Mannose-Modified AuNSs@Ag_2S for Visualized and Synergistic Photothermal/Photodynamic Tumor Therapy

*Y*ingying Zhong, Jiajing Lin, Yuanyuan Liu, Xin Liu*

Britton Chance Center for Biomedical Photonics at Wuhan National Laboratory for Optoelectronics – Hubei Bioinformatics & Molecular Imaging Key Laboratory, Systems Biology Theme, Department of Biomedical Engineering, College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan 430074, China

Experiment Section

Materials and instruments

All chemicals and solvents from commercial sources were not further purified. Gold(III) chloride trihydrate was from Shanghai Macklin Biochemical Technology Co., Ltd., sodium sulfide, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), N-hydroxysuccinimide (NHS), 1-ethyl-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), 1,3-Diphenylisobenzofuran (DPBF) were from Energy Chemical, Bovine serum protein, 4-aminophenyl α-D-mannopyranoside (98% purity) was from Shanghai Yuanye Bio-Technology Co., Ltd, amino-PEG1k-mannose, amino-PEG2k-mannose was from Xi'an Qiyue Biology, Cy5.5-NHS was from Wuhan Duofluor Biotechnology, phosphate buffer solution (PBS, pH = 7.4 , 0.1 M) was from Biosharp life sciencs, penicillin-streptomycin mix, DMEM and RPMI 1640 cell culture medium were from Gibico, fetal bovine serum was from Zhejiang Tianhang Biotechnology Co., Ltd, dialysis bag (MWCO: 10k Da) was purchased from Beijing Solarbio Science & Technology Co., Ltd., reactive oxygen reagent kit(DCFH-DA), Cell Counting Kit-8(CCK8), live/dead cells reagent kit(calcein/propidium iodide) and Hematoxylin-Eosin (HE) Stain Kit were from Beyotime Biotechnology, Concanavalin A(ConA) enzyme immunoassay kit was from Wuhan Feiyue Biotechnology Co., Ltd, CT26 cells and hela cells were

obtained from the Wuhan Procell Life Technology Co., Ltd. Balb/c mice (female, 4 weeks, 10-14 g) were obtained from Hubei Biont Biological Technology Co., Ltd (Hubei, China).

The instruments used in the experiments included ZS90 ZetaSizer (Malvern, UK), UV-1900i UV–visible spectrophotometer (Shimadzu, Japan), EasIR-9 infrared (Wuhan Guide, China), Tecnai G2 F30 (FEI, Holland), Multiskan sky High microplate reader (Thermo, USA), MDL-III-808–2.5 W laser (Changchun New Industries, China), FV1000-IX81 confocal microscope (Olympus, Japan), TI80 inverted fluorescence microscope (Shanghai Jingxin Industrial Development Co. Ltd., China), Maldi-TOF MS 5800 Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (AB SCIEX, USA), WFX-200 atomic absorption spectrophotometer (Nikon, Japan), Countstar Mira FL cell counter (ALIT, China), BC-2800ve automated hematology(Mindray, China), FL were homemade by the Wuhan National Laboratory for Optoelectronics.[1]

Experimental procedure

Synthesis of AuNSs@Ag_2S

AuNSs were synthesized using a seedless method[2]: 30.0 mL of ultrapure water (Millipore, 18.2 MΩ, ddH2O) was added to 20.0 mL of HEPES buffer (pH = 6.0, 0.1 M), followed by the addition of 200 μL of 50 mM chloroauric acid solution. The solution was slowly stirred and then allowed to stand at room temperature for about 30 minutes, during which it turned blue-green. The solution was centrifuged at 10,000 rpm for 15 minutes and washed three times with ultrapure water. Subsequently, 10.0 mL of 50 mg mL^{-1} BSA was added, stirred overnight at room temperature, centrifuged at 10000 rpm for 15 min, washed three times with ultrapure water to remove free BSA, and redispersed in 5.0 mL of ultrapure water. 1.0 mL of 10 mM $AgNO_3$ was added and stirred at room temperature for 24 hours to fully form the protein-metal complex. The complex was centrifuged at 10,000 rpm for 10 minutes to remove free $AgNO_3$ and re-dispersed in 5.0 mL of ultrapure water. The solution was adjusted to pH 12 with NaOH, and 1.0 mL of 20.0 mM Na_2S solution was injected and stirred at 55 °C for 4 h. Finally, the product was dialyzed in ultrapure water for 24 h and then stored at 4°C after freeze-drying.

Synthesis of Man-(CH_2)n-AuNSs@Ag_2S

2.0 mg of AuNSs@Ag_2S was solubilized in 1.0 mL of PBS (pH = 7.4, 0.1 M), and EDC/NHS was added to activate the carboxyl group of BSA, then 20 mg of 4-aminophenyl α-D-mannopyranoside, amino-PEG1k-mannose, and amino-PEG2k-mannose were added, respectively. After stirring at room temperature for 24 h, the free mannose was removed by centrifugation at 10000 rpm for 15 min, washed three times with ultrapure water, and then freeze-dried to obtain Man-$(CH_2)_4$-AuNSs@Ag_2S, Man-$(CH_2)_{1k}$-AuNSs@Ag_2S, Man-$(CH_2)_{2k}$-AuNSs@Ag_2S.

2.0 mg of AuNSs@Ag_2S was taken and added to 1.0 mL of PBS buffer solution to re-dissolve, and EDC/NHS was added to activate the carboxyl group of BSA, and then mixed with 4-aminophenyl α-D-mannopyranoside, amino-PEG1k-mannose, amido-PEG2k-mannose Man (50.0 mg) and Cy5.5-NHS (0.5 mg) under stirring overnight to obtain Cy5.5-labeled Man-$(CH_2)_n$-AuNSs@Ag_2S.

Quantitative analysis of total sugar content by phenol-sulfuric acid method

Mannose standard solutions with concentrations of 0, 20.0, 40.0, 60.0, 80.0, and 100.0 mg/L, along with 0.4 mg/mL Man-(CH_2)n-AuNSs@Ag_2S test solutions, were prepared. Mixed 1.0 mL of the standard or test solution with 1.0 mL of 6% phenol solution and 5.0 mL of concentrated sulfuric acid. Reacted the mixture for 20 minutes at room temperature. The absorbance at 490 nm was recorded by UV-Vis spectrophotometer. The standard solution results were used to create a standard curve, allowing for the determination of mannose content in the material.

Determining releases with the ConA enzyme immunoassay kit

Man-(CH_2) n- AuNSs@Ag_2S (1.0 mL, 200.0 μg mL^{-1}) was conjugated with excessive ConA (2.0 mg). The aggregates were washed three times with PBS, then high concentrations of mannose were added to releases the ConA. The supernatant was obtained by centrifugation. The supernatant was desalted and dried to obtain the solid releases. Re-dissolve the solid releases in sample diluent for later use. Added 100 μL of the sample to be tested to the remaining wells and incubated at 37°C for 90 minutes. Washed the enzyme plate 3 times, added 100 μL of biotinylated antibody solution to each well, and incubated the enzyme plate at 37℃ for 60 minutes. Washed

the enzyme plate 3 times, added 100 μL of HRP enzyme conjugate solution to each well and incubated at 37°C for 30 minutes. Washed the enzyme plate 3 times, added 100μL of substrate solution (TMB) to each well and incubated at 37℃ for about 15 minutes. Added 50 μL of stop solution to each well to terminate the reaction. Immediately measured the optical density (OD) at 450nm of each well within 5 minutes by a microplate reader.

Photothermal response of Man-PEG$_{2k}$-AuNSs@Ag$_2$S

600 μL of Man-PEG$_{2k}$-AuNSs@Ag$_2$S solutions of different concentrations were added into specific containers and then subjected to 1.0 W cm^{-2} 808nm laser for the temperature test for 5 min.

The photothermal conversion efficiencies (η) were measured in accordance with the previously described method[3-5]:

$$\eta = \frac{hs(T_{Max} - T_{Surr}) - Q_{dis}}{I(1 - 10^{-A808})} \quad (1)$$

h: heat transfer coefficient, *s*: the surface area of the container, *Qdis*: heat dissipated from the laser mediated by the solvent and container, I: laser power, A: absorbance at 808 nm. The value of *hs* is determined from the following equation (2):

$$hs = \frac{mc}{\tau_s} \quad (2)$$

m: the mass of the solution containing the photoactive material, *c*: the specific heat capacity of the solution, *τs*: the associated time constant, *τs* can be determined from the following equation (3):

$$t = -\tau s ln\theta \quad (3)$$

ϑ : dimensionless parameter, known as the driving force temperature, ϑ can be calculated using the following equation (4):

$$\theta = \frac{T - T_{Surr}}{T_{Max} - T_{Surr}} \quad (4)$$

T_{Max} : the maximum steady state temperature, T_{Surr} : the environmental temperature.

Cell culture

CT26 cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum and 1% penicillin-streptomycin solution at 37°C in a 5% CO_2 environment. HeLa cells were cultured in DMEM medium containing 10% fetal bovine serum and 1% penicillin-streptomycin solution at 37°C in a 5% CO_2 environment.

Cell target

CT26 cells or hela cells were inoculated into confocal dishes and allowed to grow fully adherent to the wall. The culture medium was sequentially replaced with medium containing 100.0 μg mL^{-1} of Cy5.5-Man-$(CH_2)_n$-AuNSs@Ag_2S for 2 hours or medium containing 100.0 μg mL^{-1} of Cy5.5-Man-$(CH_2)_2$k-AuNSs@Ag_2S supplemented with 5.0 mM mannose for 2 hours.

Reactive oxygen detection experiments with Man-$(CH_2)_{2k}$-AuNSs@Ag_2S probe

A 1.0 W cm^{-2} 808 nm laser was used to irradiate 100.0 μg mL^{-1} Man-$(CH_2)_{2k}$-AuNSs@Ag_2S containing 8 mM of DPBF, and the change of the absorption peak of DPBF at 420 nm was recorded by a UV-vis spectrometer.

CT26 cells were inoculated into confocal dishes and allowed to grow fully adherent to the wall. The culture medium was sequentially replaced with medium containing 100.0 μg mL^{-1} of Man-$(CH_2)_{2k}$-Ag_2S for 2 hours or medium containing 100.0 μg mL^{-1} of Man-$(CH_2)_{2k}$-Ag_2S supplemented with N-acetylcysteine for 2 hours. The culture medium was replaced with culture medium containing DCFH-DA and incubated for 30 min after laser treatment. Finally, observed and photographed under a confocal microscope.

Cytotoxicity experiments of Man-$(CH_2)_{2k}$-AuNSs@Ag_2S

The cytotoxicity of Man-$(CH_2)_{2k}$-AuNSs@Ag_2S was investigated by cck8. CT26 cells were homogeneously inoculated in 96-well plates, and incubated with Man-$(CH_2)_{2k}$-AuNSs@Ag_2S probes at different concentrations (0, 50, 100, 150, 200 μg mL^{-1}) for 2 h. The culture medium was removed and washed gently with PBS for three times to remove free probes, and 180 μL of culture medium and 20 μL of cck8 solution were added to each well and continued the incubation for 4 h. The absorption at 450 nm was measured in each well.

In vitro therapeutic experiments of Man-(CH_2)n-AuNSs@Ag_2S

The light-mediated therapy capabilities of Man-$(CH_2)_{2k}$-AuNSs@Ag_2S probes with different concentrations were investigated by CCK8. 5×10^3 CT 26 cells were homogeneously inoculated in 96-well plates, and cells were incubated with Man-$(CH_2)_{2k}$-AuNSs@Ag_2S probes at different concentrations (0, 50.0, 100.0, 150.0, 200.0 μg mL^{-1}) for 2 h. The culture medium was replaced with fresh culture medium to remove free probes, and each well was irradiated with 1.0 W cm^{-2} 808nm laser for 5 min. 20 μL cck8 solution were added to each well, and the absorption at 450 nm was measured in each well after 4 h of incubation.

The light-mediated therapy capabilities of Man-$(CH_2)_{2k}$-AuNSs@Ag_2S probes with different conditions were investigated by CCK8. 96-well plates were inoculated with 5×10^3 CT26 cells per well, a total of 4 groups (dark, PDT, PTT, PDT+PTT). After the cells were fully adhered to the wall, the culture medium was replaced with culture medium containing 200.0 μg mL^{-1} Man-$(CH_2)_{2k}$-AuNSs@Ag_2S, and incubated for 2h, respectively. The PTT group was co-incubated with N-acetylcysteine to eliminate the effects of reactive oxygen species. The culture medium was replaced with fresh culture medium to remove free probes, and each well was irradiated with 1.0 W cm^{-2} 808nm laser for 5 min. The PDT group was irradiated on ice to minimize thermal effects. 20 μL cck8 solution were added to each well, and the absorption at 450 nm was measured in each well after 4 h of incubation.

In order to visualize the apoptosis of CT26 cells more intuitively, the cells were uniformly inoculated in confocal culture dishes. After the cells were completely attached to the wall, culture medium containing 200.0 μg mL^{-1} of Man-$(CH_2)_{2k}$-AuNSs@Ag_2S were added, and incubated for 2 h. After incubation, the culture medium was aspirated out and washed with PBS to remove the free probes. The PTT group was co-incubated with N-acetylcysteine to eliminate the effects of reactive oxygen species. The cells were irradiated with a laser at a wavelength of 808 nm and a power of 1.0 W cm^{-2} for 5 min, followed by staining with calcein/propidium iodide for 30 min and recording by confocal microscopy. The PDT group was irradiated on ice to minimize thermal effects.

In vivo targeted fluorescence and light-mediated therapy of Man-$(CH_2)_{2k}$-AuNSs@Ag_2S

Four-week Balb/c nude mice were subcutaneously inoculated with 1×10^6 CT26 cells. When the tumor volume reached approximately 200 mm^3, the mice were imaged using a near-infrared fluorescence imaging system after tail vein injection of Man-$(CH_2)_{2k}$-AuNSs@Ag_2S or AuNSs@Ag_2S. At the same time, the heart, liver, spleen, lungs, kidneys, and tumors of the tumor-bearing mice were collected and imaged using the near-infrared fluorescence imaging system.

The tumor-bearing mice were divided into five groups, group I was left untreated, group II was injected with PBS, group III and group V were injected with Man-$(CH_2)_{2k}$-AuNSs@Ag_2S, group IV was injected with AuNSs@Ag_2S. Six hours after injection, mice in groups I, II, III, and V were irradiated with a 1.0 W cm^{-2} 808 nm laser for 5 minutes. After the treatment, the body weight and tumor volume of the mice were measured every two days until day 14 of the treatment period. Then, one mouse from each group was dissected, the tumor was excised, and pathological section staining was performed. The tissues were fixed with 4% paraformaldehyde at room temperature for 24 hours, then dehydrated and embedded in paraffin. The tissue sections were then prepared and stained with H&E.

Hemolysis Test

Mouse blood containing EDTA.K2 was thoroughly rinsed with 1% saline and centrifuged repeatedly until the supernatant was clear. The resulting blood cells were then resuspended in 0.5 mL PBS. Ultrapure water (0.5 mL) and PBS (0.5 mL) served as the positive and negative control groups, respectively. Blood cell suspensions were mixed with varying concentrations of Man-$(CH_2)_{2k}$-AuNSs@Ag_2S solutions (0.5 mL). These mixtures, along with the controls, were incubated for 4 hours and subsequently centrifuged. The calculation formula is as follows[6]：

$$\text{hemolysis } \% = \frac{A_{sample} - A_{control(-)}}{A_{control(+)} - A_{control(-)}}$$

Statistical Analysis

Values are expressed as mean ± standard deviation (SD), and the data was

repeated at least 3 times. Unpaired t-test was used to check the difference between the two groups. It was considered statistically significant when the p value was < 0.05. All statistical analyses were performed using GraphPad Prism 9.5 (GraphPad software).

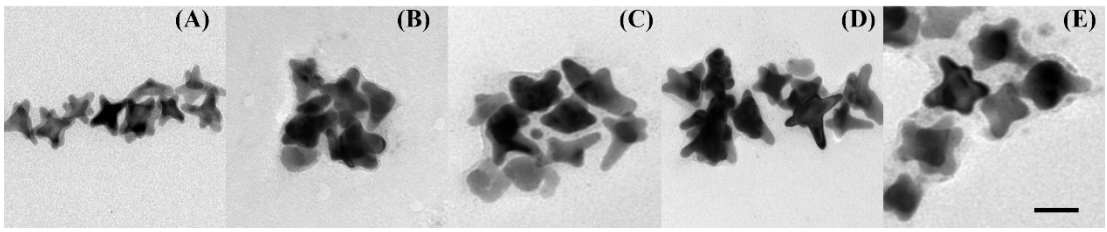

Figure S1. Transmission electron micrographs of AuNSs(A), AuNSs@Ag_2S(B), Man-$(CH_2)_4$-AuNSs@Ag_2S(C), Man-$(CH_2)_{1k}$-AuNSs@Ag_2S(D), Man-$(CH_2)_{2k}$-AuNSs@Ag_2S(E), (Scale bar=50 nm).

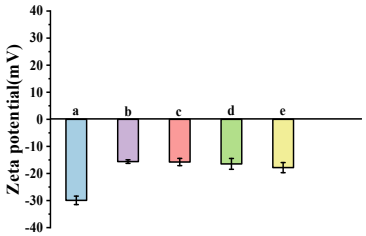

Figure S2. The zeta potential of AuNSs(a), AuNSs@Ag_2S(b), Man-$(CH_2)_4$-AuNSs@Ag_2S(c), Man-$(CH_2)_{1k}$-AuNSs@Ag_2S(d), Man-$(CH_2)_{2k}$-AuNSs@Ag_2S(e)

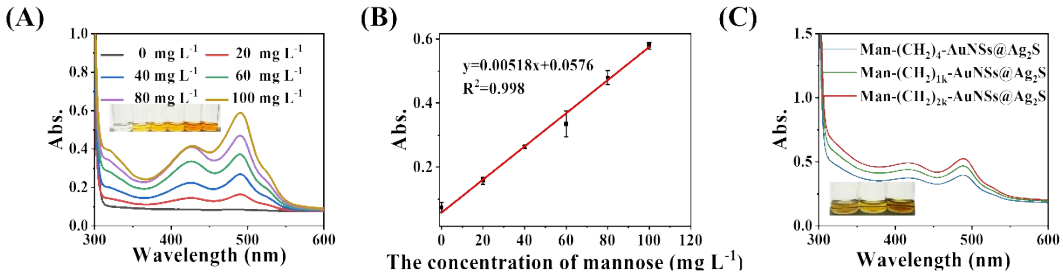

Figure S3. (A) UV-Vis absorption spectra of different concentrations of mannose after treatment with phenol- sulfuric acid solution; (B) Linear regression equation between the concentration of mannose (0-100 mg/L) and its absorbance at 490 nm. The error bars represent the standard deviation of three parallel tests; (C) UV-Vis absorption spectra of Man-$(CH_2)_4$-AuNSs@Ag_2S, Man-$(CH_2)_{1k}$-AuNSs@Ag_2S, and Man-$(CH_2)_{2k}$-AuNSs@Ag_2S after sulfuric acid-phenol treatment (all materials were diluted by a factor of 5 prior to treatment)

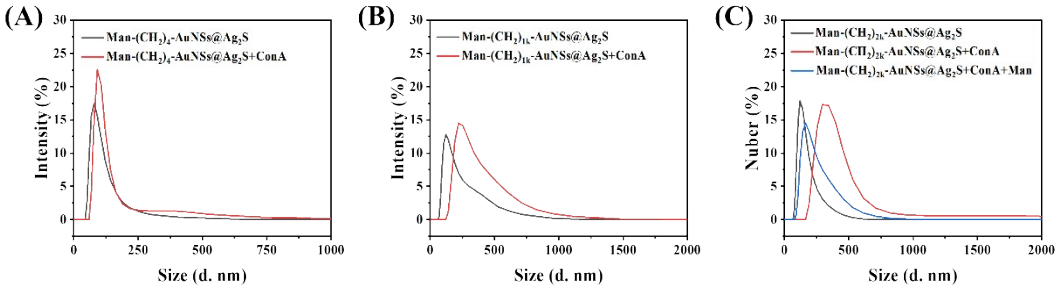

Figure S4. The hydrodynamic size of (A)Man-$(CH_2)_4$-AuNSs@Ag_2S and Man-$(CH_2)_4$-AuNSs@Ag_2S+ConA, (B) Man-$(CH_2)_{1k}$-AuNSs@Ag_2S and Man-$(CH_2)_{1k}$-AuNSs@Ag_2S+ConA, (C) Man-$(CH_2)_{2k}$-AuNSs@Ag_2S, Man-$(CH_2)_{2k}$-AuNSs@Ag_2S+ConA and Man-$(CH_2)_{2k}$-AuNSs@Ag_2S+ConA+Man.

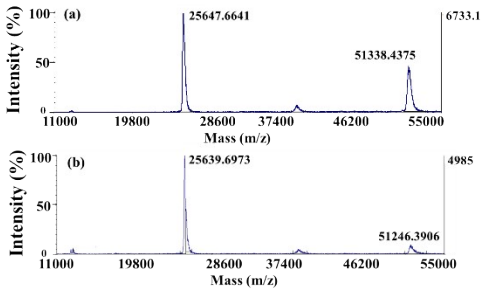

Figure S5. Mass spectra of ConA standard(a) and ConA released from Man-(CH_2)n-AuNSs@Ag_2S aggregates with high concentrations of mannose(b)

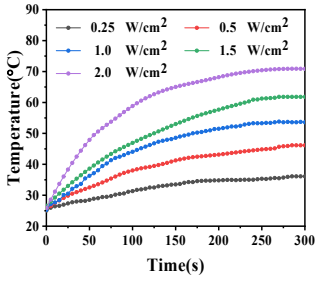

Figure S6 . Temperature-time curves of Man-$(CH_2)_{2k}$ -AuNSs@Ag_2S with different laser power densities (0.25, 0.5, 1.0, 1.5, 2.0 W/cm^2)

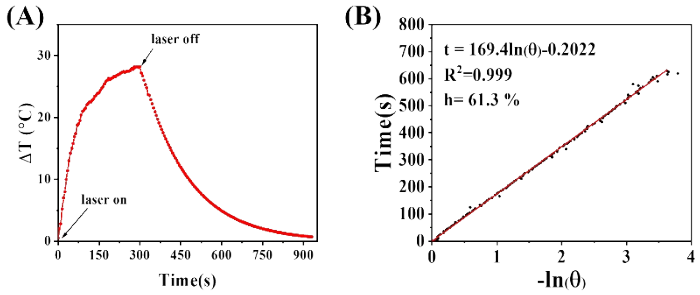

Figure S7. heating-cooling curve of Man-$(CH_2)_{2k}$-AuNSs@Ag_2S

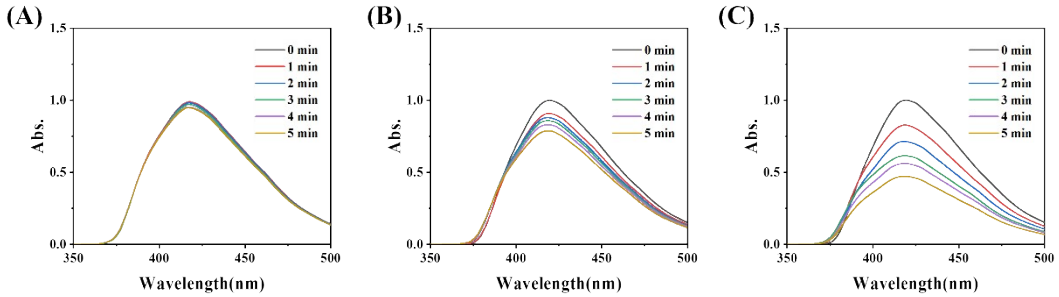

Figure S8. UV-vis spectrum of DPBF mixed with PBS(A), Man-$(CH_2)_{2k}$-Ag_2S(B) and Man-$(CH_2)_{2k}$-AuNSs@Ag_2S(C).

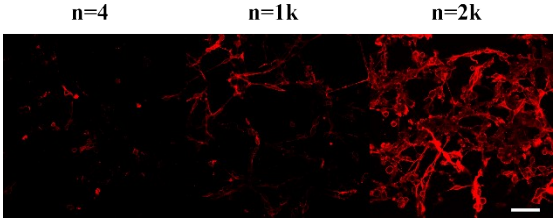

Figure S9. CLSM images of Man-(CH_2) n-AuNSs@Ag_2S targeting CT26 cells (scale bar = 100 μm)

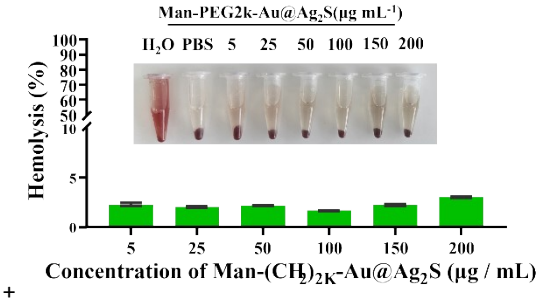

Figure S10. Hemolytic percentage of red blood cells incubated with PBS solution of Man-$(CH_2)_{2k}$-AuNSs@Ag_2S at different concentrations

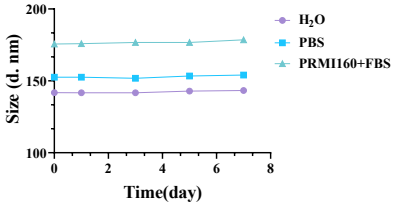

Figure S11. hydrodynamic sizes of Man-$(CH_2)_{2k}$-AuNSs@Ag_2S dispersed in various media (water, PBS, RPMI 1640 and RPMI 1640+FBS) over a 7-day incubation period.

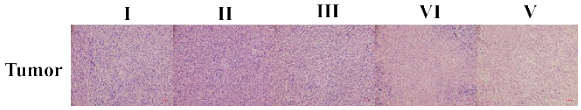

Figure S12. H&E staining of the whole tumors of the mice with different treatments.

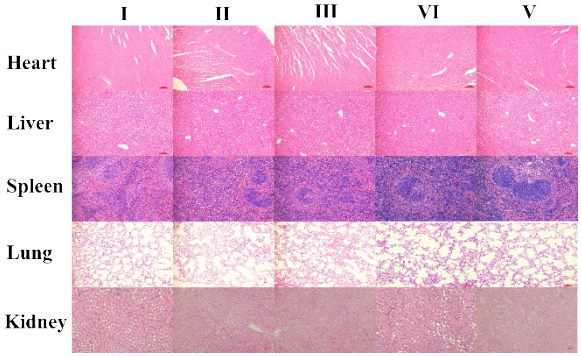

Figure S13. H&E staining of organ sections from mice with different treatments.

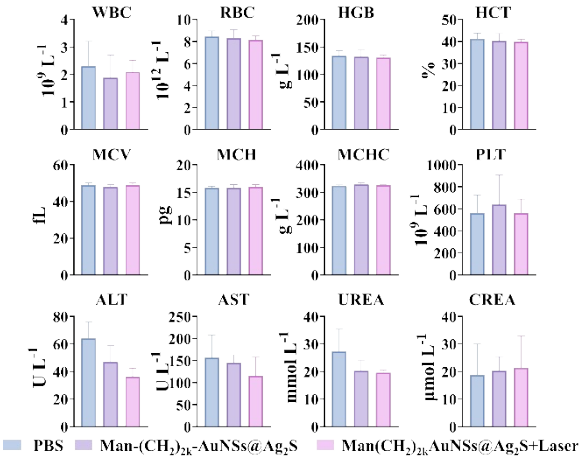

Figure S14. Routine blood tests were performed after 14 days in the different treatment groups (n = 5).

Table S1. Results for determining releases with the ConA enzyme immunoassay kit (n=3)

Sample		Mass of releases (μg)	RSD (%)
Man-$(CH_2)_{2k}$-AuNSs@Ag_2S	1	616.75	4.1
	2	614.83	3.4
	3	612.49	1.6
Man-$(CH_2)_{1k}$-AuNSs@Ag_2S	1	548.70	3.8
	2	538.57	4.8
	3	544.42	4.8
Man-$(CH_2)_4$-AuNSs@Ag_2S	1	506.95	4.1
	2	518.63	1.3
	3	504.98	2.5

Reference

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