Targeted Photothermal Ablation of Murine Melanomas with Melanocyte-Stimulating Hormone Analog – Conjugated Hollow Gold Nanospheres

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Abstract

Purpose: To develop melanoma-targeted hollow gold nanospheres (HAuNS) and evaluate their potential utility for selective photothermal ablation in melanoma.

Experimental Design: A new class of photothermal coupling agents based on HAuNS was synthesized. HAuNS were stabilized with polyethylene glycol (PEG) coating and attached with α—melanocyte-stimulating hormone (MSH) analog, [Nle⁴,D-Phe⁷]α—MSH (NDP-MSH), which is a potent agonist of melanocortin type-1 receptor overexpressed in melanoma. The intracellular uptake of the NDP-MSH–conjugated PEGylated HAuNS (NDP-MSH-PEG-HAuNS) and the distribution of β-arrestin were examined in murine B16/F10 melanoma cells. The biodistribution of NDP-MSH-PEG-HAuNS was assessed at 4 hours post i.v. injection in tumor-bearing nude mice. Photothermal ablation effect of the nanoparticles was evaluated both histologically using excised tissue and functionally by [¹⁸F]fluorodeoxyglucose positron emission tomography.

Results: NDP-MSH-PEG-HAuNS consist only of a thin gold wall with hollow interior (outer diameter, 43.5 ± 2.3 nm; shell thickness, 3-4 nm), which displays strong and tunable resonance absorption in near-IR region (peak, 808 nm). The nanoparticles were specifically taken up by melanoma cells, which initiated the recruitment of β-arrestins, the adapters to link the activated G-protein–coupled receptors to clathrin, indicating the involvement of receptor-mediated endocytosis. This resulted in enhanced extravasation of NDP-MSH-PEG-HAuNS from tumor blood vessels and their dispersion into tumor matrix compared with nonspecific PEGylated HAuNS. Successful selective photothermal ablation of B16/F10 melanoma with targeted HAuNS was confirmed by histologic and [¹⁸F]fluorodeoxyglucose positron emission tomography evaluation at 24 hours post near IR–region laser irradiation at a low-dose energy of 30 J/cm².

Conclusion: NDP-MSH-PEG-HAuNS have the potentials to mediate targeted photothermal ablation of melanoma.

Malignant melanoma is one of the most lethal cancers. Its incidence is increasing rapidly, making it a significant public health problem (1). Although the disease is considered to be highly treatable upon early diagnosis, the prognosis upon onset of metastasis is dire, typified by a 13% of 5-year survival rate once distant malignancy has occurred (2). Besides surgery, radiation therapy, and chemotherapy, other novel strategies including photothermal sensitization and photothermal ablation therapy with near IR-region laser light have been explored for the treatment of melanoma (2–6). Here, the development of novel photothermal coupling agents will be necessary for increasing the photothermal ablation efficiency, decreasing the energy dose of the laser light, and minimizing the potential for damage to surrounding normal tissues.

Nanostructures of noble metals such as gold can exhibit a strong optical extinction at near IR–region wavelengths (700-850 nm), wherein optical absorption in tissue is minimal and penetration is optimal (7–13). The efficiency of photothermal ablation can be significantly enhanced by integrating the light-absorbing material into the target tissue to mediate selective photothermal effects. Several studies have explored the use of gold nanostructures for cancer photothermal ablation, including nanorods (11), nanocages (10), and “core/shell” structures (7–9, 12, 13). However, a major and challenging requirement for successful biomedical applications of nanomaterials is efficient in vivo delivery to the target sites after systemic administration (14, 15).

Recently, a second-generation nanostructure based on hollow gold nanospheres (HAuNS) has been fabricated (16). These gold nanostructures have the unique combination of...
Affinity (IC50 = 0.21 nmol/L; refs. 26, 27). We hypothesized (23–25), and binds to melanocortin type-1 receptor with high efficient photothermal ablation of melanoma in a murine tumor model. The newly developed nanoparticles are particularly relevant to clinical translation for photothermal ablation of melanoma because these lesions are accessible to near-IR light penetration. Targeted delivery of nanoparticles to melanoma could increase the efficacy, decrease the energy dose of the laser, and minimize the potential for damage to surrounding normal tissues. In addition, noninvasive [18F]fluorodeoxyglucose positron emission tomography can be useful in monitoring early treatment response after photothermal ablation, which can have significant implication in the clinic in guiding repeat ablation procedures.

Materials and Methods

Materials. All N\(^{\beta}\)-9-fluorenylmethoxy carbonyl amino acids, 2(1H-benzotriazole-1-yl)1,3,3-tetramethyluronium hexafluorophosphate, 1-hydroxybenzotriazole, N\(^{\beta}\)-disuccinimidyl carbonate, diisopropylthylamine, and Rink amide resin (4-(2,4-dimethoxyphenyl-fluorenylmethoxycarbonyl-aminomethyl)phenoxyl resin were purchased from Nova-Biochem. The following side chain protecting groups were used: 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl (Dde) for Lys [Lys(N\(^{\beta}\)-Dde)], 1-butoxycarbonyl for Trp [Trp(N\(^{\beta}\)-butoxycarbonyl)], 2,2,4,6,7-pentamethylidyhydro-benzofuran-5-sulfonyl (Pbf) for Arg [Arg(N\(^{\beta}\)-2,2,4,6,7-pentamethylidyhydro-benzofuran-5-sulfonyl)], triptyl for His [His(N\(^{\beta}\)-trityl)], and t-butyloxyl for Glu [Glu(t-butyloxyl)]. Ser [Ser(t-butyloxyl)] and Tyr [Tyr(t-butyloxyl)]. Trifluoroacetic acid, triethylsilane, and piperidine were purchased from Chem-Impex International. Methoxy--PEG--thiol (SH; molecular weight, 5,000) was purchased from Nektar. NH\(_2\)-PEG-COOH-HCl (molecular weight, 5,000) was purchased from JenKem Technology. Cobalt chloride hydrate, sodium borohydride, chloroauric acid trihydrate, hydroxyamine, and dialysis bag (molecular weight cutoff, 1,000) were purchased from Fisher Scientific. N-succinimidyl S-acetyltiothiocarbonate (SATC), hydrazine, 4,6-diamidino-2-phenylindole (DAPI), N-(2-(2-(aminoethoxy)-ethoxy)ethoxy)-lipidic acid, FITC, diethylformamide, and all the other solvents were purchased from Sigma-Aldrich Chemical. PD-10 columns were purchased from Amersham-Pharmacia Biotech. All the chemicals and solvents were at least American Chemical Society grade and were used without further purification.

Conjugation of PEG and NDP-MSH to HAuNS. HAuNS were synthesized according to Schwartzberg et al. (16) with some modifications (see supplementary information). Lys\(^{\beta}\)-NH\(_2\)-protected NDP-MSH peptide, Ser-Tyr-Ser-Nle-Glu-His-d-Arg-Trp-Trp-Lys-Dde-Pro-Val-NH\(_2\) {Lys\(^{\beta}\)-Dde}[NDP-MSH], was synthesized manually using Rink amide resin and N\(^{\beta}\)-fluorenylmethoxycarbonyl chemistry. The peptide was conjugated to one terminus of a heterodifunctional PEG precursor, N-hydroxysuccinimidyl--PEG--SATA (molecular weight, 5,000), through its N-terminal amine and activated ester in N-hydroxysuccinimidyl--PEG--SATA. After removal of the protection group Dde in [Lys\(^{\beta}\)-Dde]NDP-MSH with 2% hydrazine in dimethylformamide, the sulfhydryl group on the other terminus of NDP-MSH-PEG-SATA was released by treatment with 0.5 mol/L hydroxylamine in PBS (Supplementary Fig. S1). [Lys\(^{\beta}\)-Dde]NDP-MSH with 2% hydrazine in dimethylformamide, the sulfhydryl group on the other terminus of NDP-MSH-PEG-SATA was released by treatment with 0.5 mol/L hydroxylamine in PBS (Supplementary Fig. S1). [Lys\(^{\beta}\)-Dde]NDP-MSH with 2% hydrazine in dimethylformamide, the sulfhydryl group on the other terminus of NDP-MSH-PEG-SATA was released by treatment with 0.5 mol/L hydroxylamine in PBS (Supplementary Fig. S1). [Lys\(^{\beta}\)-Dde]NDP-MSH with 2% hydrazine in dimethylformamide, the sulfhydryl group on the other terminus of NDP-MSH-PEG-SATA was released by treatment with 0.5 mol/L hydroxylamine in PBS (Supplementary Fig. S1). [Lys\(^{\beta}\)-Dde]NDP-MSH with 2% hydrazine in dimethylformamide, the sulfhydryl group on the other terminus of NDP-MSH-PEG-SATA was released by treatment with 0.5 mol/L hydroxylamine in PBS (Supplementary Fig. S1).

Tagging HAuNS with FITC. In our preliminary studies, we found that, although HAuNS could be readily visualized by acquiring their light-scattering signal using a microscope equipped with a dark-field condenser, the scattered light from melanin pigment in B16/F10 cells interfered with unambiguous identification of HAuNS. Therefore, we introduced a fluorescent dye to HAuNS through lipidic acid (Supplementary Fig. S2). Briefly, FITC (197 mg; 0.51 mmol) was first conjugated to N-(2-(2-(aminoethoxy)ethoxy)ethyl)-lipidic acid (116 mg; 0.34 mmol) in 2 ml of anhydrous dimethylformamide in the presence of diisopropylthylamine (0.57 ml). After removal of the solvent under reduced pressure, the crude product was purified using silica gel chromatography to yield N-(2-(2-(3-fluoresceinhydroxydo)ethoxy)ethoxy)ethyl)-lipidic acid (FITC-lipidic acid; 125 mg; 51.0%).
For FITC labeling of nanoparticles, FITC-lipoic acid (50 μg/mL; 1 mL) was mixed with 9 mL aqueous solution of HAuNS (8.5 × 10^{12} particles per milliliter) in the presence of NDP-MSH-PEG-SH (50 μg/mL) and PEG-SH (500 μg/mL) or PEG-SH alone (500 μg/mL). The products were purified by centrifugation and resuspension steps as described in the preceding section.

**Characterization of HAuNS.** For transmission electron microscopy, aqueous solution of HAuNS was deposited on copper grid without negative staining. The nanoparticles were allowed to adhere on the grid for 1 h, after which they were briefly rinsed with deionized water and air dried. The samples were then examined using a transmission electron microscope (JEM 1010, JEOL USA) at an accelerating voltage of 80 kV. Digital images were obtained using the Amt Imaging System (Advanced Microscopy Techniques Corp.). The average diameter of HAuNS and thickness of the shell were determined by measuring up to 45 individual particles.

The UV-Vis spectroscopy of HAuNS was recorded on a Beckman Coulter DU-800 UV-Vis spectrophotometer with a 1-cm optical path length quartz cuvette. The concentration of gold atoms of a HAuNS solution was analyzed by inductively coupled plasma mass spectroscopy (Galbraith). The stability of HAuNS in PBS and serum was investigated by incubating HAuNS in PBS, PBS containing 10% goat serum, or full serum for up to 24 h. The formation of aggregates over time was observed visually.

**Immunogold staining.** About 1.0 × 10^{12} NDP-MSH-PEG-HAuNS or PEG-HAuNS were incubated with rabbit anti-α-MSH polyclonal antibodies (1:100; ICN Biomedicals, Inc.) in 1 mL of PBS containing 1% bovine serum albumin and 10% albumin at 37°C for 1 h. The particles were passed through a Sepharose CL-4B column (Sigma) to separate unbound antibodies. The antibody-stained HAuNS were further incubated with 5-nm colloidal gold-conjugated goat anti–rabbit immunoglobulin G (1:100; Sigma) at 37°C for 1 h. The samples were directly visualized using transmission electron microscopy (JEM 1010 microscope) at an accelerating voltage of 80 kV. NDP-MSH-PEG-HAuNS incubated with colloidal gold-conjugated goat anti–rabbit immunoglobulin G were washed and resuspended with PBS. The antibody-stained HAuNS and negative control were then washed and resuspended with PBS at room temperature.

**Cell uptake and trafficking.** B16/F10 cells (American Type Culture Collection, 5,000 per well) were seeded in a 96-well plate 1 d before the experiment. The cells were then incubated with FITC-tagged NDP-MSH-PEG-HAuNS or FITC-tagged PEG-HAuNS in RPMI-1640 without phenol red (–3 × 10^{-7} M/mL) at 37°C for 1 h, followed by fixation in 4% paraformaldehyde. For inhibition study, the cells were incubated with free NDP-MSH (200 μg/mL) for 30 mins before addition of FITC-tagged NDP-MSH-PEG-HAuNS. After washing and fixation, cell nuclei were stained with 4’-6-diamidino-2-phenylindole (DAPI). To evaluate β-arrestin activation and recruitment, cells were incubated with NDP-MSH-PEG-HAuNS or PEG-HAuNS for 15 mins and then subjected to β-arrestin immunohistochemistry using goat anti–β-arrestin-2 polyclonal antibodies and donkey anti–goat immunoglobulin G tetramethyl rhodamine isothiocyanate conjugate (both from Santa Cruz Biotechnology, Inc.). The cellular fluorescence was examined under a Zeiss Axios Observer.Z1 fluorescence microscope (Carl Zeiss Microimaging GmbH). For transmission electron microscopy, the cellular uptake study was done in a 24-well plate for 15 mins at 37°C. The cells were fixed with a cocktail containing 2% paraformaldehyde and 3% glutaraldehyde, and samples were prepared using standard procedures for biological samples.

**In vitro photothermal ablation of melanoma cells.** B16/F10 cells were seeded onto a 96-well plate at a density of 10,000/well 1 d before the irradiation experiment. Cells were washed three times with RPMI-1640 without phenol red. The following treatments were used: NDP-MSH-PEG-HAuNS plus near IR–region laser, PEG-HAuNS plus near IR–region laser, near IR–region laser alone, and NDP-MSH-PEG-HAuNS alone. For treatments, cells were incubated with NDP-MSH-PEG-HAuNS or PEG-HAuNS (100 μL; 3 × 10^{12} particles/mL) at 37°C for 1 h. Thereafter, cells were washed three times with PBS to remove unbound HAUSS. Cells were then resupplied with RPMI-1640 containing 10% fetal bovine serum. Cells were irradiated with near IR–region laser light centered at 808 nm at an output power of 32 W/cm² for 3 mins (15PLUS laser, Diomed) and then incubated at 37°C for 24 h. The diode laser was coupled to a 1-m, 2-mm core fiber, which delivered a circular laser beam of 2 mm in diameter, covering the central area of the microplate well. Power calibration was done automatically.

Twenty-four hours after laser treatment, cells were washed three times with Hanks’ balanced salt solution and stained with calcine AM (Invitrogen) for visualization of live cells and with ethidium homodimer (EthD-1, Invitrogen) for visualization of dead cells. Cells were examined using an Olympus Fluoview FV1000 confocal laser scanning microscope (FV1-ASW) equipped with filter sets specific for excitation/emission wavelengths at 494/517 nm for calcine and 528/617 nm for EthD-1.

**Biodistribution-counting fluorescent HAUSS clusters.** All experiments involving animals were done in accordance with the guidelines of the Institutional Animal Care and Use Committee. Nude mice were inoculated s.c. with B16/F10 murine melanoma cells (5 × 10^5) in one flank of the abdomen. When tumors had grown to 4 to 6 mm in average diameter, the mice were randomly allocated into 2 groups (n = 5). Each group of mice received an i.v. injection of 2.5 × 10^{12} FITC-labeled NDP-MSH-PFG-HAuNS or PEG-HAuNS. Mice were killed 4 h after injection. Tumor, liver, spleen, kidney, lung, heart, and brain were removed and cryosectioned into 5-μm slices. The sections were stained with the following markers: rabbit anti–mouse melanocortin type-1 receptor polyclonal antibodies (Millipore), rat anti–mouse CD31 monoclonal antibody (Millipore), and/or rabbit anti–CD68 polyclonal antibodies (Santa Cruz Biotechnology). The secondary antibodies were Alexa Fluor 488–conjugated goat anti–rabbit immunoglobulin G and Alexa Fluor 594–conjugated goat anti–rat immunoglobulin G (Invitrogen). Cell nuclei were counterstained with DAPI. The slides were examined under a Zeiss fluorescence microscope equipped with an Apotome module. For quantification of the HAuNS, green fluorescent spots representing nanoparticle aggregates were counted in 5 randomly selected 0.035-mm² fields per slice at ×200 magnification. The tissue sections from five mice per group were examined. Values are presented as mean ± SD.

**Radiolabeling.** A chelation agent, S-2-[(4-[5-{1,2}dithiolane-3-pentanamide]benzyl)diethylenetriamine pentaacetic acid (DTPA-TA), was synthesized and attached to the surface of HAuNS (see supplementary information). For conjugation of radiometal chelator to nanoparticles, DTPA-TA (1 mg/mL; 10 μL) was mixed with 1 mL aqueous solution of HAuNS (1 × 10^{13} particles per milliliter) for 4 h at room temperature. This was followed by conjugation of NDP-MSH-PFG-SH and PEG-SH to the nanoparticles according to previously described procedures. For radiolabeling, aliquots of NDP-MSH-PFG-HAuNS(DTPA) or PEG-HAuNS(DTPA) (1 × 10^{12} particles per milliliter) were mixed with 0.1 mL sodium acetate solution (pH 5.5) mixed with an aqueous solution of 111InCl₃ (–200 μCi) for 30 mins. The radiolabeled HAuNS was then purified by centrifugation at 8,000 rpm for 5 mins and washed 3 times with PBS. The radiolabeling efficiency and the stability of labeled nanoparticles were analyzed using instant TLC (ITLC; see supplementary information).

**Biodistribution-radioactivity counting.** Mice bearing s.c. B16/F10 melanoma were randomly allocated into two groups (n = 5). Mice in group 1 were injected with 111In-labeled NDP-MSH-PFG-HAuNS [NDP-MSH-PFG-HAuNS(111In-DTPA)] and mice in group 2 were injected with 111In-labeled PEG-HAuNS [PEG-HAuNS(111In-DTPA)], both at a dose of 2 × 10^{12} particles per mouse (40 μCi per mouse in 0.2 mL). Mice were killed by CO₂ overexposure 4 h after injection. Blood, heart, liver, spleen, kidney, lung, and tumor tissues were removed and weighed, and radioactivity was measured with a Cobra gamma counter (Packard). Uptakes of 111In-labeled nanoparticles in various organs were calculated as percentage of injected dose per gram of tissue (%ID/g).

In vivo photothermal ablation and 18F-fluorodeoxyglucose positron emission tomography (PET). Nude mice were inoculated s.c. with B16/
F10 cells in both flanks of the abdomen. The mice were injected i.v. with $^{18}$F-fluorodeoxyglucose (200 μCi; 0.2 ml) before laser treatment. The animals were anesthetized with 2% isoflurane (Baxter), and PET images were acquired 30 mins after radiotracer injection using a Rodent R4 microPET scanner (Concorde Microsystems, Inc.). The mice were then randomly divided into three groups ($n = 3$). Mice in each group received an i.v. injection of NDP-MSH-PEG-HAuNS (2.5 × 10$^{11}$ particles per mouse), PEG-HAuNS (2.5 × 10$^{12}$ particles per mouse), or saline control. Four hours later, the tumor on one flank of each mouse was randomly selected for laser irradiation (808 nm; 0.5 W/cm$^2$; 1 min). A 5-m, 600-μm core LCM-002 laser collimating fiber (BioTex, Inc.) was used to transfer laser power from the laser unit to the target; this delivered a circular laser beam of 1 cm in diameter, covering the surface area of the tumor. Twenty-four hours after laser treatment, the mice again received an i.v. injection of $^{18}$F-fluorodeoxyglucose, and PET images were acquired as aforementioned. After the experiments, the mice were killed, and the tumors were removed and weighed. PET images were reconstructed using ASIPro VM 6.3.3.0 software provided by Concorde Microsystems, Inc. Counts per pixel per minute in the regions of interest were converted to microcuries using a calibration curve derived from scanning standard activity phantoms in the microPET scanner. $^{18}$F-fluorodeoxyglucose uptake in each tumor was divided by the weight of the tumor to obtain %ID/g.

For histologic evaluation, 15 additional tumor-bearing mice were randomly divided into 3 groups ($n = 5$). Mice in groups 1, 2, and 3 were injected with NDP-MSH-PEG-HAuNS, PEG-HAuNS, or saline control, respectively, and were treated with near IR–region laser as described in the proceeding section. Tumors were removed and cryosectioned for H&E staining. The slices were examined under a Zeiss microscope. The images were taken using a Zeiss AxioCam MRc5 color camera, and the extent of tumor necrosis, expressed as a percentage of the entire tumor area, was analyzed with Zeiss AxioVision software (version 4.6.3).

Statistical analysis. Comparisons of the number of green spots representing aggregates of HAuNS in tissue sections, tissue uptake of $^{111}$In-labeled nanoparticles (%ID)/g, $^{18}$F-fluorodeoxyglucose uptake (%ID)/g, and necrotic area as a percentage of tumor area were made using two-tailed Student’s t test or ANOVA (for all groups). Differences between groups were considered statistically significant if $P < 0.05$.

Results and Discussions

Synthesis and characterization of NDP-MSH-PEG-HAuNS. NDP-MSH was conjugated to HAuNS through a PEG linker in the presence of excess of sulfhydryl methoxy-PEG (molecular weight, 5,000; molar ratio NDP-MSH-PEG to PEG, 1:10). A small-molecular-weight peptide, NDP-MSH, was conjugated to HAuNS using a fluorescein was conjugated to HAuNS using a fluorescent dye that is used to probe cell membrane integrity. Twenty-four hours after near IR–region irradiation (32 W/cm$^2$; 3 minutes), most B16/F10 cells treated with NDP-MSH-PEG-HAuNS were stained red with EthD-1 and there was absence of green calcein AM staining in these cells (Fig. 3A). In comparison, only a small fraction of cells were stained red with EthD-1 after treatment with PEG-HAuNS followed by near IR–region laser. The other control treatment groups (near IR-region laser alone, NDP-MSH-PEG-HAuNS alone) showed no observable damage to the cancer cells. At higher magnification, most of the cells treated with PEG-HAuNS plus near IR–region
laser were polygonal (Fig. 3B, top row) with few cells stained red with EthD-1. However, after treatment with NDP-MSH-PEG-HAuNS plus near IR–region laser, cell membranes were lysed and cell nuclei condensed (Fig. 3B, bottom row, arrows). These results indicate that NDP-MSH-PEG-HAuNS mediated selective photothermal destruction of melanoma cells.

Previous studies showed that the threshold temperature in the range of 70°C to 80°C was required for the destruction of tumor cells with the used of gold nanoparticles (33). Thermally induced cellular injury/death above 40°C resulted from protein denaturation (34). There are two general effects of protein denaturation that one would expect to be particularly harmful to cells: direct inactivation of protein function and disruption of complex structures (35). Inactivation of enzyme activity, membrane receptors, and ion transporters has been shown to occur during hyperthermia (36). This can consist of membrane permeability changes, depolymerization of complex structures (e.g., disruption of cytoskeletal elements), and aggregation (e.g., aggregation of membrane proteins and binding of proteins to the nuclear matrix and other cytoskeletal structures). At higher temperature (i.e., 85-90°C), DNA and RNA also denature and unfold (34). In our study, the negative staining of calcein AM in B16/F10 melanoma cells treated with NDP-MSH-PEG-HAuNS plus near IR–region laser indicated the complete lost of ubiquitous intracellular esterase activity. A positive staining with EthD-1 in cells treated with the targeted HAUuNS and laser indicated the disruption of cell membrane. The inactivation of enzyme activity and disruption of cell membrane permeability could be attributed to the protein denaturation induced by photothermal effect. Further studies will be needed to investigate the temperature change in real time during near IR–region laser irradiation and protein denaturation and DNA unfolding transitions in relation to the observed temperature profile to clarify the mechanism of photothermal ablation mediated by HAuNS.

**In vivo targeting to murine melanoma.** Figure 4 compares distribution of NDP-MSH-PEG-HAuNS and PEG-HAuNS in melanocortin type-1 receptor–positive B16/F10 tumors grown s.c. in nude mice. Significantly higher uptake of FITC-tagged NDP-MSH-PEG-HAuNS than of FITC-tagged PEG-HAuNS in the tumor 4 hours after i.v. injection of nanoparticles was observed. Although PEG-HAuNS were scattered adjacent to the tumor vasculature, NDP-MSH-PEG-HAuNS were found throughout the tumor matrix, including in areas that were >200 μm away from the nearest blood vessels (Fig. 4A). The distributions of FITC-tagged NDP-MSH-PEG-HAuNS and FITC-tagged PEG-HAuNS in the other major organs are presented in Fig. 4B and Supplementary Fig. 5. For
Fig. 2. Specific uptake of NDP-MSH-PEG-HAuNS in B16/F10 cells. A, uptake of FITC-tagged HAuNS in B16/F10 cells. Cell nuclei were counterstained with DAPI (blue). Bar, 20 μm. B, distribution of β-arrestin expression in relation to FITC-tagged HAuNS in B16/F10 cells. In cells incubated with NDP-MSH-PEG-HAuNS, the HAuNS (green) colocalized with β-arrestin (red) in polarized fashion (yellow, arrows), whereas in cells incubated with PEG-HAuNS, β-arrestin was evenly distributed in the cytoplasm and did not colocalize with the HAuNS. Cell nuclei were counterstained with DAPI (blue). Bar, 10 μm. C, transmission electron microscopy images of B16/F10 cells incubated with NDP-MSH-PEG-HAuNS or PEG-HAuNS. The electron-dense NDP-MSH-PEG-HAuNS were seen in coated pits (arrowheads), early endosomes (arrow), and cytoplasm. PEG-HAuNS were found only outside the cell membrane.
NDP-MSH-PEG-HAuNS, the relative fluorescence intensity in different tissues was in the order of tumor > spleen > liver >> lung ≈ kidney > heart ≈ brain. For PEG-HAuNS, the order was spleen > liver >> tumor ≈ lung ≈ kidney ≈ heart ≈ brain (Supplementary Fig. S5). More NDP-MSH-PEG-HAuNS were taken up by the tumors than by the liver and the spleen at 4 hours after injection. Uptakes of HAuNS in the liver and the spleen were primarily mediated by macrophages, as confirmed by colocalization of HAuNS with macrophages stained positively for CD68 (Supplementary Fig. S6).

For accurate quantitative analysis, HAuNS were further labeled with the γ-emitter $^{111}\text{In}$, which has a desirable decay half-life ($t_{1/2} = 67.3$ hours). Radiolabeling was accomplished through incubation of $^{111}\text{InCl}_3$ with HAuNS tagged with the chelation agent, DTPA-TA. Instant TLC analysis showed that the radiochemical purities of NDP-MSH-PEG-HAuNS(DTPA-$^{111}\text{In}$) and PEG-HAuNS(DTPA-$^{111}\text{In}$) were >99% (Supplementary Fig. S7). After incubation in full mouse serum for 24 hours, the radiochemical purity of NDP-MSH-PEG-HAuNS(DTPA-$^{111}\text{In}$) and PEG-HAuNS(DTPA-$^{111}\text{In}$) remained >95%.

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**Fig. 3.** Cell viability after near IR region laser irradiation. B16/F10 cells were treated with different HAuNS and near IR region light centered at 808 nm (32 W/cm²; 3 mins). A, after treatment with PEG-HAuNS plus near IR region laser, near IR region laser alone, or NDP-MSH-PEG-HAuNS alone, cells retained normal morphology, and few dead cells were observed. In contrast, after treatment with NDP-MSH-PEG-HAuNS plus near IR region laser, most cells were dead. Viable cells were stained green with calcein AM; dead cells were stained red with EthD-1. Circled area labeled with asterisk, laser-irradiated area; bar, 100 μm. B, microphotographs of cells treated with PEG-HAuNS plus near IR region laser (top) and NDP-MSH-PEG-HAuNS plus near IR region laser (bottom) at higher magnification. Cells treated with PEG-HAuNS plus near IR region laser were viable and polygonal, whereas most cells treated with NDP-MSH-PEG-HAuNS plus near IR region laser were rounded and lost membrane integrity (arrows). DIC, differential interference contrast. Bar, 20 μm.
Supplementary Fig. S7), indicating that the radiolabel on HAuNS was stable.

Biodistribution data obtained at 4 hours after i.v. injection of radiolabeled HAuNS are summarized in Fig. 4C. The results confirmed significantly higher uptake of NDP-MSH-PEG-HAuNS(DTPA-111In) in the tumor than that of PEG-HAuNS(DTPA-111In) (12.6 ± 3.1%ID/g versus 4.3 ± 1.2%ID/g). In agreement with the results obtained by counting the particle clusters in tissue slices, mice injected with NDP-MSH-PEG-HAuNS(DTPA-111In) showed the highest uptake in the tumor. Kidney, liver, lung, and spleen were other major organs taken up significant amount of HAuNS, but there was no significant difference between the group injected with NDP-MSH-PEG-HAuNS(DTPA-111In) and the control group injected with nontargeted PEG-HAuNS(DTPA-111In). The radioactivity of both nanoparticles in the blood pool was relatively high at 4 hours after injection, suggesting the long blood half-lives of PEGylated HAuNS. The tumor-to-blood uptake ratio of NDP-MSH-PEG-HAuNS(DTPA-111In) was about 1.0, whereas that of PEG-HAuNS(DTPA-111In) was only 0.37.

Potential mechanisms for increasing nanoparticle delivery and dispersion in the tumor include increase in extravasation and interstitial transport (37). The observed transvascular distribution of PEG-HAuNS can be attributed to passive accumulation (18, 20). However, the tumor-to-blood uptake ratio of nontargeted PEG-HAuNS was much lower than one, suggesting that the enhanced permeability and retention effect was not significant at the time of analysis (i.e., 4 hours after injection). We believe that an active transport mechanism, operating through receptor-mediated endocytosis, is responsible for enhanced extravasation and dispersion of NDP-MSH-PEG-HAuNS into the tumor matrix. Our assertion is supported by the fact that the tumor-to-blood ratio of NDP-MSH-PEG-HAuNS(DTPA-111In) was 2.7-fold higher than that of PEG-HAuNS(DTPA-111In). This assertion is further substantiated by Z-stack images of tumor sections at higher magnification, which revealed colocalization of NDP-MSH-PEG-HAuNS but not PEG-HAuNS with melanocortin type-1 receptor (Fig. 4D, arrowheads; yellow and orange colors resulting from overlapping green and red colors), confirming melanocortin type-1 receptor–mediated endocytosis of NDP-MSH-PEG-HAuNS in vivo.

Fig. 4. Biodistribution and intratumoral distribution of FITC-tagged HAuNS. Tissue and tumors were removed 4 h after i.v. injection of HAuNS. A, representative fluorescence micrographs of cryosectioned B16/F10 melanoma. Microvessels (red) were stained with rat anti–mouse CD31 monoclonal antibody. Cell nuclei were counterstained with DAPI (blue). Significantly more HAuNS (green) were found in the tumors of mice injected with NDP-MSH-PEG-HAuNS than in the tumors of mice injected with PEG-HAuNS. NDP-MSH-PEG-HAuNS were distributed throughout the tumor matrix in the interstitial space, whereas PEG-HAuNS were distributed around tumor vessels (arrow). Bar, 100 μm. B, biodistribution of FITC-tagged HAuNS in different tissues. Data were calculated as the number of particle aggregates per square millimeter visual area at ×200, and values are presented as mean ± SD (n = 5). Bars, SD. *, P < 0.01. Microphotographs of FITC-tagged HAuNS in other major organs are shown in Supplementary Fig. S5. C, biodistribution of NDP-MSH-PEG-HAuNS(DTPA-111In) and PEG-HAuNS(DTPA-111In). Data were plotted as %ID/g. Mean ± SD (n = 5). *, P < 0.01. D, Z-stack images of tumor sections at higher magnification. Melanocortin type-1 receptor was stained with rabbit anti–mouse melanocortin type-1 receptor polyclonal antibody (pseudocolored red). Blood vessels were stained with rat anti–mouse CD31 monoclonal antibody (pseudocolored blue). FITC-tagged HAuNS were green. NDP-MSH-PEG-HAuNS but not PEG-HAuNS colocalized with melanocortin type-1 receptor (yellow and orange, arrowheads), indicating melanocortin type-1 receptor–mediated endocytosis of NDP-MSH-PEG-HAuNS in vivo. Asterisks, the lumens of tumor vasculature with discontinuous CD31 staining; bar, 10 μm.
**Fig. 5. In vivo photothermal ablation with targeted NDP-MSH-PEG-HAuNS induced selective destruction of B16/F10 melanoma in nude mice.**

**A.** [*18F*]fluorodeoxyglucose PET imaging shows significantly reduced metabolic activity in tumors after photothermal ablation in mice pretreated with NDP-MSH-PEG-HAuNS but not in mice pretreated with PEG-HAuNS or saline. [*18F*]fluorodeoxyglucose PET was conducted before (0 h) and 24 h after near IR region laser irradiation (0.5 W/cm² at 808 nm for 1 min), which was commenced 4 h after i.v. injection of HAuNS or saline. Tumor Arrowheads, tumors irradiated with near IR region light. [*18F*]fluorodeoxyglucose uptakes (%ID/g) before and after laser treatment are shown graphically at the bottom. Bars, SD (n = 3). *, P < 0.01 for %ID/g posttreatment versus %ID/g pretreatment.

**B.** Histologic assessment of tumor necrosis. Representative photographs of whole tumors stained with H&E 24 h after near IR region laser irradiation. Bar, 500 μm. Representative microphotographs at high magnification show tumor cells characterized by extensive pyknosis (arrows), karyolysis (arrowheads), cytoplasmic acidophilia, and degradation of the extracellular matrix of the tumor (asterisks) in mice treated with NDP-MSH-PEG-HAuNS plus laser. In mice treated with PEG-HAuNS plus laser, such features were observed mostly in areas close to the surface. Bar, 50 μm. The necrotic area as a percentage of the tumor is shown in the bar graph. *, P < 0.05. Bars, SD (n = 5).
Our results raise a number of interesting questions that must be further addressed. For example, what is the role of particle size in successful active targeting? We believe that HAUuNS used in this study are ideally suited for targeted delivery not only because they are sufficiently small to pass through the pores of tumor vessels but also because intracellular uptake of gold nanoparticles by mammalian cells is optimal when particle size is ~50 nm (38). In our recent work with HAUuNS coated with monoclonal antibodies, only a moderate, albeit statistically insignificant, increase in tumor uptake of antibody coated HAUuNS was observed (17). It is reasonable to speculate that the much smaller size of peptide as compared with antibody used as the targeting moiety is responsible for successful active targeting showed in the current work. Another question is related to the role of receptor-mediated endocytosis in extravasation and interstitial transport of NDP-MSH-PEG-HAuNS. The fact that NDP-MSH-PEG-HAuNS were rapidly transported to extravascular fluid space as far as 200 μm beyond the nearest microvessels can hardly be explained by a simple diffusion mechanism. Clearly, further work is needed to elucidate the role of diffusion and/or transcytosis in enhancing extravasation and interstitial transport of targeted HAUuNS in B16/F10 melanoma.

In vivo photothermal ablation of murine melanoma. Having shown that NDP-MSH-PEG-HAuNS were selectively delivered to B16/F10 melanoma, we next asked whether targeted delivery of NDP-MSH-PEG-HAuNS could translate into selective photothermal ablation of melanoma in vivo. We used [18F]fluorodeoxyglucose PET to assess changes in metabolic activity after photothermal ablation. In mice injected with NDP-MSH-PEG-HAuNS, microPET showed markedly reduced tumor [18F]fluorodeoxyglucose uptake 24 hours after near IR–region laser treatment; the percentage of injected dose per gram of tumor (%ID/g) decreased by 86% ([P = 0.0088] compared with the pretreatment value (Fig. 5A). In contrast, in mice injected with PEG-HAuNS or saline, tumor [18F]fluorodeoxyglucose uptake was similar before and after near IR–region laser treatment. Moreover, tumor [18F]fluorodeoxyglucose uptake was similar for tumors inoculated at contralateral sites not irradiated with near IR–region laser, indicating that reduced metabolic activity of tumors treated with NDP-MSH-PEG-HAuNS plus laser was not caused by NDP-MSH-PEG-HAuNS alone (Fig. 5A).

Histologic examination confirmed that NDP-MSH-PEG-HAuNS plus laser caused significantly greater necrotic response than did PEG-HAuNS plus laser, saline plus laser, or saline only (Fig. 5B). About 66% of tissues were necrotic, characterized as pyknosis, karyolysis, cytoplasmic acidophilia, and degradation and corrosion of the extracellular matrix of the tumor (Fig. 5B). Only a small fraction of necrotic tissues was found in the tumors treated with PEG-HAuNS plus near IR–region laser (7.9%). Thus, selective photothermal destruction of the target tumors mediated by NDP-MSH-PEG-HAuNS was confirmed histologically (using excised tissue) and functionally ([18F]fluorodeoxyglucose PET imaging). Early effects on metabolic activity as assessed by noninvasive [18F]fluorodeoxyglucose PET imaging can be useful in monitoring response to photothermal ablation therapy. Long-term studies to evaluate the antitumor activity of targeted HAUuNS in combination with near IR–region laser irradiation will need to be carried out to further confirm the short-term results obtained in the current studies.

The use of targeted HAUuNS as photothermal coupling agents is particularly attractive in photothermal ablation of melanomas. Clinical data for the treatment of choroidal melanoma with an 810-nm light used an output power of at least 300 mW for 1 minute (3.0-mm spot in diameter), corresponding to a dose of ~255 J/cm² (4). In another study in a murine cutaneous melanoma model similar to the one used in the current study, a light dose of 1,000 J/cm² was applied with a Nd:yttrium-lanthanum-fluoride laser at 1,047 nm (2). In our photothermal ablation experiment, s.c. murine melanoma in mice injected with targeted HAUuNS was exposed to near IR–region light centered at 808 nm for 1 minute at an output power of 0.5 W/cm², which corresponded to a light dose of 30 J/cm². Reduced laser power is highly desirable to avoid unnecessary damage to surrounding normal tissues. Finally, HAUuNS contains nothing else other than pure gold. This is advantageous compared with other metal core/shell nanostructures that contain such materials as silica in the core (7–9). Colloidal gold has been safely used to treat rheumatoid arthritis for decades (39, 40). Gold colloids have little toxicity or other adverse effects in vivo (41, 42). Nevertheless, the long-term fate of HAUuNS (as with other nanoparticles) after systemic injection requires further investigation.

Conclusions

Our current work establishes targeted HAUuNS for in vivo photothermal ablation. The combination of spherical shape, small size (average diameter ~40 nm), absence of silica core, and tunable and strong absorption bands in near-IR region makes these HAUuNS ideally suited for photothermal ablation applications. Using a small-molecular-weight peptide as a targeting ligand and attaching it at the end of PEG chains, we showed for the first time receptor-mediated active targeting of melanoma and efficient photothermal ablation with photothermal coupling agents in vivo. We further showed that noninvasive [18F]fluorodeoxyglucose PET can be useful in monitoring early treatment response after photothermal ablation. This can have significant implication in the clinic in guiding repeat ablation procedures.

Although our current study has shown promising results in selective photothermal ablation of melanoma using targeted HAUuNS, much work remains to be done to advance this technology further into the clinic. For example, more detailed preclinical studies with regard to excetration/clearance, safety, and efficacy of targeted HAUuNS need be documented. The physicochemical properties of HAUuNS (including particle size and surface characteristics) may be further improved to minimize their retention in the liver, the spleen, and the kidney. Studies correlating intratumoral distribution of HAUuNS, temperature map, and thermal damage in vivo using noninvasive imaging techniques will be another area warrant future investigations.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Dr. Juri Gelovani for the helpful discussions, Stephanie Deming for editing the manuscript, Marites Melancon and Zhi Cheng for the assistance with nanoshell preparation, Kenneth Dunner for the assistance and use of the transmission electron microscopy facility, which, together with the Small Animal Imaging Facility, was supported by Cancer Center Support Grant CA16672 to M. D. Anderson Cancer Center.
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