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 a 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 (r2) of 193 and 353 mM−1 s−1 versus Feridex™ of 171 and 300 mM−1 s−1, using 1.5 T and 7 T MR scanners, respectively. Specific targeting of the synthesized C225-SPIO@Au NS was tested in vitro using A431 cells and oral cancer cells, FaDu, OSC19, and HNS, 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/cm2 for 3 min, 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.

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.

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(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].

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-sulfhydryl (PEG-SH, molecular weight [MW] 5000), N-succinimidyl 3-acetylthioacetate (SATA), and phosphate-buffered saline (PBS; pH 7.4) were purchased from Sigma-Aldrich (St. Louis, MO). p-Isothiocyanatoethyl-dithiethylenetriaminepentacetic acid (p-SCN-Bz-DTPA) was obtained from Macrocyclics (Dallas, TX). PD-10 columns were purchased from GE Healthcare (Piscataway, NJ). Trisodium citrate dihydrate (obtained from Macrocyclics (Dallas, TX). PD-10 columns were purchased from GE Healthcare (Piscataway, NJ). Trisodium citrate dihydrate (obtained from Macrocyclics (Dallas, TX). PD-10 columns were purchased from GE Healthcare (Piscataway, NJ).

2.2. Synthesis of SPIO@Au NS

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 overlay to form an Au NS. Thiolated PEG-SH was attached to the surface of the SPIO@Au NS by adding 5 μM of PEG-SH (final concentration) to 1 × 10⁸ particles/mL in deionized water. After 2 h, the solution was centrifuged for 5 min at 8000 rpm and washed three times with deionized water. The resulting solution was resuspended in PBS (0.1 M).

2.3. Synthesis of C225-SPIO@Au NS

C225-SPIO@Au NS was synthesized according to previously published procedures [13,14]. Briefly, an aqueous solution of C225 (2.5 mg, 0.017 μmol; 5 mg/mL) was first allowed to react with SATA (0.077 mg, 0.332 μmol) 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 colour (BioRad Laboratories, Hercules, CA) as an indicator to guide the collection of antibody-containing fractions. C225-ATA was treated with hydroxylamine (50 mM, 50 μL) 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@Au NS (1 × 10⁸ particles/mL) to a final antibody concentration of 5 μg/mL. The suspension was then stirred for 1 h at room temperature. Thereafter, PEG-SH was added to the antibody-coated SPIO@Au NS 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@Au NS were centrifuged at 8000 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 °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 μmol; 5 mg/mL) was first allowed to react with SATA (0.077 mg, 0.332 μmol) at room temperature for 1 h. The resulting conjugate, C225-ATA, was purified by passing it through a gel filtration PD-10 column, using protein-dye colour (BioRad Laboratories, Hercules, CA) 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 μmol) at 4 °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, diethylentriamine pentaacetic acid (DTPA)-thiooctaacetic acid (DTPA-TA) was conjugated on the surface of SPIO@Au NS. DTPA-TA was synthesized as described by Zhang et al. [15]. PEG-SH was then added to the DTPA-TA treated with SPIO@Au NS 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 (Radionabeling).

2.5. Characterization of nanoparticles

For the TEM study, bare (uncoated) SPIO@Au NS and C225-SPIO@Au NS 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@Au NS 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@Au NS solution was analyzed by inductively coupled plasma mass spectroscopic analysis (ICP-MS) (Gaithersburg 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 10⁻¹⁷ emu. For the magnetization measurement, both the C225-coated and bare SPIO@Au NS were in the form of dry powders. Gelatin cups were used as the containers for the powder samples.

2.6. Quantification of antibodies per SPIO@Au NS

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 Cu^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@Au NS at 562 nm in the BCA assay was subtracted from the absorbance measured for plain SPIO@Au NS of an equal particle concentration to correct for 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 (HNS, FaDu, and OSC19) 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), 100 unit/mL of penicillin and 100 mg/mL of streptomycin.

For microscopic imaging to assess in vitro binding, HNS, FaDu, OSC19, 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@Au NS (100 μL, 1 × 10^11 particles/mL) and PEG-SPIO@Au NS (100 μL, 1 × 10^11 particles/mL) or C225-SPIO@Au NS plus C225 (250 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 Axios Observer.Z1 fluorescence microscope (Carl Zeiss Micromaging GmbH, Göttingen, Germany). Gold nanoshells were examined under a dark-field condenser illuminated with a halogen light source, and the fluorescence of the cell nuclei was detected with a Chroma DAPI filter (Chromato Technology Corp., Bellows Falls, VT) illuminated by a Xenon XBO light source (OSSRAM 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 with 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 onto 6-well plates and allowed to incubate with C225-SPIO@Au NS (2 mL, 1 × 10^11 particles/mL) or PEG-SPIO@Au NS (2 mL, 1 × 10^11 particles/mL) or C225-SPIO@Au NS plus C225 (100 μg/mL) at 37 °C for 4 h and then washed three times 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 1100 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 placed in a 5-mm gel tube. After curing under a UV lamp (NMR, brunette 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 relaxation measurements. All measurements were made at room temperature with a 1.5 T MRI clinical scanner (Excite HDxt; GE Healthcare Technologies, Waukesha, WI) and a 7 T small animal MRI system (BioSpec; Bruker Biospin Corp., Billerica, MA). T2 relaxation at 1.5 T used a spin-echo acquisition utilizing ten-echo-times (TE) ranging from 15 ms to 400 ms. Other scan parameters were: repetition time (TR) = 4000 ms, field of view = 7 cm, slice thickness = 3 mm, and acquisition matrix = 256 × 128. At 7 T, 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 (R2 in mm^2 s^−1) were obtained from a linear least-square determination of the slopes of 1/T2 versus [Fe]. These calculations were performed using MATLAB software (MathWorks, Natick, MA).

For in vitro cell binding measurements using MRI, 5 μL NMR tubes cut in 3-cm lengths containing cells in 0.5% agarose were imaged by 7 T MRI using a three-dimensional fast spin echo acquisition with TR/TE = 5000/65 ms, number of signal averages = 3, field of view = 3.5 cm × 3.0 cm, and acquisition matrix = 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 (S225/431) was calculated by subtracting the signal intensity of C225-SPIO@Au NS, PEG-SPIO@Au NS, and C225 + SPIO@Au NS 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.
3. Results and discussion

3.1. Synthesis and characterization of C225-SPIO@Au NS 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@Au NS was synthesized (C225-SPIO@Au NS) to target EGFR's overexpressed in head and neck cancers. The schematic diagram of C225-SPIO@Au NS 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@Au NS 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@Au NS. 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@Au NS 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@Au NS, complete coverage of the gold surface onto the SPIO@Au NS 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@Au NS. Antibody-conjugated SPIO@Au NS 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.

Fig. 1. (A) Structure and (B) high-resolution TEM images of C225-SPIO@Au NS showing the morphology and composition. Bar, 20 nm.
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@Au NS showed increased absorption in the NIR region (Fig. 2A), and the absorbance spectra in the extinction maximum ($\lambda_{\text{max}}$) 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@Au NS. 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@Au NS 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@Au NS were almost completely reversible, except for an extremely small irreversibility at very low fields (within $\pm200$ 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

![Graph A](image1.png)

![Graph B](image2.png)

Fig. 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 1.5 T and experimental 7.0 T MRI scanners.

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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-Fe₂O₃ 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 environments 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].

Fig. 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 µL, $1.0 \times 10^{11}$ particles/mL), PEG-SPIO@Au NS (100 µL, $1.0 \times 10^{10}$ particles/mL), or C225 (500 µg/mL) plus C225-SPIO@Au NS for 30 min at 37 °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 pseudocoloured green. Bar, 20 µm. (B) $T_2$-weighted MRI of EGFR-positive A431 cells suspended in 0.5% agarose after being incubated with [1] medium (cells alone), [2] C225 (500 µg/mL) plus C225-SPIO@Au NS, [3], PEG-SPIO@Au NS (100 µL, $1.0 \times 10^{10}$ particles/mL), [4] C225-SPIO@Au NS (100 µL, $1.0 \times 10^{11}$ particles/mL) or [5] C225-SPIO@Au NS at $1.0 \times 10^{10}$ 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 $T_2$-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 ($p < 0.001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Several gold-shelled iron oxide nanoparticles showed favorable transverse relaxivity [12,18,19]. We previously reported that SPIO@Au NS exhibited high transverse relaxivity, r₂, and a large r₂/r₁ ratio and therefore could be imaged by MRI to obtain T₂-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 systems (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@Au NS. These data suggest that dual-function SPIO@Au NS 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@Au NS, PEG-SPIO@Au NS, and C225-SPIO@Au NS plus a large excess of EGFR, exhibited a strong signal in extranuclear areas in the cells. Few spots were seen in cells treated with PEG-SPIO@Au NS, indicating there was less uptake of PEG-SPIO@Au NS in the cells. Moreover, uptake of C225-SPIO@Au NS in those cells was efficiently blocked by the anti-EGFR antibody C225 (Fig. 4A, bottom).

Because SPIO@Au NS is multifunctional, we are able to evaluate the selective binding of SPIO@Au NS though 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 7 T 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@Au NS, PEG-SPIO@Au NS, or C225 + C225-SPIO@Au NS. Cells incubated with C225-SPIO@Au NS (Fig. 4B) were substantially darker than those incubated with the non-targeting control, PEG-SPIO@Au NS (Figs. 4B,3), and the blocking group (C225 + C225-SPIO@Au NS, Figs. 4B,2). The decrease in signal intensity was quantified using Image J software and was then compared with the signal intensity of C225-SPIO@Au NS without cells (Fig. 4B,Fig. 5), which was taken as 100%. A statistically significant decrease in binding was observed when C225-SPIO@Au NS was compared with PEG-SPIO@Au NS and C225 + C225-SPIO@Au NS (Fig. 4C).

3.5. In vivo biodistribution

To enable quantitative analysis, we labeled SPIO@Au NS conjugates with the gamma emitter ¹¹¹In, which has a desirable physical half-life (t₁/₂ = 67.3 h). Radiolabeling of the nanoparticles was accomplished by incubating ¹¹¹InCl₃ with DTPA-SPIO@Au NS conjugates in sodium acetate buffer (pH, 5.5) at room temperature. Unlabeled ¹¹¹In and ¹¹¹In-DTPA-antibody were removed by centrifugation and washing steps. The radiochemical purities for both

<table>
<thead>
<tr>
<th>A</th>
<th>C225-SPIO@AuNS (%ID/g)</th>
<th>PEG-SPIO@AuNS (%ID/g)</th>
<th>C225 + C225-SPIO@AuNS (%ID/g)</th>
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<tbody>
<tr>
<td>Blood</td>
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<tr>
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<td>0.10 ± 0.04</td>
<td>0.32 ± 0.19</td>
<td>0.62 ± 0.58</td>
</tr>
<tr>
<td>Tumor</td>
<td>2.91 ± 0.71</td>
<td>0.83 ± 0.13</td>
<td>0.78 ± 0.37</td>
</tr>
</tbody>
</table>

Fig. 5. (A) Biodistribution of ¹¹¹In-labeled DTPA-C225-SPIO@Au NS and DTPA-PEG-SPIO@Au NS (both at a dose of 1 × 10¹⁵ particles/mL, 10 μCi/mouse in 0.20 mL). Highest uptakes were at the liver, spleen and kidney. (B) Tumor uptake was highest with ¹¹¹In-DTPA-C225-SPIO@Au NS than with ¹¹¹In-DTPA-PEG-SPIO@Au NS and C225 + C225-SPIO@Au NS (blocking group). The difference is statistically significant (p < 0.001). Mean ± SD %ID/g (n = 5).

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was positioned. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

$^{111}$In-DTPA-C225-SPIO@Au NS and $^{111}$In-DTPA-PEG-SPIO@Au NS were greater than 95%.

The biodistribution of $^{111}$In-labeled antibody-SPIO@Au NS 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@Au NS, PEG-SPIO@Au NS, and C225 + C225-SPIO@Au NS 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@Au NS had a significantly higher uptake value (2.91 ± 0.71 %ID/g) in the tumor than did $^{111}$In-labeled PEG-SPIO@Au NS (0.83 ± 0.13 %ID/g) or the blocking group, C225 + C225-SPIO@Au NS (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/cm² laser caused selective photothermal ablation. Cells incubated with C225-SPIO@Au NS and PEG-SPIO@Au NS 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@Au NS, caused cell death lysis; none of the other groups (irradiation alone, PEG-SPIO@Au NS, and C225 + C225-SPIO@Au NS) 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 Au NS 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@Au NS 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.

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References


