Targeted photothermal ablation of murine melanomas with melanocyte-stimulating hormone analog-conjugated hollow gold nanospheres

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Supplementary Information

Synthesis of Hollow Gold Nanospheres (HAuNS). HAuNS were synthesized by the cobalt nanoparticle-mediated reduction of chloroauric acid according to Schwartzberg et al (15). Cobalt template nanoparticles were synthesized by adding 2500 μL of sodium borohydride (1 M) into 1000 mL of deoxygenated aqueous solution of sodium citrate (2.8 mM) and cobalt chloride (0.4 mM). The slightly pink solution turned brown upon the addition of sodium borohydride, indicating the formation of cobalt nanoparticles. The solution was allowed to stand at room temperature for 45 min under constant argon flow until complete hydrolysis of the sodium borohydride. In a separate vial, 600 mL of aqueous solution of chloroauric acid (0.04 mM) was deoxygenated by bubbling with argon gas. Subsequently, 800 mL of cobalt nanoparticle solution was transferred to the chloroauric acid solution under argon protection and magnetic stirring. The cobalt immediately reduced the gold ions onto the surface of cobalt nanoparticles while at the same time it was oxidized to cobalt oxide. Any remaining cobalt core was further oxidized by air, resulting in the final formation of HAuNS.

Synthesis of NDP-MSH Peptide. The reaction scheme for the synthesis of the partially protected NDP-MSH peptide, H-Ser-Tyr-Ser-Nle-Glu-His-*d*-Phe-Arg-Trp-Gly-Lys(Dde)-Pro-Val-NH_2, is shown in **Figure S1**. The peptide was synthesized manually using N^{α}-Fmoc chemistry. Briefly, Rink amide resin (4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy resin, substitution 0.6 mmol/g) was swollen in dichloromethane:DMF (1:1) overnight. The resin was washed with DMF (3 × 10 mL), and the N^{α}-Fmoc protecting group was removed by 25% piperidine in DMF (1 × 5 min and 1 × 20 min). The resin was washed with DMF (3 × 5 mL) and then with

dichloromethane (3 × 5 mL), and the first N^{α}-Fmoc amino acid was coupled using preactivated ester (3 equiv of N^{α}-Fmoc amino acid, 3 equiv of HOBt, and 3 equiv of HBTU) in DMF solution containing 6 equiv of DIEA and stirred at room temperature for 60 min under an inert atmosphere. At the end of the coupling, the resin was washed with DMF (3 × 10 mL) and dichloromethane (3 ×10 mL), and a Kaiser ninhydrin test was done to check the extent of coupling. If the test was positive, the coupling was repeated for another hour. If double coupling did not result in a negative Kaiser test, the resin was washed with DMF and dichloromethane, and the unreacted amino groups were capped using acetic anhydride in DMF for 30 min. When coupling was complete, the resin was once again washed with DMF followed by dichloromethane, and the same procedure was repeated to couple the remaining amino acids.

The cleavage cocktail consisting of tifluoroacetic acid (TFA, 8.5 mL), triethylsilane (0.7 mL), and water (0.8 mL) was chilled on ice. The cold solution was then added to the resin-bound protected peptide (1 g, 0.4 mmol) in a clean glass vial with a Teflon-coated screw cap. The glass vial was capped, and the reaction mixture was gently agitated for 2.5 h at room temperature using a mechanical shaker. The reddish solution was then filtered through a Pasteur pipette plugged with cotton wool. The resin was washed with TFA (4-5 mL), and the solution was filtered off. The volume of the combined cleavage cocktail was reduced to 4 mL using a stream of argon gas, and the crude peptide was precipitated out by the addition of diethyl ether (40 mL) to give a white solid. The organic layer was decanted off after centrifugation of the peptide for 5 min at 12,000 rpm. The precipitate was washed with diethyl ether (3 × 40 mL) and dried in vacuum overnight. The crude peptides were purified using a Hewlett-Packard 1100-

series high-performance liquid chromatography instrument with a reverse-phase column (Vydac, 21.2 × 250 mm, 7 μm, 300 Å). The peptides were eluted with a linear acetonitrile/0.1% TFA in water gradient at a flow rate of 10.0 mL/min. Separations were monitored with a Hewlett-Packard 1100-series UV/Vis detector at 230 nm and 280 nm. The peptide was validate by liquid chromatography-mass spectrometry on an Agilent 1100 Series LC/MSD-TOF instrument (Santa Clara, CA) equipped with a Vydac C18 column (4.6 x 250 mm, 7 μm, 300 Å). The peptides were eluted with water containing 0.1% TFA and acetonitrile containing 0.1% TFA varying from 0% to 90% over 30 min at a flow rate of 1 mL/min. The partially protected NDP-α-MSH, H-Ser-Tyr-Ser-Nle-Glu-His-d-Phe-Arg-Trp-Gly-Lys(Dde)-Pro-Val-NH_2 {[Lys^{11}(Dde)]NDP-MSH} showed a single peak with retention time 15.0 min, *m/z* = 1769.9678 for $[M+H]^+$ ($C_{86}H_{121}N_{21}O_{20}$, calculated 1769.0096).

Synthesis of NDP-MSH-PEG-SATA and NDP-MSH-PEG-SH. The heterodifunctional PEG precursor containing protected SATA on one terminus and the activated ester *N*-hydroxysuccinimide (NHS) on the other terminus (SATA-PEG-NHS) was synthesized from HOOC-PEG-NH_2 HCl. HOOC-PEG-NH_2 HCl (0.5 g) was first reacted with *N*-hydroxysuccinimide S-acetylthioacetate (40 mg) in DCM (2.6 ml) in the presence of TEA. (50 μL). Dichloromethane was removed under vacuum after 1 h of reaction. The product was purified by dialysis against deionized water (molecular weight cut-off, 1000) overnight and freeze-dried to yield HOOC-PEG-SATA. To prepare activated ester, TEA (0.069 mmol, 9.6 μL) was added to 1 mL of DMF solution containing HOOC-PEG-SATA (0.023 mmol, 120 mg) and *N′,N′*-disuccinimidyl carbonate (0.035 mmol, 8.8 mg). The mixture was stirred at room temperature overnight. After removal of solvent under

reduced pressure, the crude reaction mixture was diluted with DCM and washed with 0.1 N aq HCl and then with brine. The organic layer was dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The crude residue was purified by chromatography on silica gel (Hexanes:EtOAc, 2:8) to give succinimidyl ester, NHS-PEG-SATA (82 mg, 65%) as an oil.

For peptide conjugation, NHS-PEG-SATA (0.015 mmol, 82 mg) was dissolved in 1 mL of DMF together with 12 mg of [Lys^{11}(Dde)]NDP-MSH (0.0068 mmol) and 50 µL of diisopropylethylamine (DIPEA) (**Fig. S1**). The reaction mixture was stirred at room temperature overnight. After removal of solvent, the residue was dissolved with 0.5 mL of water and purified through a Sephadex G-50 (GE Healthcare) gel permeation chromatography column using water as the eluting solvent. The eluted PEG fractions (1.5 mL each) containing peptide (UV absorption at 280 nm) were collected, and the product, [Lys^{11}(Dde)]NDP-MSH-PEG-SATA, was validated by LC-MS.

To remove Dde protection group on the side chain of Lys and to release the active sulfhydryl group, 1 mg of [Lys^{11}(Dde)]NDP-MSH-PEG-SATA was treated with 1 mL of DMF containing 2% hydrazine at room temperature for 10 min. The organic solvent was removed under vacuum, 450 µL of PBS containing 10 mM of EDTA (pH 7.0) was added, and then 50 µL of hydroxylamine (0.5 M) was added. The reaction mixture was stirred at room temperature for 2 h under argon protection. The final product, NDP-MSH-PEG-SH, was purified by passage through a PD-10 column eluted with PBS containing 10 mM EDTA (pH 7.0).

Synthesis of FITC-Lipoic Acid. *N*-(2-(2-(2-aminoethoxy)ethoxy)ethyl)-lipoic acid (116 mg, 0.34 mmol) in 2 mL of anhydrous DMF was allowed to react with fluorescein isothiocyanate (FITC, 197 mg, 0.51 mmol) in the presence of DIPEA (0.57 mL) at room temperature under argon protection for 12 h. After removal of the solvent under reduced pressure, the crude product was purified using silica gel chromatography (methanol in chloroform, from 5% to 10%) to yield *N*-(2-(2-(2-(3-fluoresceinthioureido)ethoxy)ethoxy)ethyl)-lipoic acid (FITC-lipoic acid) (125 mg, 51.0%). Calcd. mass for $C_{35}H_{40}N_3O_8S_3$ $[M+H]^+$: 726.1978; Found: 726.2188.

Photothermal Effect in Aqueous Solution

The laser was a continuous-wave GCSLX-05-1600m-1 fiber-coupled diode laser (DHC, China Daheng Group, Beijing, China) with a center wavelength of 808 ± 10 nm. It was powered by a DH 1715A-5 dual-regulated power supply (DHC, China Daheng Group). A 5-m, 600-μm core BioTex LCM-001 optical fiber (BioTex Inc., Houston, TX) was used to transfer laser power from the laser unit to the target. This fiber had a lens mounting at the output that allowed the laser spot size to be changed by changing the distance from the output to the target. The output power was independently calibrated using a handheld model 840-C optical power meter (Newport Corporation, Irvine, CA) and was found to be 1 W for a spot diameter of 3.5 mm (~8 W/cm^2) and a 2-amp supply current. The end of the optical fiber was attached to a retort stand using a movable clamp and positioned directly above the sample cell. For measuring temperature change mediated by HAuNS, NIR laser light (808 nm) was delivered through a quartz cuvette containing the HAuNS (100 μL). A thermocouple was inserted into the solution perpendicular to the path of the laser light. The temperature was measured over 15 min. Water was used as a control. **Fig.**

S3 shows temperature change as a function of exposure time. Exposure of an aqueous solution of PEG-HAuNS (7.3×10^{10} HAuNS/mL) to NIR laser light (808 nm, 8 W/cm^2) for 4 min elevated the temperature of the solution from 25ºC to 41.5ºC (an increase of 16.5ºC). Without HAuNS, little temperature change was observed. The same level of temperature change was observed with an aqueous solution of superparamagnetic iron oxide-silica-core AuNS under the same experimental conditions but at 100 times higher nanoshell concentration (7.5×10^{12}).[2] These data indicated that HAuNS acted as an efficient photothermal coupling agent.

Synthesis of *S*-2-(4-[5-{1,2}Dithiolane-3-pentanamide]benzyl)diethylenetriamine pentaacetic acid (DTPA-TA).

Thioctic acid (440 mg) and NH_2-Bz-DTPA(*t*-butyl ester) (710 mg, 0.91 mmol) were dissolved in 8 mL of anhydrous dichloromethane. 1, 3-Diisopropylcarbodiimide (159 mg, 1.26 mmol), 0.43 mL of pyridine, and a catalytic amount of 4-dimethylaminopyridine (DMAP) were added to the reaction solution. The reaction mixture was stirred at room temperature overnight. The reaction solution was washed two times by using deionized water, and the organic phase was dried with sodium sulfate and concentrated. The crude product was purified via silica chromatography (eluent: Ethyl acetate/Hexane 3:1) to provide the *tert*-butyl-protected titled compound as yellow powder (0.95 g). To remove the protective groups of carboxylic acid, the resulted was dissolved in 2 mL of dichloromethane, and then 2 mL of TFA was added slowly. After stirring under Argon at room temperature, the solvent and TFA were evaporated in reduced vacuum, and then dissolved in methanol and precipitated to cold ethyl ether to yield the titled compound as

yellow solid (410 mg). ESI-MS: Calcd. For $(M+H)^+$ $C_{29}H_{43}N_4O_{11}S_2$: 687.79; Found: 687.22.

Instant Thin Layer Chromatography (ITLC)

The instant ITLC strips were developed with PBS (pH 7.4) containing 4 mM EDTA and quantified using a Bioscan IAR-2000 TLC Imaging Scanner (Washington, D.C.). For the study of labeling stability, NDP-MSH-PEG-HAuNS(DTPA-^{111}In) or PEG-HAuNS(DTPA-^{111}In) were suspended in the mouse serum and incubated at 37ºC for 24 h. Free $^{111}In^{3+}$ moved to the solvent front (Rf = 0.9), and the nanospheres remained at the original spot (Rf = 0.0). One hundred percent of radioactivity was associated with NDP-MSH-PEG-HAuNS and PEG-HAuNS after labeling procedures (Fig. S7A and C). After incubation in mouse serum for 24 h, NDP-MSH-PEG-HAuNS and PEG-HAuNS lost 2.3% and 2.8% of associated radioactivity (presumably free $^{111}In^{3+}$) respectively, indicating that the radiolabels on both nanoparticles were very stable (Fig. S7B and D).

Figures

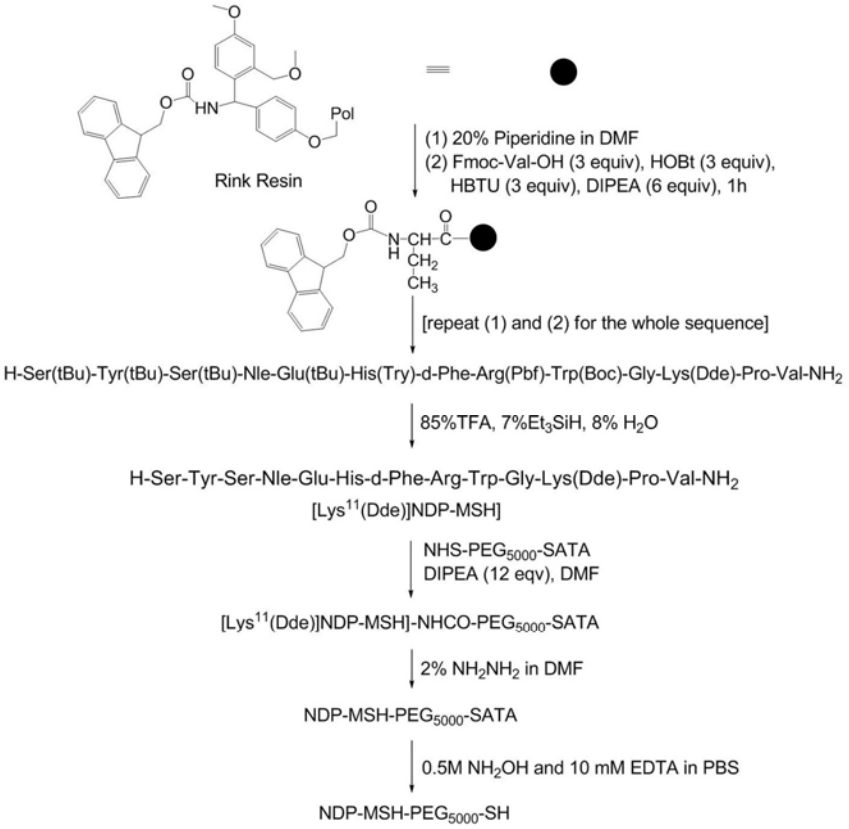

Fig. S1

Fig. S1. Reaction scheme for the synthesis of partially protected NDP-MSH peptide, [Lys^{11}(Dde)]NDP-MSH], and NDP-MSH-PEG-SH.

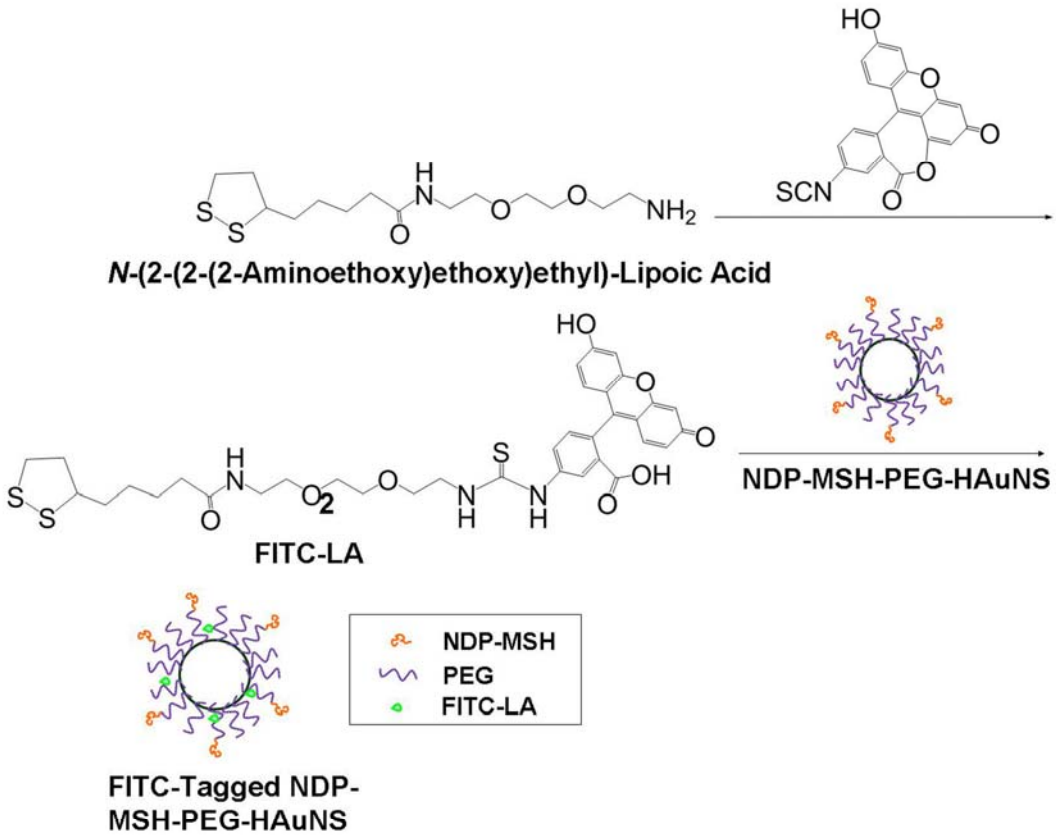

Fig. S2

Fig. S2. Reaction scheme for the synthesis of FITC-tagged HAuNS. LA, lipoic acid.

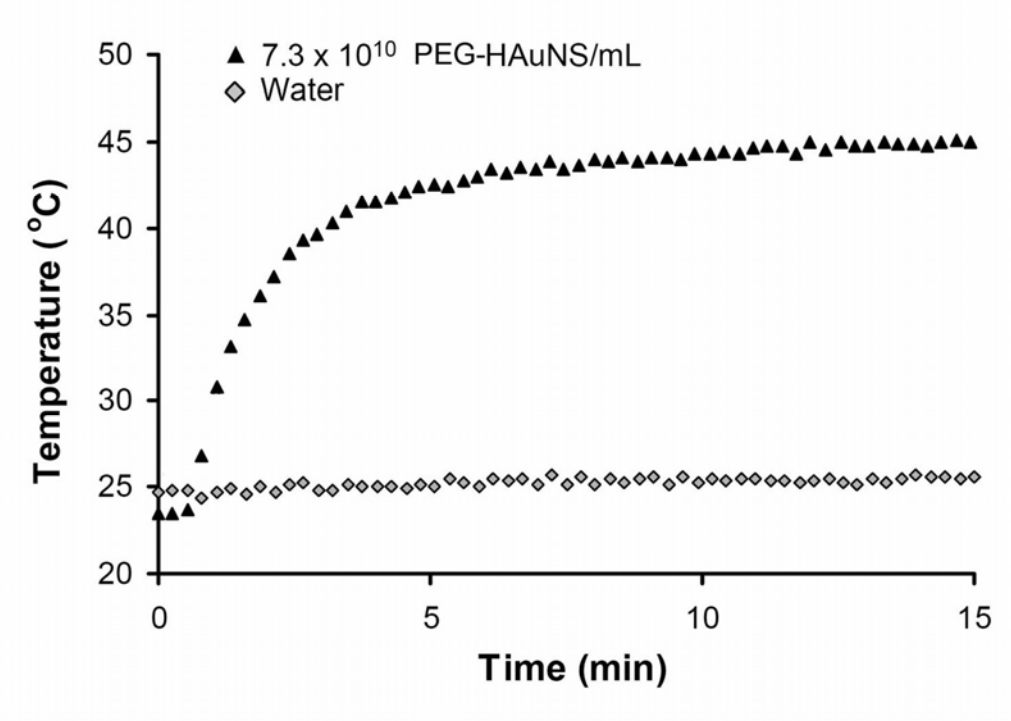

Fig. S3

Fig. S3. Temperature-time profile of aqueous PEG-HAuNS solution exposed to NIR light (808 nm) at 8 W/cm^2.

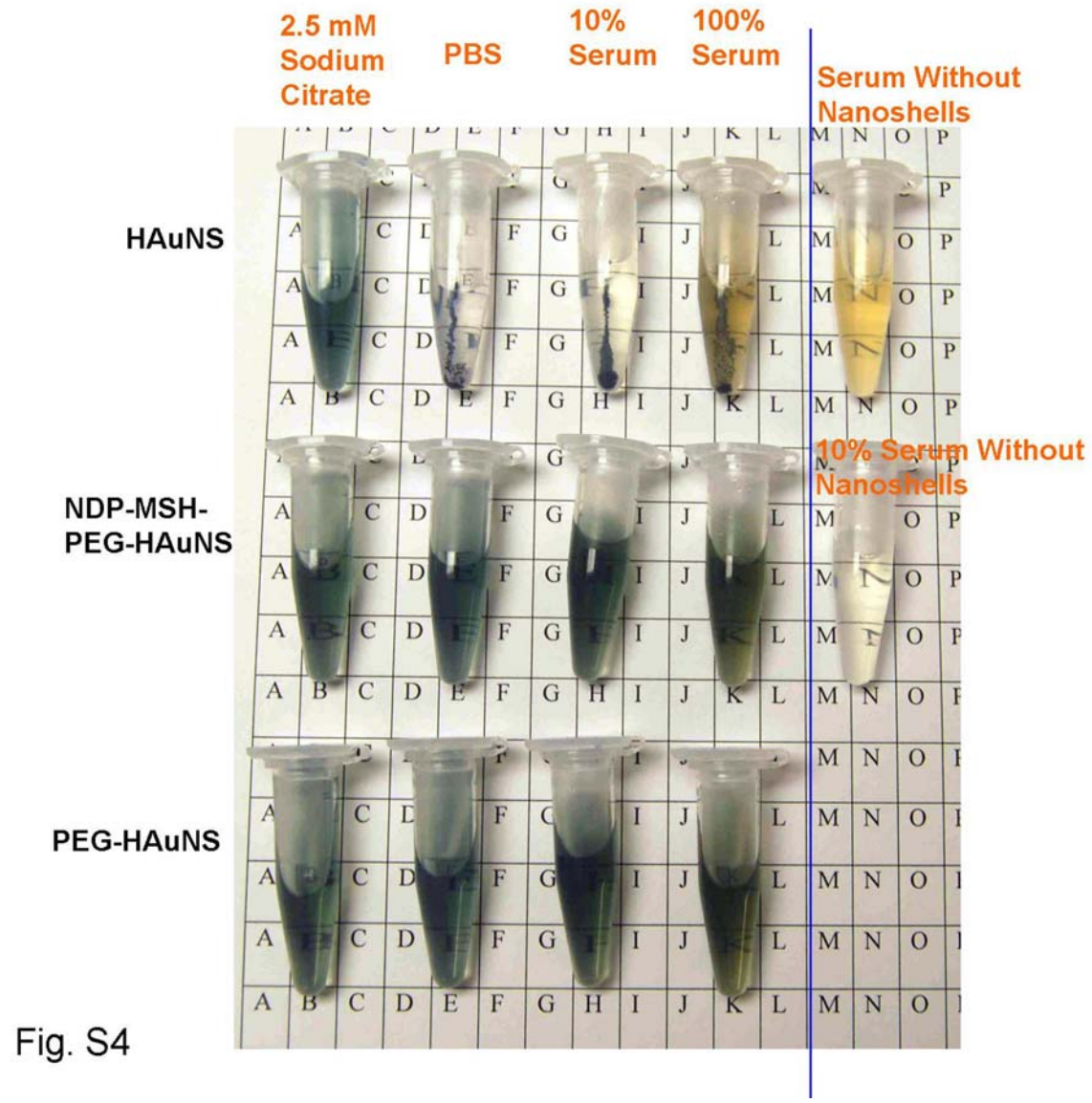

Fig. S4

Fig. S4. Colloidal stability of uncoated HAuNS, NDP-MSH-PEG-HAuNS, and PEG-HAuNS. The nanoparticles were incubated in different solutions at 37ºC for 24 h. Uncoated HAuNS aggregated in PBS (pH 7.4), PBS containing 10% goat serum, and 100% goat serum. Both NDP-MSH-PEG-HAuNS and PEG-HAuNS were stable under the same conditions.

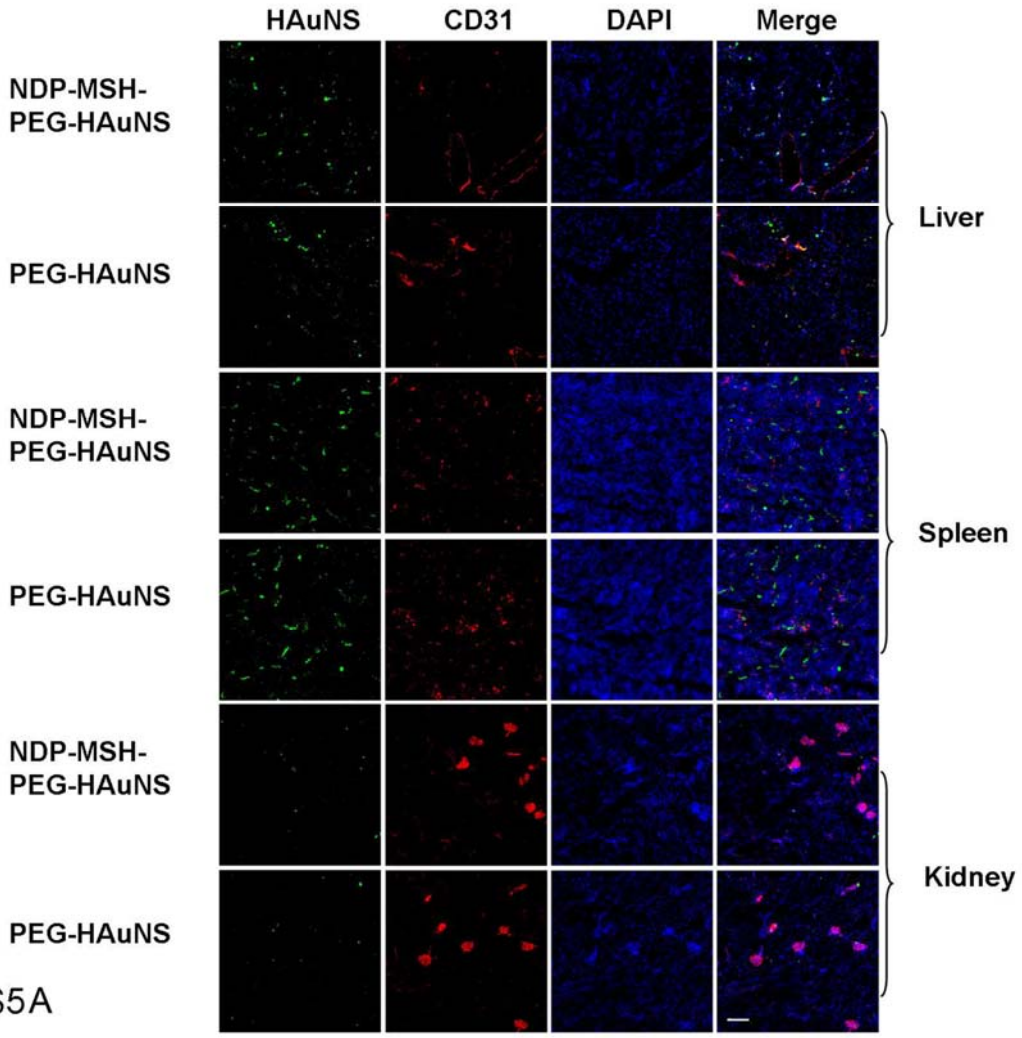
HAuNS
CD31
DAPI
Merge
NDP-MSH-PEG-HAuNS
PEG-HAuNS
NDP-MSH-PEG-HAuNS
PEG-HAuNS
NDP-MSH-PEG-HAuNS
PEG-HAuNS
Liver
Spleen
Kidney

Fig. S5A

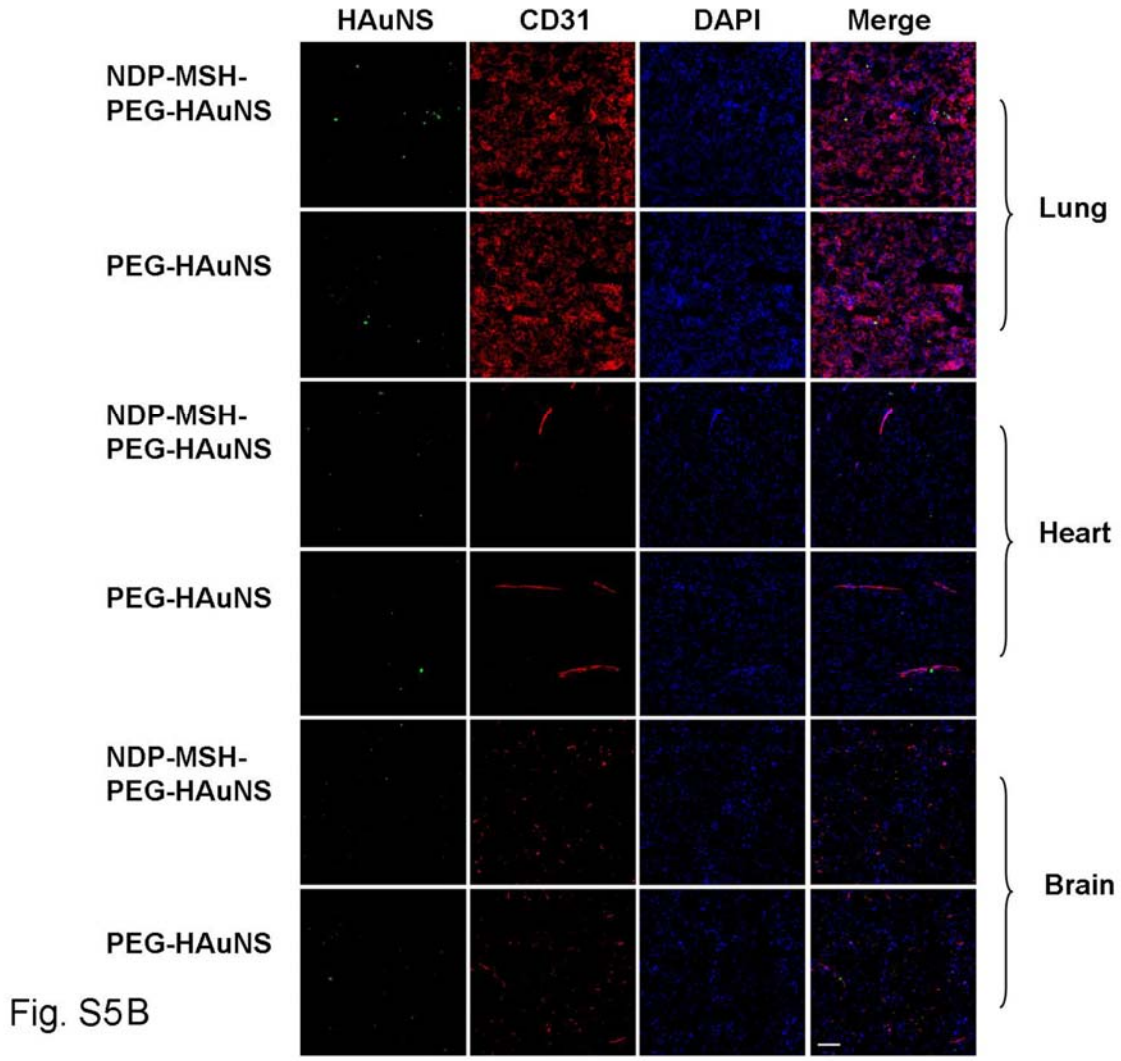

Fig. S5. Representative fluorescence micrographic images of major organs taken from B16/F10 melanoma-bearing mice injected with either FITC-tagged NDP-MSH-PEG-HAuNS or FITC-tagged PEG-HAuNS (2.5×10^{12} particles/mouse). Tissues were removed 4 h after injection and cryosectioned into 5-μm slices as described in Methods. Blood vessels (red) were stained with rat anti-mouse CD31 monoclonal antibody. Cell nuclei were counterstained with DAPI (blue). Bar = 100 μm.

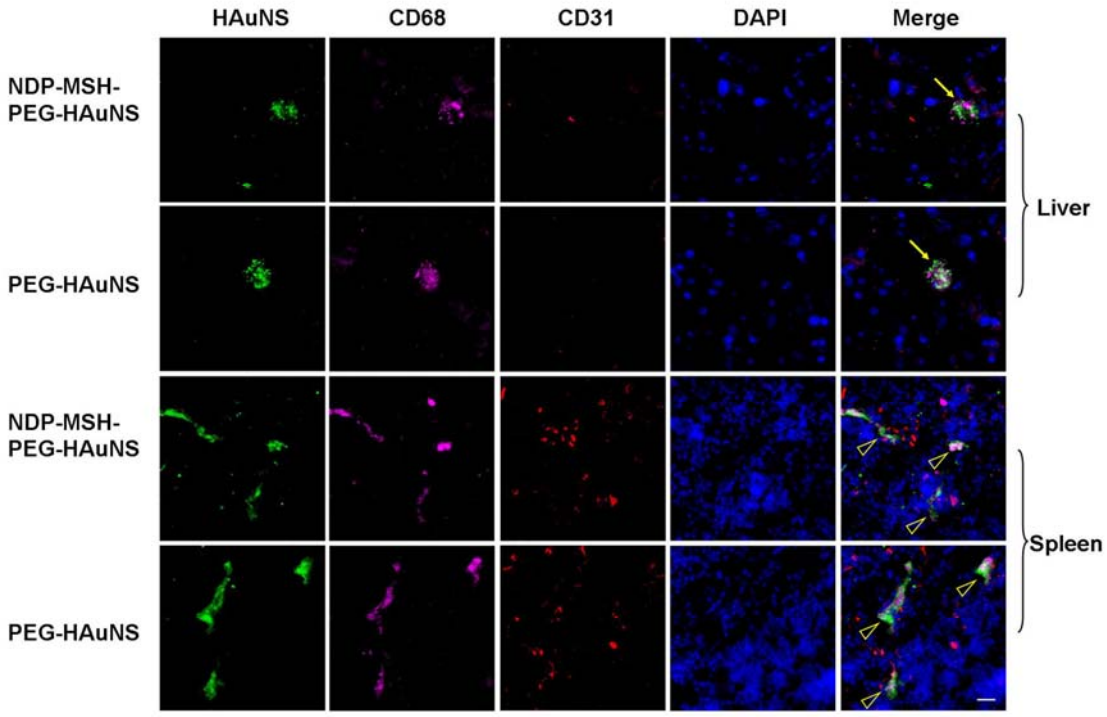

Fig. S6

Fig. S6. Colocalization of HAuNS with macrophages in liver and spleen. Tissues taken from nude mice injected with either FITC-tagged NDP-MSH-PEG-HAuNS or FITC-tagged PEG-HAuNSHGNS were removed 4 h after injection and cryosectioned into 5-μm slices as described in Methods. Kupffer cells in liver and macrophages in spleen (pseudocolored magenta) were stained with rabbit anti-CD68 polyclonal antibodies. Blood vessels (red) were stained with rat anti-mouse CD31 monoclonal antibody. Cell nuclei were counterstained with DAPI (blue). Both FITC-tagged NDP-MSH-PEG-HAuNSHAuNS and FITC-tagged PEG-HAuNS (green) colocalized with CD68-positive cells in liver (arrows, white color) and spleen (open arrowheads, white color), indicating that these particles were captured by the cells of the monophagocytic system. Bar = 20 μm.

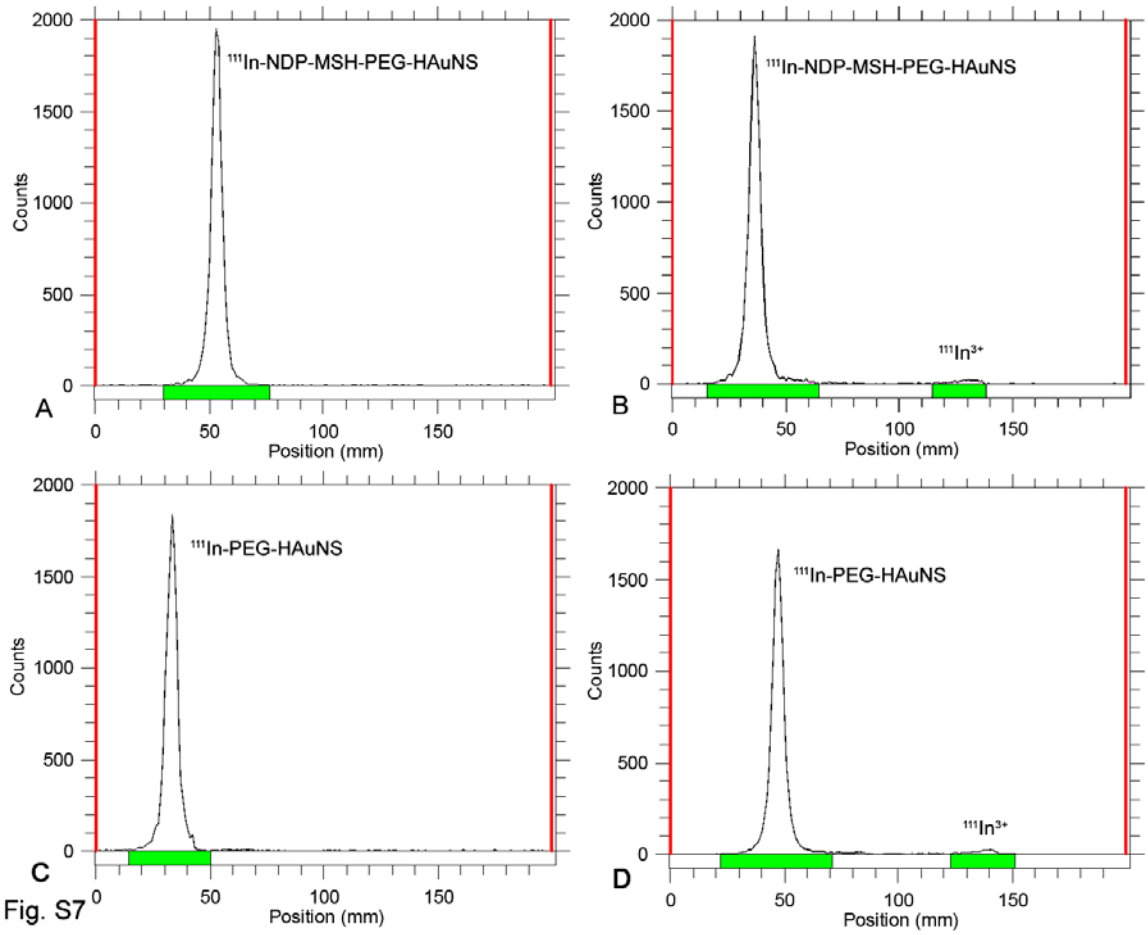

Fig. S7

Fig. S7. Instant thin layer chromatograph of NDP-MSH-PEG-HAuNS(DTPA-^{111}In) before (A) and 24 h after (B) incubation in full mouse serum at 37ºC. ITLC of PEG-HAuNS(DTPA-^{111}In) before (C) and 24 h after (D) incubation in full mouse serum at 37ºC.

References

1. Schwartzberg AM, Olson TY, Talley CE, Zhang JZ. Synthesis, characterization, and tunable optical properties of hollow gold nanospheres. J Phys Chem B 2006;110:19935-44.
2. Ji X, Shao R, Elliott AM*, et al.* Bifunctional gold nanoshells with a superparamagnetic iron oxide-silica core suitable for both MR imaging and photothermal therapy. J Phys Chem C 2007;111:6245-51.