 mg/mL) for stirring (6 hours, room temperature). The Ag@P(MAA-*co*-EGDMA)-HA core-shell nanoparticles were purified by centrifugation-decantation-resuspension cycle in several times.

Preparation of Ag@*S*-nitrosothiols-HA core-shell nanoparticles

100 mg of Ag@P(MAA-*co*-EGDMA)-HA core-shell nanoparticles and excess equivalent of NAP-thiolactone to the free amino groups on the interface P(MAA-*co*-EGDMA) polymeric layer were dispersed in 40 mL of chloroform and stirring for 3

hours at room temperature. After the reaction, the nanoparticles were centrifuged and dispersed in 10 mL of H_2O/CH_3OH mixture (V/V, 1/1) at 0 ^oC. 1 mL of hydrochloric acid (1 M) and 1 mLof sodium nitrite (0.5 M) were successively added into the mixture, reacted for 90 mins, purified by centrifugation-decantation-resuspension cycle for three times. After being dried in a vacuum oven at room temperature for 48 h, the Ag@*S*-nitrosothiols-HA core-shell nanoparticles were stored in a freezer in absence of light irradiation.

Characterization and analysis of the nanoparticles

The morphology, particle size, and size distribution of the nanoparticles were characterized by TEM (JEOL JEM-2100, Japan). All of the TEM size data reflect the averages of the particles, which are calculated by the formulae as follow.

$$U = D_W / D_n, \quad D_n = \sum_{i=1}^{k} n_i D_i / \sum_{i=1}^{k} n_i, \quad D_w = \sum_{i=1}^{k} n_i D_i^4 / \sum_{i=1}^{k} n_i D_i^3$$

where, U is the polydispersity index, D_n is the number-average diameter, D_w is the weight-average diameter, D_i is the particle diameter of the determined nanoparticles.

Fourier transform infrared spectra (FTIR) were carried out by a Bruker Alpha FTIR spectrometer over potassium bromide pellets and the diffuse reflectance spectra were scanned.

The elemental analyses (EA) were determined by a Perkin Elmer 2400 to determine the carbon, hydrogen, and nitrogen contents of the nanoparticles. In order to check the sulfur and silver element contents, experiments were cross-performed on an ICP-9000 (N+M) instrument (Inductive Coupled Plasma Emission Spectrometer, Thermo Jarrell-Ash Crop, USA).

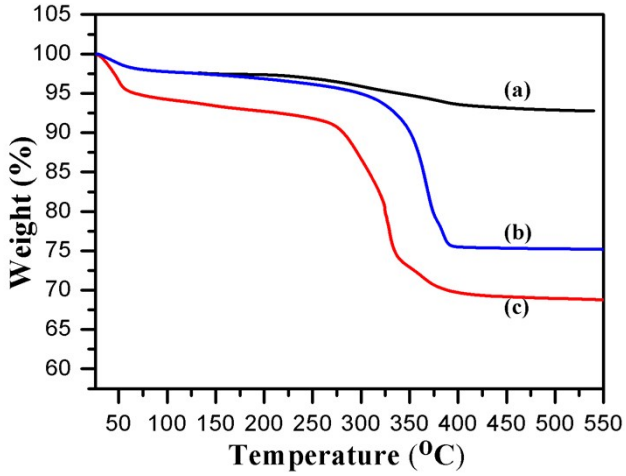
X-ray photoelectron spectrometer (Mg as exciting source, ESCALAB MK II), and the X-ray diffractometer (2θ ranging from 10° to 90°, PERSEE, XD-3) were applied.

Tab. S1 Analytical data of the nanoparticles.

Entry	Sample name	D_n[a] (nm)	U[a]	D_h[a] (nm)	C[b] (%)	H[b] (%)	N[b] (%)	S[c] (%)
1	Ag@SiO_2 NPs	89.72	1.05	102.03	-	-	-	-
2	**Ag**@SiO_2-P(MAA-*co*-EGDMA) NPs	156.82	1.05	406.23	-	-	-	-
3	**Ag**@P(MAA-*co*-EGDMA)-HA NPs	141.41	1.06	485.53	50.69	7.66	9.28	-
4	Ag@*S*-nitrosothiols-HA NPs	142.33	1.06	492.20	52.26	8.31	11.25	5.64

a) D_n and U refer to the number-average diameter and the size distribution index of the particles by TEM, D_h refers to hydrodynamic diameters by dynamic laser scattering (DLS); b) Referring to the *CHN* elemental characterization data; c) Referring to the *S* elemental analyze data by *ICP*.

Fig. S1 TGA characterization of the Ag@SiO_2 NPs (**a**), **Ag**@SiO_2-P(MAA-*co*-EGDMA) NPs (**b**), and Ag@*S*-nitrosothiols-HA NPs (**c**), respectively.



NO release behavior of the nanoparticles

The NIR light triggered release characters of the Ag@*S*-nitrosothiols-HA nanoparticles or the SiO_2@*S*-nitrosothiols-HA nanoparticles were used for determination of NO release behavior with respective to the various triggers (such as ascorbate, light, copper, temperature, thiol, etc). The kinetic of NO release were determined by Griess kit assay in PBS buffer (pH 7.4) with an initial temperature of 37 ^{o}C under a laser (2 W, 808 nm, continuous irradiation for 3 mins each 50 mins). A typical procedure was as follow: 5 mg of nanoparticles in 5 mL of PBS buffer were added into a sealed bottle in dark and mildly stirred at 37 ^{o}C after ultrasonic dispersion. At each pre-determined time interval point, continuous irradiation was applied for 3 mins. After that, the mixture was centrifugated and 1.0 mL of supernatant was taken out of the system (Then 1.0 mL of fresh PBS buffer was added for further investigation of NO release). Finally, 100 μL of the sampled supernatant was added with 100 μL of Griess reagent I and 100 μL of Griess reagent II. The azo compound was formed as purple color and the concentrations were determined by the absorbance at the wavelength of 540 nm on a UV-vis spectrometer. $T_{[NO]}$ (The total amount of NO-releasing) and the NO release kinetics were determined according to a working curve.

The NO release behavior of the Ag@*S*-nitrosothiols-HA nanoparticles in a bovine serum or PBS buffer without laser irradiation was also performed in a similar procedure.

In the experiment of the temperature variation investigation, 20 μg/mL of the **A**g@*S*-nitrosothiols-HA NPs or the SiO_2@*S*-nitrosothiols-HA NPs were dispersed in PBS buffer (pH 7.4) and mildly stirred with an initial temperature of 37 ^{o}C. At every

pre-determined time interval point, irradiation was applied and the temperature was recorded.

***In vitro* cells survival investigation**

The cells for experiments were cultured in the Dubecco's modified Eagle's medium (DMEM, Gibco) containing 10 % of fetal bovine serum (Gibco) and 1% of penicillin/streptomycin at 37 ^{o}C in a humidified CO_2 (5 %) atmosphere. Cells were passaged when reached 80 % of confluence.

The cytotoxicity of the nanoparticles and reagents, including the **A**g@P(MAA-*co*-EGDMA)-HA NPs scaffolds, **A**g@*S*-nitrosothiols-HA NPs, bare Ag@*S*-nitrosothiols NPs, SiO_2@*S*-nitrosothiols-HA NPs, or PYRRO-NO were determined by a modified WST-1 cell proliferation and cytotoxicity assay kit against different cells (HepG2 cells and normal hepatocellular cells) with laser or without laser. WST-1 is a tetrazolium salt which may react with thiols to produce false positive for cell viability. Although only trace free thiols might exist in the *S*-nitrosothiol nanoparticles, the WST-1 assay was performed with the *S*-nitrosothiol nanoparticles in absence of cells in order to eliminate possible disturbances in presence of thiol groups.

Briefly, different cells were seeded in 96-well plates with a density of 5000 cells/well and incubated at 37 ^{o}C in DMEM media plus 10% FBS, 100 units/mL of penicillin and 100 μL/mL streptomycin (200 μL) for 24 h. The cells were then incubated in a culture medium (200 μL) containing the **A**g@P(MAA-*co*-EGDMA)-HA NPs scaffolds, **A**g@*S*-nitrosothiols-HA NPs, bare Ag@*S*-nitrosothiols NPs, SiO_2@*S*-nitrosothiols-HA NPs, or PYRRO-NO, respectively. The amounts of nanoparticles or reagents were

ranged from 50 to 400 μg/mL. After incubation with laser (NIR laser, 808 nm, continuous irradiation for 3 mins every 50 mins at 2.0 W/cm^2) or without laser for 24 h, the medium was replaced by fresh DMEM without FBS (100 μL) and WST-1 solution (10 μL). The plate was incubated for a further 1 h at 37 ^{o}C to allow viable cells for reducing WST-1 into an orange formazan crystal. The luminance was determined at a wavelength of 450 nm on a Bio-Rad microplate reader. (SD, n=3).

Tumor targeting performance and systemic toxicity investigation *in vivo*.

All animal experiments were carried out in strict accordance with the ethics committee guidelines of the National Institutes of Health (NIH) and Shandong Provincial Hospital Guide.

Biodistribution assay

The BALB/c mice bearing HepG2 tumor (around 6 weeks old) were randomly divided into 5 groups (5 mice per group). Each group mice were intravenously injected everyday with saline (Control 1), Ag@P(MAA-*co*-EGDMA)-HA NPs without laser (Control 2), Ag@P(MAA-*co*-EGDMA)-HA NPs with laser, Ag@*S*-nitrosothiols-HA NPs without laser, or Ag@*S*-nitrosothiols-HA NPs with laser (10 mg/kg for every two days), respectively. After 4 hours, the treated mice were sacrificed, and the major organs were collected and digested with aqua regia at boiling temperature. Then, the solution was evaporated, and the precipitate was suspended in an aqueous solution containing 0.5 % (v/v) nitric acid. The suspension was centrifuged to remove any undigested debris. The Ag content in the supernatant was analyzed and characterized by an *ICP-MS* instrument (Thermo Jarrell-Ash Crop, USA).

In vivo tumor growth, survival unit, mice weight, and toxicity investigation

After the similar treatment to the above mentioned administration, the tumor volumes of the 5 mice groups were calculated everyday by the formulae, $V = ab^2/2$, where a is the length, and b is the width. The relative tumor inhibition was calculated by the ration of V/V_0 (V_0, the tumor volume of the **Control 1**). Meanwhile, the mice weight and the survival units of each groups treated with various reagents were also recorded.

After 40 days administration, the treated mice were euthanized and the major organs including heart, kidney, liver, lung, spleen of the each mice groups were collected, fixed into 10 % formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Finally, the typical H&E staining images were observed under an Olympus fluorescence microscope (Japan, IX71).

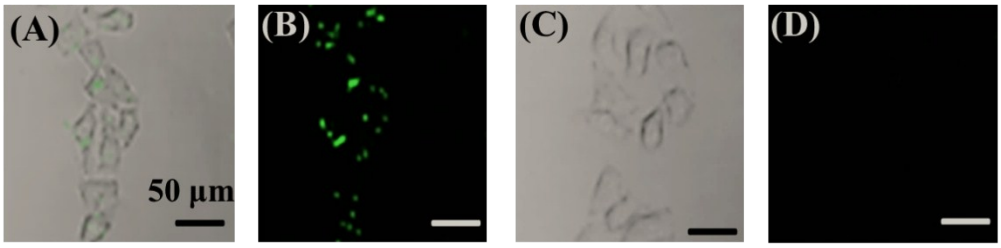


Fig. S2 Confocal fluorescence microscope of HepG2 tumor cells treated with DAF-2DA. HepG2 cells incubated with **A**g@*S*-nitrosothiols-HA NPs (50 mg/mL) for 0.5 h, washed by PBS buffer, dealt by DAF-2 DA (10 mM), and then incubated in medium for 1 h (**A, B**). HepG2 cells treated with DAF-2DA (10 mM), then incubated in medium for 1 h (**C, D**) (Excitation, 490 nm; Emission, 510 nm).

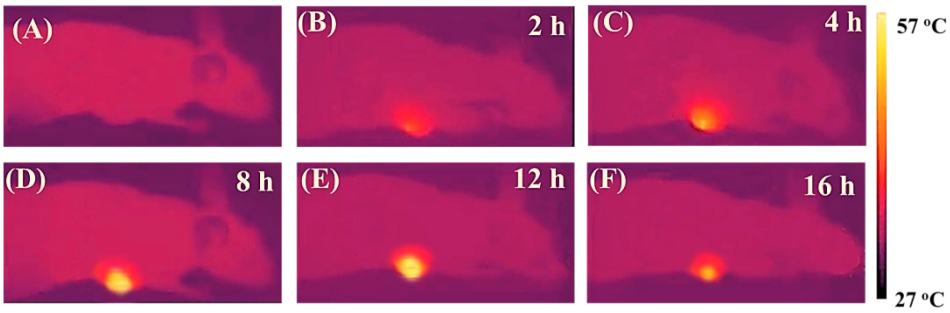


Fig. S3 Thermal images of the mice under laser irradiation (808 nm, 2.0 W/cm^2, a continuous irradiation for 3 mins at each 50 mins). Mice treated with saline (**A**, Control), or treated with Ag@*S*-nitrosothiols-HA NPs at 2 h (**B**), 4 h (**C**), 8 h (**D**), 12 h (**E**), and 16 h (**F**), respectively.