Published in final edited form as:

Biomaterials. 2011 October ; 32(30): 7600–7608. doi:10.1016/j.biomaterials.2011.06.039.

Targeted multifunctional gold-based nanoshells for magnetic resonance-guided laser ablation of head and neck cancer

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Abstract

Image-guided thermal ablation of tumors is becoming a more widely accepted minimally invasive alternative to surgery for patients who are not good surgical candidates, such as patients with advanced head and neck cancer. In this study, multifunctional superparamagnetic iron oxide coated with gold nanoshell (SPIO@Au NS) that have both optical and magnetic properties was conjugated with the targeting agent, C225 monoclonal antibody, against epidermal growth factor receptor (EGFR). C225-SPIO@Au NS have an average diameter of 82 ± 4.4 nm, contain 142 ± 15 antibodies per nanoshell, have an absorption peak in the near infrared (~ 800 nm), and have transverse relaxivity (r_2) of 193 and $353 \text{ mM}^{-1}\text{s}^{-1}$ versus Feridex of 171 and $300 \text{ mM}^{-1}\text{s}^{-1}$, using 1.5T and 7T MR scanners, respectively. Specific targeting of the synthesized C225-SPIO@Au NS was tested *in vitro* using A431 cells and oral cancer cells, FaDu, OSC-19, and HN5, all of which overexpress EGFR. Selective binding was achieved using C225-SPIO@Au NS but not with the non-targeting PEG-SPIO@Au NS and blocking group (excess of C225 + C225-SPIO@Au NS). *In vivo* biodistribution on mice bearing A431 tumors also showed selective targeting of C225-SPIO@Au NS compared with the non-targeting and blocking groups. The selective photothermal ablation of the nanoshells shows that without laser treatment there were no cell death and among the groups that were treated with laser at a power of 36 W/cm^2 for 3 minutes, only the cells treated with C225-SPIO@Au NS had cell killing ($p < 0.001$). In summary, successful synthesis and characterization of targeted C225-SPIO@Au NS demonstrating both superparamagnetic and optical properties has been achieved. We have shown both *in vitro* and *in vivo* that these nanoshells are MR-active and can be selectively heated up for simultaneous imaging and photothermal ablation therapy.

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Keywords

Theranostics; Gold nanoshells; Magnetic resonance imaging; Laser ablation; Ultrasmall paramagnetic iron oxide (SPIO)

1. Introduction

Head and neck cancer recur locally and/or regionally in as many as 50% of patients treated with standard therapies including surgery, radiation, and/or chemoradiation [1]. Treating recurrent disease with further surgery and/or radiotherapy is often not feasible owing to limitations associated with the extent of the disease and the limits of radiation that normal tissues can receive. Therefore, for these patients, there is a compelling need for an effective but less-invasive approach to therapy that provides highly conformal treatment of the tumor and that can be repeated as needed to manage locoregional recurrence.

Image-guided laser ablation of head and neck tumors is a promising minimally invasive technique currently being investigated as an alternative to conventional surgical interventions. Laser ablation uses small laser fibers, placed directly into the tumor, to induce heating and destroy the tumor tissue. While the region of damage is small and localized around the fiber, the laser-induced heating is not confined exclusively to the tumor tissue, and thus, there is a chance that nearby tissue can be damaged. To help overcome this limitation and increase the conformality of therapy delivery, we propose the use of tumor-targeted nanoparticles, which are preferentially targeted to the tumor and, thereby enhance the safety and efficacy of the overall procedure.

Laser ablation has been shown to be mediated efficiently by gold nanoshells (AuNS), or other nanoparticulate materials, that can be made to preferentially home to tumor tissue and strongly absorb near-infrared (NIR) light [2]. Absorption in the NIR region (650–1100 nm) is highly desirable because water and naturally occurring fluorochromes have the lowest absorption in this region, and therefore light can penetrate deeper into the tissues. Several types of nanoparticles, including nanospheres [2, 3] and nanorods [4, 5], have been shown to work both *in vitro* and *in vivo*. Clinical trials are currently being conducted to investigate the use of gold nanospheres surrounded by silica in NIR photothermal therapy for refractory head and neck cancers [6]. However, to ensure the safety and efficacy of this type of thermal ablation therapy, one must know that the nanoparticles have been delivered and be able to monitor the extent of therapy in real time. Therefore, the successful translation of photothermal ablation into clinical use/applications will highly benefit from (1) selective delivery of a sufficient amount of AuNS to the tumor tissue to mediate a photothermal effect, (2) accurate visualization of NS accumulation for pretreatment planning, and (3) noninvasive real-time monitoring of the spatiotemporal heat profile and response to therapy in a given target volume. Since EGFR is overexpressed in more than 90% of oral squamous cell carcinomas (OSCCs), this tumor type represents an excellent model for preclinical studies of nanoparticle-targeted thermoablation and an ideal disease for which preclinical findings may be translated into clinical trials [7].

Magnetic resonance imaging (MRI) is a useful modality for providing image guidance of particle-mediated thermoablation as the exquisite soft-tissue contrast of MRI makes identifying lesions and critical structures possible for planning and targeting therapy. In addition, MRI is sensitive to nanoscale contrast agents such as superparamagnetic iron oxide (SPIO) nanoparticles, and can provide real-time temperature-sensitive feedback during therapy [8]. In addition, medical lasers currently cleared by the Food and Drug

Administration can be adapted easily to the MRI environment at 1.5T and 3.0T (the field strength of current clinical scanners) [9].

The purpose of our study is to demonstrate the specificity of the synthesized targeted SPIO@Au NS in targeting EGFR-positive tumors both in vitro and vivo, and to show its efficacy for laser ablation therapy. We hypothesize that multifunctional gold nanoshells encapsulated with superparamagnetic iron oxide (SPIO@Au NS) and conjugated with cetuximab (C225 monoclonal antibody) targeted to EGFR will selectively accumulate within head and neck cancer, which can enhance heating and thermally induce tumor destruction, thus increasing the safety and efficacy of therapy. In this study we created multifunctional SPIO@Au NS that have both optical and magnetic properties, which could be exploited for imaging and therapy. The core-shell nanoparticles comprise a SPIO core surrounded with silica and a gold shell (SPIO@Au NS) that is conjugated with the targeting agent, C225 monoclonal antibody. We further evaluated this agent's (C225-SPIO@Au NS) ability to selectively bind to tumor cells overexpressing EGFR and mediate photothermal ablation.

2. Materials and methods

2.1. Materials

Monoclonal anti-EGFR antibody C225 was obtained from ImClone Systems (New York, NY). C225 is a chimeric human-mouse immunoglobulin G1 that binds EGFR with high affinity [10, 11]. Methoxy-polyethylene glycol-sulphydryl (PEG-SH, molecular weight [MW] 5,000), *N*-succinimidyl *S*-acetylthioacetate (SATA), and phosphate-buffered saline (PBS; pH, 7.4) were purchased from Sigma-Aldrich (St. Louis, MO).

p-Isothiocyanatobenzyl-diethylenetriaminepentaacetic acid (*p*-SCN-Bz-DTPA) was obtained from Macrocyclics (Dallas, TX). PD-10 columns were purchased from GE Healthcare (Piscataway, NJ). Trisodium citrate dihydrate (>99%) and chloroauric acid trihydrate (American Chemical Society reagent grade) were purchased from Fisher Scientific (Pittsburg, PA) and used without further purification. SPIO (EMG 304) was purchased from FerroTec (Bedford, NH).

All the chemicals and solvents were at least American Chemical Society grade and were used without further purification. Indium-111 radionuclide was obtained from Perkin Elmer (Waltham, MA).

2.2. Synthesis of SPIO@AuNS

SPIO@Au NS was synthesized using methods described by Ji et al. [12]. Briefly, SPIO nanoparticles of about 10 nm in diameter were coated with amorphous silica using the sol-gel process. Next, gold nanocrystal seeds (2–3 nm) were introduced onto an amine-functionalized silica surface and used to nucleate the growth of a gold overlayer to form an AuNS. Thiolated PEG-SH was attached to the surface of the SPIO@Au NS by adding 5 μ M of PEG-SH (final concentration) to 1×10^{12} particles/mL NS in deionized water. After 2 h, the solution was centrifuged for 5 min at 8,000 rpm and washed three times with deionized water. The resulting solution was resuspended in PBS (0.1 M).

2.3. Synthesis of C225-SPIO@AuNS

C225-SPIO@AuNS was synthesized according to previously published procedures [13, 14]. Briefly, an aqueous solution of C225 (2.5 mg, 0.017 μ mol; 5 mg/mL) was first allowed to react with SATA (0.077 mg, 0.332 μ mol) at room temperature for 1 h. The resulting conjugate, C225-acetylthioacetate (C225-ATA), was purified by passing it through a gel

filtration PD-10 column and using protein-dye color (Bio-Rad Laboratories, Hercules, CA) as an indicator to guide the collection of antibody-containing fractions. C225-ATA was treated with hydroxylamine (50 mM, 50 μ L) at room temperature for 2 h to expose free SH. After passing through the PD-10 column, the resulting C225-SH was added to an aqueous solution of SPIO@AuNS (1.0×10^{13} particles/mL) to a final antibody concentration of 5 μ g/mL. The suspension was then stirred for 1 h at room temperature. Thereafter, PEG-SH was added to the antibody-coated SPIO@AuNS to a final concentration of 0.2 mg/mL, and the mixture was reacted for an additional 1 h to ensure the gold surface was completely covered. C225-SPIO@AuNS were centrifuged at 8,000 rpm for 5 min, and the resulting pellet was washed twice with deionized water. The C225 present in the supernatant liquid was tested with the protein dye. After the second wash, C225 was not detected in the supernatant. The purified products were resuspended in 0.1 mM PBS and stored at 4 $^{\circ}$ C until further use.

2.4. Synthesis of DTPA-C225-ATA and DTPA-PEG-ATA

An aqueous solution of C225 (2.5 mg, 0.017 μ mol; 5 mg/mL) was first allowed to react with SATA (0.077 mg, 0.332 μ mol) at room temperature for 1 h. The resulting conjugate, C225-ATA, was purified by passing it through a gel filtration PD-10 column, using the protein-dye color as an indicator to guide the collection of antibody-containing fractions. The purified C225-ATA was then reacted with *p*-SCN-Bz-DTPA (0.216 mg, 0.332 μ mol) at 4 $^{\circ}$ C overnight in an aqueous solution in which the pH value was adjusted to 8 using 0.1 M sodium bicarbonate. The product was then purified by passing it through a PD-10 column to remove excess *p*-SCN-Bz-DTPA and other small-molecular-weight contaminants to yield DTPA-C225-ATA.

DTPA-PEG-ATA, used as a nonspecific antibody control, was synthesized as described by Melancon et al.[13]. Briefly, diethylenetriamine pentaacetic acid (DTPA)-thioacetic acid (DTPA-TA) was conjugated on the surface of SPIO@AuNS. DTPA-TA was synthesized as described by Zhang et al [15]. PEG-SH was then added to the DTPA-TA treated with SPIO@AuNS to enhance its colloidal stability. After purification, the NS were resuspended in sodium acetate buffer (pH, 5) and labeled with indium-111 radionuclide as described in Section 2.11 (Radiolabeling).

2.5. Characterization of nanoparticles

For the TEM study, bare (uncoated) SPIO@AuNS and C225-SPIO@AuNS as prepared were applied to a 100-mesh, polyvinyl formal resin- and carbon-coated nickel grid. 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 TEM (JEM 2100F, JEOL Ltd.) at an accelerating voltage of 200 kV. The average diameter and thickness of the NS were determined by measuring up to 50 individual SPIO@AuNS particles. The elemental composition of the nanoparticles was analyzed using the JEM 2100F TEM equipped with an energy-dispersive x-ray spectrometer with an ultrathin window.

The UV-Vis spectroscopy of the NSs was recorded on a Beckman Coulter DU800 UV-Vis spectrophotometer with a 1.0-cm optical path-length quartz cuvette. The concentration of gold and iron in the SPIO@AuNS solution was analyzed by inductively coupled plasma mass spectroscopic analysis (ICP-MS) (Galbraith Laboratories Inc., Knoxville, TN).

We measured the magnetization at 300 K and in a magnetic field (*H*) up to 50 kOe using a superconducting quantum-interference device magnetometer (Magnetic Property Measurement System [MPMS]; Quantum Design Inc., San Diego, CA), which can measure magnetic moments as low as 10^{-7} emu. For the magnetization measurement, both the C225-

coated and bare SPIO@Au NS were in the form of dry powders. Gelatin capsules were used as the containers for the powder samples.

2.6. Quantification of antibodies per SPIO@AuNS

The number of antibody molecules attached to each NS particle was estimated using the bicinchoninic acid (BCA) total protein assay according to the manufacturer's protocol (Micro BCA Protein Assay Kit; Thermo Scientific, Rockford, IL). In this procedure, we prepared standards containing the C225 antibody (concentration range = 0.5–20 µg/mL) and unknowns (C225-SPIO@Au NS and PEG-SPIO@Au NS). These standards and unknowns were then reacted with Ca^{2+} followed by BCA, which produces strong absorbance at 562 nm. The absorbance values were measured at 562 nm with a Spectramax microplate spectrophotometer (Molecular Devices Corp., Sunnyvale, CA). The absorbance of antibody-coated SPIO@AuNS at 562 nm in the BCA assay was subtracted from the absorbance measured for plain SPIO@AuNS of an equal particle concentration to correct for the SPIO@Au NS background. The amount of antibody present was extrapolated from the linear curve from the C225 standards.

2.7. Cell culture and in vitro cell binding

Head and neck cancer cell lines overexpressing EGFR (HN5, FaDu, and OSC-19) were kindly provided by Dr. Jeffrey Myers of The University of Texas MD Anderson Cancer Center. Human squamous carcinoma A431 cells, which also overexpress EGFR, were obtained from American Type Culture Collection (Manassas, VA). All the cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 in Dulbecco's modified Eagle's medium and nutrient mixture F-12 Ham (DMEM/F12) containing 10% fetal bovine serum (Invitrogen, Grand Island, NY).

For microscopic imaging to assess *in vitro* binding, HN5, FaDu, OSC-19, and A431 cells were seeded onto an 8-well Lab-Tek II Chamber Slide (10,000 cells/well) (Thermo Scientific, Rochester, NY). After 24 h, the cells were washed three times with Hank's balanced salt solution (HBSS) and incubated with C225-SPIO@AuNS (100 µL, 1×10^{11} particles/mL), PEG-SPIO@Au NS (100 µL, 1×10^{11} particles/mL), or C225-SPIO@Au NS plus C225 (500 µg/mL) at 37 °C for 1 h. Thereafter, the cells were washed three times with HBSS and fixed with 70% ethanol for 30 min, and the cell nuclei were stained with 4',6'-diamidino-2-phenylindole (DAPI) for 5 min. Cells were then washed three times with PBS, mounted, and examined under a Zeiss Axio Observer.Z1 fluorescence microscope (Carl Zeiss MicroImaging GmbH, Göttingen, Germany). Gold nanoshells were examined under a dark-field condenser illuminated by a halogen light source, and the fluorescence of the cell nuclei was detected with a Chroma DAPI filter (Chroma Technology Corp., Bellows Falls, VT) illuminated by a Xenon XBO light source (OSRAM GmbH, Augsburg, Germany). The images were collected using a Hamamatsu B/W chilled charge-coupled camera (Hamamatsu Photonics K. K., Hamamatsu City, Japan) and processed by Image-Pro Plus 4.5.1 software (Media Cybernetics, Inc., Bethesda, MD). With the dark-field condenser, the microscope is sensitive only to scattered light, resulting in light-scattering images.

For MRI assessment of *in vitro* cell binding, A431 cells (1×10^6) were seeded in 6-well plates and allowed to incubate with C225-SPIO@AuNS (2 mL, 1×10^{11} particles/mL), PEG-SPIO@Au NS (2 mL, 1×10^{11} particles/mL), or C225 + C225-SPIO@AuNS (2 mL, 1×10^{11} particles/mL) for 2 h at 4 °C. After 2 h, the cells were washed with PBS to remove unbound nanoshells. Subsequently, the cells were scraped from the bottom of the petri dish, dispersed in PBS (500 mL), and centrifuged at 1,100 rpm for 5 min. The supernatant was then removed, and the pellet containing the cells with nanoshells were resuspended in 0.5% agarose gel (volume total = 500 µL) and was placed inside a 5-mm nuclear magnetic

resonance tube (NMR, height of the tube = 3 cm). The samples were allowed to solidify inside the refrigerator and were directly used for MRI.

2.8. MRI

Solutions of C225-SPIO@Au NS and PEG-SPIO@Au NS were prepared in water at iron concentrations of 0.008, 0.015, 0.03, 0.06, and 0.12 mM Fe for relaxivity measurements. All measurements were made at room temperature with a 1.5T MRI clinical scanner (Excite HDxt; GE Healthcare Technologies, Waukesha, WI) and a 7T small animal MRI system (Biospec; Bruker Biospin Corp., Billerica, MA). T2 relaxivity at 1.5T used a spin-echo acquisition utilizing ten echo-times (TE) ranging from 15 ms – 400 ms. Other scan parameters were: repetition time (TR) = 4,000 ms, field of view = 7 cm, slice thickness = 3 mm, and acquisition matrix = 256×128 . At 7T, a fast-spin-echo sequence was used with 24 TE values ranging from 15 ms to 360 ms. Other scan parameters were: TR = 1000 ms, field of view = 3.2 cm, slice thickness = 1 mm, and acquisition matrix = 64×64 . T2 values were calculated as the slopes from a linear least-square line fit to the log of the mean measured MR signal in a region of interest versus TE. Similarly, the associated relaxivities (R_2 in $\text{mM}^{-1}\text{s}^{-1}$) were obtained from a linear least-square determination of the slopes of $1/T_2$ versus [Fe]. These calculations were performed using MATLAB software (MathWorks, Natick, NJ).

For *in vitro* cell binding measurements using MRI, 5 mm NMR tubes cut in 3-cm lengths containing cells in 0.5% agarose were imaged by 7T MRI using a three-dimensional fast spin echo acquisition with TR/TE = 5000 ms/65 ms, number of signal averages = 3, field of view = 3.5 cm $\times$ 3.0 cm, and acquisition matrix of 256×192 . Quantification of the change in T2-weighted signal was performed by inverting the grayscale and measuring the mean signal in a region of interest using Image J software (available at <http://rsb.info.nih.gov/ij>; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD). The normalized signal change in T2-weighted MR images (S_{T2-w}) was calculated by subtracting the signal intensity of C225-SPIO@AuNS, PEG-SPIO@AuNS, and C225+ C225-SPIO@AuNS by the signal intensity of the cells alone and then dividing the result by the signal intensity of C225-SPIO@Au NS without the cells and multiplying by 100. Differences between groups were considered statistically significant for $p < 0.05$ using a two-tailed Student's *t* test.

2.9. Photothermal effect in SPIO@AuNS conjugates

The laser used 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. The laser was powered by a DH 1715A-5 dual-regulated power supply (DHC, China Daheng Group). A 5-m-long, 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 adjusting the distance from the output to the target. The output power was independently calibrated using a handheld optical power meter (Newport model 840-C) and was found to be 1 W for a spot diameter of 3.5 mm ($\sim 8 \text{ W/cm}^2$) and a 2-A supply current. The end of the optical fiber was attached to a retort stand by a movable clamp and then positioned directly above the sample cell. For measuring temperature change mediated by C225-SPIO@AuNS and PEG-SPIO@Au NS, NIR laser light (808 nm) was delivered through a quartz cuvette containing the nanoshells (100 μL). A thermocouple was inserted into the solution perpendicular to the path of the laser light. The temperature was measured over a period of 20 min. Water was used as a control.

2.10. In vitro photothermal ablation of tumor cells

A431 cells were seeded onto a 96-well plate at a density of 10,000/well, 1 day before the irradiation experiment. Cells were washed three times with HBSS buffer. The following treatments were used: no treatment, NIR laser alone, C225-SPIO@AuNS (100 μ L, 1×10^{11} particles/mL) alone, PEG-SPIO@Au NS (100 μ L, 1×10^{11} particles/mL) alone, PEG-SPIO@Au NS (100 μ L, 1×10^{11} particles/mL) plus NIR laser, C225 + C225-SPIO@AuNS (100 μ L, 1×10^{11} particles/mL) plus laser, and C225-SPIO@AuNS (100 μ L, 1×10^{11} particles/mL) + laser. For treatment with SPIO@AuNS, cells were incubated with C225-SPIO@AuNS, C225 + C225-SPIO@Au NS, or PEG-SPIO@Au NS (100 μ L, 1×10^{11} particles/mL) at 37 °C for 1 h. Thereafter, cells were washed three times with HBSS to remove any unbound nanoshells. Cells were then resupplied with DMEM/F12 medium containing 10% fetal bovine serum. Cells were irradiated with NIR laser light centered at 808 nm at an output power of 36 W/cm² for 3 min (15PLUS laser; Diomed, Andover, MA) and then incubated at 37 °C for 24 h. The diode laser was coupled to a 1-m-long, 2-mm-core fiber, which delivered a circular laser beam 2 mm in diameter, covering the central area of the microplate well. Power calibration was performed automatically.

Twenty-four hours after laser treatment, cells were washed three times with HBSS and stained with calcein AM (Invitrogen, CA) for visualization of live cells. Cells were examined using the Zeiss fluorescence microscope equipped with FITC filter sets specific for calcein.

2.11. Radiolabeling

Aliquots of DTPA-C225-SPIO@AuNS or DTPA-PEG-SPIO@Au NS (1×10^{13} particles/mL, 0.2 mL) in 0.1 M sodium acetate solution (pH, 5.5) were mixed with an aqueous solution of ¹¹¹InCl₃ (~200 μ Ci) for 30 min. The radiolabeled SPIO@AuNS was then purified by centrifugation at 4,000 rpm for 5 min and then washed three times with PBS. Radiolabeled nanoshells were analyzed using instant thin-layer chromatography (ITLC). The ITLC strips were developed with PBS (pH, 7.4) containing 4 mM EDTA and then quantified using a TLC Imaging Scanner (Bioscan, Washington, DC). Free ¹¹¹In³⁺ moved to the solvent front (R_f = 0.9), and the nanoparticles remained at the original spot (R_f = 0.0).

2.12. Biodistribution

All experiments involving animals were performed in accordance with the guidelines of the institutional animal care and use committee. Solid A431 tumors were grown subcutaneously in the right thigh of nude mice (20–25 g; Harlan Sprague Dawley, Indianapolis, IN) by injecting 1×10^6 viable tumor cells suspended in PBS. When tumors had grown to 6–8 mm in average diameter (~2 weeks), the mice were randomly allocated into two groups (four mice per group). Mice in group 1 were injected with ¹¹¹In-DTPA-C225-SPIO@Au NS, and mice in group 2 were injected with ¹¹¹In-DTPA-PEG-SPIO@AuNS (both at a dose of 1×10^{13} particles/mL, 10 μ Ci/mouse in 0.20 mL). Mice were killed by carbon dioxide exposure and cervical dislocation 24 h after injection. Blood, heart, liver, spleen, kidney, muscle, and tumor tissues were removed and weighed, and radioactivity was measured with a Cobra gamma counter (Packard, Downers Grove, IL). Uptakes of nanoshells in various organs were calculated as the percentage of the injected dose per gram of tissue (%ID/g).

2.13. Statistical analysis

Differences in biodistribution data were analyzed using a two-tailed unpaired Student's *t* test, with *p* < 0.05 considered statistically significant.

3. Results and discussion

3.1. Synthesis and characterization of C225-SPIO@AuNS conjugates

Core-shell magnetic nanomaterials composed of iron oxide coated with a layer of gold are unique because the gold not only reduces the toxicity of the iron oxide core, but also because of their potential theranostic (therapy and diagnostic) applications. Although many core-shell magnetic nanoparticles have been synthesized and have shown their potential as theranostic agents, the delivery of these agents into the target tumor tissues, remains a great challenge [8]. To increase the effectiveness of these magnetic gold-based nanostructures, one must carefully control their physicochemical properties including size, shape, morphology, charge, and surface chemistry. In the above study, an SPIO@Au NS was synthesized, having an average diameter of the SPIO@Au NS and the thickness of the Au shell of approximately 82.4 ± 4.4 nm and 7.8 ± 2.2 nm, respectively, as measured from TEM images. This size is desirable for evading the reticuloendothelial system [15].

Another strategy for increased accumulation in the tumor is through active targeting. For example, OSCC overexpresses EGFR [7], and therefore attaching an anti-EGFR monoclonal antibody C225 (cetuximab) into the magnetic gold-shelled nanoparticles increases their delivery to and residence within the tumor. In our study, C225-conjugated SPIO@AuNS was synthesized (C225-SPIO@Au NS) to target EGFR's overexpressed in head and neck cancers. The schematic diagram of C225-SPIO@AuNS is shown in Fig. 1A. The particles have a SPIO core followed by a layer of silica that is coated with gold on the surface. The C225 monoclonal antibody is then covalently conjugated to the gold surface through -SH groups introduced to the antibody. Since the surface of SPIO@AuNS is gold, a similar protocol for the attachment of the C225 antibody to hollow gold nanoshells was adapted [13]. In this procedure, C225 monoclonal antibody was first functionalized with the activated ester SATA to introduce SH-protected S-acetylthioacetate groups (C225-ATA). It was then deprotected using hydroxylamine to expose the -SH moiety, which would readily attach to the surface of the gold to give C225-SPIO@AuNS. PEG-SPIO@Au NS was prepared by readily attaching PEG-SH into a suspension of SPIO@Au NS.

For biodistribution studies, attachment of the chelator, DTPA, is an additional required step. The radiometal chelator DTPA was subsequently conjugated to C225-ATA through an isothiocyanate-mediated coupling reaction. The DTPA-containing antibody was finally conjugated to the gold surface of SPIO@AuNS through the S-Au bond after free SH in the antibody was released by treating DTPA-antibody-ATA with hydroxylamine (similar to the previous procedure). Once the C225 was attached to the SPIO@AuNS, complete coverage of the gold surface onto the SPIO@AuNS was ensured by treating the antibody-coated SPIO@Au NS with an excess of PEG-SH.

High-resolution TEM images equipped with an energy dispersive x-ray spectrophotometer showed and confirmed the presence of gold, iron, and silica (Fig. 1B) in the synthesized SPIO@AuNS. Antibody-conjugated SPIO@AuNS exhibited excellent colloidal stability, as no apparent aggregation was observed when the nanoshells were stored in PBS buffer at 4 °C over a period of 2 weeks. The SPIO@Au NS concentration was calculated from the volume of each NS and from the gold concentration, which was determined by ICP-MS. Each nanoshell particle was estimated to be covered by approximately 142 ± 15 antibody molecules, as determined by the BCA protein assay.

3.2. Optical and heating properties of SPIO@Au NS conjugates

Gold nanostructures are ideally suited as photothermal coupling agents. In addition to enhanced absorption, cross-sections of the gold nanostructures that ensure effective laser therapy at relatively low laser output energies, the surface plasmon resonance (SPR)

absorption band of the core-shell-structured nanoparticles is readily tunable in the NIR region, which makes it possible to deliver thermal doses to deeper tissues.

SPIO@AuNS showed increased absorption in the NIR region (Fig. 2A), and the absorbance spectra in the extinction maximum ($\lambda_{\max}$) showed that antibody conjugation made no difference in the absorption profile of SPIO@Au NS (Fig. 2A). This absorption in the NIR regions was efficiently converted into heat, as shown in Fig. 2B. The heating properties of the conjugates were also determined by treating solutions of C225-SPIO@Au NS, PEG-SPIO@Au NS, and saline with a 808-nm laser. Results showed that the temperature rose to 65 °C and that there was no apparent difference in the heating profile with and without antibody on the surface of the SPIO@AuNS. The temperature of the solution dropped immediately after the NIR laser was turned off. Deionized water, which was used as a control, did not have any heating when treated with laser.

3.3. Magnetic properties of SPIO@AuNS conjugates

Fig. 3A shows the room-temperature magnetic hysteresis $M(H)$ loops for the C225-coated and uncoated SPIO@Au NS, measured in fields between -50 kOe and 50 kOe. The insets show these hysteresis loops in the zoomed-in region between $H = -800$ Oe and 800 Oe to check the irreversibility in the low-field region. The $M(H)$ loops for both the C225-coated and uncoated SPIO@AuNS were almost completely reversible, except for an extremely small irreversibility at very low fields (within ± 200 Oe), indicating that both samples exhibited superparamagnetic characteristics at room temperature (300 K). The coercivity (H_c) of the $M(H)$ loop, defined as the magnetic field at which the magnetization is zero, was around 25 Oe and 27 Oe for the C225-conjugated and -unconjugated nanoshells, respectively. Such a small value of coercivity is very common for γ -Fe₂O nanoparticles with sizes ranging between 5 and 100 nm.

Usually, for magnetic nanoparticles with sizes ranging from 5 to 30 nm, H_c increases with particle size and with a decrease in temperature. This phenomenon can be explained by the theory that the increase in particle size can lead to an increase in blocking temperature, which has been confirmed by many published studies. The very small H_c value suggests that our nanoparticles are almost perfectly superparamagnetic [16].

MRI measures the changes in magnetization of hydrogen protons in water molecules in a magnetic field after a pulse of radiofrequency has hit them. Protons from different chemical environment relax at different rates (relaxation time), resulting in clearly defined anatomical images. In clinical settings, a contrast agent is often used to differentiate between normal and abnormal tissues. Signal intensity arises largely from the local values of the longitudinal relaxation rate of water protons, $1/T_1$, and the transverse rate, $1/T_2$. Pulse sequences that emphasize changes in $1/T_2$ are referred to as T_2 -weighted, the signal of which decreases with increasing $1/T_2$. Iron oxide nanoparticles are used to enhance T_2 -weighted images, owing to their large transverse relaxivity values (r_2 refers to the amount of increase in $1/T_2$ per millimole of contrast agent). To date, a wide variety of particles have been produced, differing in size (hydrodynamic particle sizes vary from 10 to 500 nm) and the type of coating material used (such as dextran, starch, albumin, silicones, and PEG) [17].

Several gold-shelled iron oxide nanoparticles showed favorable transverse relaxivity [12, 18, 19]. We previously reported that SPIO@AuNS exhibited high transverse relaxivity, r_2 , and a large r_2/r_1 ratio and therefore could be imaged by MRI to obtain T_2 -weighted images [19]. In Fig. 3B we compared the relaxivity values of C225-SPIO@Au NS and Feridex to the bare SPIO@Au NS using the clinical 1.5 T and experimental 7.0 T MRI systems. It can be seen in this table that with and without the antibody coating, the relaxivity values taken from both clinical (1.5 T) and experimental (7.0 T) MRI scanners are comparable. Feridex,

which is a clinically approved T2-weighted MRI contrast agent, has slightly lower relaxivity values than either of the SPIO@AuNS. These data suggest that dual-function SPIO@AuNS may be used in theranostic applications, in which the distribution of the nanoparticles depicted by MRI may be used to assist planning of photothermal therapy and to predict the treatment outcome.

3.4. In vitro binding assessed using light scattering and MRI

When gold nanoparticles are stimulated with light, the conduction band electrons oscillate coherently on the particles' surface, creating SPR. Light scattering occurs as energy from the light stimulates the electrons on the particles' surface to oscillate and subsequently emit photons at a single frequency. Under dark-field microscopy, this scattering of light may be captured and used to determine the presence of the gold nanoparticle *in vitro*. Fig. 4A shows the light-scattering images of the different EGFR-positive cells (FaDu, HN5, OSC19, and A431) incubated with C225-SPIO@AuNS, PEG-SPIO@AuNS, and C225-SPIO@AuNS plus a large excess of C225 (blocking). Only C225-SPIO@AuNS, which specifically targets EGFR, exhibited a strong signal in extranuclear areas in the cells. Few spots were seen in cells treated with PEG-SPIO@AuNS, indicating there was less uptake of PEG-SPIO@AuNS in the cells. Moreover, uptake of C225-SPIO@AuNS in those cells was efficiently blocked by the anti-EGFR antibody C225 (Fig. 4A, bottom).

Because SPIO@AuNS is multifunctional, we are able to evaluate the selective binding of SPIO@AuNS through the scattering of gold and also use MRI to detect the SPIO. In a separate experiment, binding to A431 cells was also evaluated using the experimental 7T MR scanner. Fig. 4B shows the T2-weighted MR image of the cells after being inoculated with the conjugates and suspended in 0.5% agar solution, C225-SPIO@AuNS, PEG-SPIO@AuNS, or C225 + C225-SPIO@AuNS. Cells incubated with C225-SPIO@AuNS (Fig. 4B-4) were substantially darker than those incubated with the nontargeting control, PEG-SPIO@AuNS (Fig. 4B-3), and the blocking group (C225 + C225-SPIO@AuNS, Fig. 4B-2). The decrease in signal intensity was quantified using Image J software and was then compared with the signal intensity of C225-SPIO@AuNS without cells (Fig. 4B-5), which was taken as 100%. A statistically significant decrease in binding was observed when C225-SPIO@AuNS was compared with PEG-SPIO@AuNS and C225 + C225-SPIO@AuNS (Fig. 4C).

3.5. In vivo biodistribution

To enable quantitative analysis, we labeled SPIO@AuNS conjugates with the gamma emitter ^{111}In , which has a desirable physical half-life ($t_{1/2} = 67.3$ h). Radiolabeling of the nanoparticles was accomplished by incubating $^{111}\text{InCl}_3$ with DTPA-SPIO@AuNS conjugates in sodium acetate buffer (pH, 5.5) at room temperature. Unlabeled ^{111}In and ^{111}In -DTPA-antibody were removed by centrifugation and washing steps. The radiochemical purities for both ^{111}In -DTPA-C225-SPIO@AuNS and ^{111}In -DTPA-PEG-SPIO@AuNS were greater than 95%.

The biodistribution of ^{111}In -labeled antibody-SPIO@AuNS at 24 h after injection, as determined by *ex vivo* measurements of radioactivity, is presented in Fig. 5A. The organs that had the highest uptakes of ^{111}In -labeled C225-SPIO@AuNS, PEG-SPIO@AuNS, and C225 + C225-SPIO@AuNS were the liver (46.56 ± 5.95 %ID/g vs. 54.78 ± 28.83 %ID/g vs. 59.88 ± 35.15 %ID/g), the spleen (25.88 ± 7.89 %ID/g vs. 30.13 ± 14.68 %ID/g vs. 29.85 ± 17.97 %ID/g), and the kidney (9.52 ± 1.29 %ID/g vs. 9.22 ± 5.10 %ID/g vs. 7.59 ± 4.99 %ID/g). ^{111}In -labeled C225-SPIO@AuNS had a significantly higher uptake value (2.91 ± 0.71 %ID/g) in the tumor than did ^{111}In -labeled PEG-SPIO@AuNS (0.83 ± 0.13 %ID/g) or

the blocking group, C225 + C225-SPIO@AuNS (0.78 ± 0.37 %ID/g). These differences were statistically significant ($p < 0.001$) (Fig. 5B).

3.6. Photothermal therapy

In addition to EGFR-specific targeting, as shown in the light-scattering and MR images, exposure of cells incubated with SPIO@Au NS conjugates to a 36 W/cm^2 laser caused selective photothermal ablation. Cells incubated with C225-SPIO@Au NS and PEG-SPIO@AuNS without laser treatment showed no cell death (Fig. 6, top). For cells incubated with SPIO@Au NS conjugates and treated with the laser, only the targeting agent, C225-SPIO@AuNS, caused cell death lysis; none of the other groups (irradiation alone, PEG-SPIO@Au NS, and C225 + C225-SPIO@AuNS) showed observable damage to the cancer cells (Fig. 6, bottom).

Selective targeting with thermal ablation of OSCC using gold nanospheres and nanorods has been shown *in vitro* and *in vivo* [20]. Kim et al. [18] synthesized magnetic core-shell gold-shelled nanoparticles that were targeted to human epidermal growth factor receptor 2, which is overexpressed in some breast cancers. However, selective targeting using MR-active gold-based nanoparticles targeted to OSCC has not been shown, and to best of our knowledge, ours is the first study to demonstrate selective laser ablation of head and neck cancer.

4. Conclusion

We believe that this image-guided approach to laser ablation using targeted nanoparticles to mediate heating may offer a promising alternative to conventional therapeutic strategies for patients with head and neck cancer. This targeted delivery of AuNS to tumor cells and tumor stroma should increase the efficacy of thermal ablation and reduce thermal damage to surrounding normal tissues. Also, the use of MRI-visible SPIO@AuNS should make it possible to estimate the spatiotemporal heat profile before NIR laser irradiation for more robust treatment planning, which will potentially improve both the safety and efficacy of therapy delivery. These are important steps that will lay the groundwork for clinical trials of MR-guided thermal ablation of head and neck cancer mediated with magnetic gold nanoshells.

Acknowledgments

We thank Kristi M. Speights, ELS, for editing our manuscript. This work was supported in part by a grant from the National Institutes of Health ([C.L.] R01 CA119387), SPOR Head and Neck Career Development Award (to M.P.M., P50CA097007), and an Odyssey Fellowship (to M.P.M.). The Odyssey Fellowship is supported by the Odyssey Program and the Cockrell Foundation for Scientific Achievement at The University of Texas MD Anderson Cancer Center. This research was also supported in part by the National Institutes of Health through MD Anderson's Cancer Center Support Grant (CA016672) for the support of our small animal facility and small animal imaging facility.

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Figure 1.
 (A) Structure and (B) high-resolution TEM images of C225-SPIO@Au NS showing the morphology and composition. Bar, 20 nm

Figure 2.

(A) Absorption spectra of SPIO@Au NS and C225-SPIO@Au NS showing the plasma resonance peak tuned to the NIR region. (B) Heating of aqueous SPIO@Au NS, C225-SPIO@Au NS and deionized water exposed to NIR light centered at 808 nm at 8 W/cm². Temperature was monitored during (12 min duration) and 5 min after laser treatment.

Figure 3.

(A) Magnetization of PEG-SPIO@Au NS and C225-SPIO@Au NS as a function of applied magnetic field of nanocomplexes at 300 K where the external magnetic field is cycled between -5000 and $+70$ Oe (F). (B) Relaxivity values of SPIO@Au NS, C225-SPIO@Au NS, and Feridex measured using the clinical 1.5T and experimental 7.0T MRI scanners.

Figure 4.

(A) Selective binding of anti-EGFR-conjugated SPIO@Au NS to EGFR-positive cells. Oral squamous carcinoma cell lines, FaDu, HN5, and OSC19 along with A431 cells were seeded onto a 96-well plate and incubated with C225-SPIO@Au NS (100 μ L, 1.0×10^{11} particles/mL), PEG-SPIO@Au NS (100 μ L, 1.0×10^{11} particles/mL), or C225 (500 μ g/mL) plus C225-SPIO@Au NS for 30 min at 37 $^{\circ}$ C. Only cells incubated with C225-SPIO@Au NS had a strong light-scattering signal with the EGFR-positive cell lines. Cells were stained with DAPI for visualization of the cell nuclei (blue). Light-scattering images of nanoshells were pseudocolored green. Bar, 20 μ m. (B) T2-weighted MRI of EGFR-positive A431 cells suspended in 0.5% agarose after being incubated with [1] medium (cells alone), [2] C225 (500 μ g/mL) plus C225-SPIO@Au NS, [3] PEG-SPIO@Au NS (100 μ L, 1.0×10^{10}

particles/mL), [4] C225-SPIO@Au NS (100 μ L, 1.0×10^{10} particles/mL), or [5] C225-SPIO@Au NS at 1.0×10^{11} particles/mL without cells was also imaged and taken as 100% uptake. (C) Quantification of the decrease in signal intensity after binding with A431 cells was done using Image J software. Calculation of the normalized signal change in T2-weighted MR images (S_{T2-w}) was done by inverting the image and then subtracting the intensity of the cells with the conjugates to the cells alone, divided by the signal intensity of C225-SPIO@Au NS without the cells multiplied by 100. *Denotes statistically significant difference between the groups ($p < 0.001$).

Figure 5.

(A) Biodistribution of ^{111}In -labeled DTPA-C225-SPIO@Au NS and DTPA-SPIO@Au NS (both at a dose of 1×10^{13} particles/mL, $10 \mu\text{Ci}/\text{mouse}$ in 0.20 mL). Highest uptakes were at the liver, spleen and kidney. (B) Tumor uptake was highest with ^{111}In -DTPA-C225-SPIO@Au NS than with ^{111}In -DTPA-PEG-SPIO@Au NS and C225 + C225-SPIO@Au NS (blocking group). The difference is statistically significant ($p < 0.001$). Mean $\pm$ SD %ID/g ($n = 5$).

Figure 6.

Cell viability after various treatments. Cells retained normal morphology with no apparent death observed (stained green with calcein CM) when cells were treated with (A) medium, (B) C225-SPIO@Au NS alone, (C) PEG-SPIO@Au NS alone, (D) blocking group (C225 + C225-SPIO@Au NS) with the laser, (E) PEG-SPIO@Au NS with NIR laser, and (F) NIR laser alone. In contrast, most cells were lysed after treatment with (G) C225-SPIO@Au NS plus NIR laser. Magnification, $\times 40$. Laser Power = 36 W/cm^2 for 3 min. L is where the laser was positioned.