Published in final edited form as:

J Control Release. 2013 November 28; 172(1): . doi:10.1016/j.jconrel.2013.07.020.

In vitro and in vivo mapping of drug release after laser ablation thermal therapy with doxorubicin-loaded hollow gold nanoshells using fluorescence and photoacoustic imaging

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Abstract

Doxorubicin-loaded hollow nanoshells (Dox@PEG-HAuNS) increases the efficacy of photothermal ablation (PTA) by not only mediating efficient PTA but also through chemotherapy, and therefore have potential utility for local anticancer therapy. However, in vivo real-time monitoring of Dox release and temperature achieved during the laser ablation technique has not been previously demonstrated before. In this study, we used fluorescence optical imaging to map the release of Dox from Dox@PEG-HAuNS and photoacoustic imaging to monitor the tumor temperature achieved during near-infrared laser-induced photothermal heating *in vitro* and *in vivo*. *In vitro*, treatment with a 3-W laser was sufficient to initiate the release of Dox from Dox@PEG-HAuNS (1:3:1 wt/wt, 1.32×10^{12} particles/mL). Laser powers of 3 and 6 W achieved ablative temperatures of more than 50 °C. In 4T1 tumor-bearing nude mice that received intratumoral or intravenous injections of Dox@PEG-HAuNS, fluorescence optical imaging (emission wavelength = 600 nm, excitation wavelength = 500 nm) revealed that the fluorescence intensity in surface laser-treated tumors 24 h after treatment was significantly higher than that in untreated tumors ($p=0.015$ for intratumoral, $p=0.008$ for intravenous). Similar results were obtained using an interstitial laser to irradiate tumors following the intravenous injection of Dox@PEG-HAuNS ($p=0.002$ at $t=24h$). Photoacoustic imaging (acquisition wavelength = 800 nm) revealed that laser treatment caused a substantial increase in tumor temperature, from 37 °C to ablative temperatures of more than 50 °C. *Ex vivo* analysis revealed that the fluorescence intensity of laser-treated tumors was twice as high as that of untreated tumors ($p=0.009$). Histological analysis confirmed that intratumoral injection of Dox@PEG-HAuNS and laser treatment caused significantly more tumor necrosis compared to tumors that were not treated with laser ($p<0.001$). On the basis of these findings, we conclude that fluorescence optical imaging and photoacoustic imaging are promising approaches to assessing Dox release and monitoring temperature, respectively, after Dox@PEG-HAuNS-mediated thermal ablation therapy.

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Keywords

targeted hollow gold nanoshells; magnetic resonance temperature imaging; photoacoustic imaging; near-infrared optical imaging; molecular imaging

1. Introduction

Given its potential as a minimally invasive treatment, near-infrared (NIR) laser-induced photothermal ablation (PTA) has been of great interest in developing safer and more effective anticancer therapies [1–3]. Photothermal agents that have NIR absorbance properties are clinically preferred for PTA therapy because they have high tissue-penetrating ability and photothermal conversion efficiency [3–5]. Photothermal agents for PTA therapy include organic compounds [6, 7], carbon-based materials [5], copper sulfide [8, 9], and noble metal-based nanostructures [10–12]. Of this last group, poly(ethylene glycol)-coated hollow gold nanospheres (PEG-HAuNS), which are nontoxic and nonimmunogenic, can be used for NIR laser-induced PTA of tumors with minimal damage to surrounding normal tissue [12–16].

One way of increasing the antitumor efficacy of PEG-HAuNS is to load the nanospheres with doxorubicin (Dox), a commercially available chemotherapeutic agent that can be released upon NIR laser irradiation [17]. Dox@PEG-HAuNS is expected to passively accumulate within tumor tissue after systemic injection due to the enhanced permeability and retention effect [18–20]. Dox-loaded PEG-HAuNS (Dox@PEG-HAuNS) increase the antitumor efficacy of PTA by efficiently mediating the effects of the high temperatures induced by NIR laser and delivering chemotherapy directly to the tumor [14]. Because of the drug's inherent fluorescence properties, the release of Dox from Dox@PEG-HAuNS and its localization can be monitored using fluorescence optical imaging. To our knowledge, however, no study has evaluated the real-time monitoring of Dox release in an animal model.

In addition to the release and localization of Dox, the temperature of the region of interest within the tumor during NIR laser-induced PTA must be accurately monitored. In preclinical studies, a thermocouple probe is inserted into the region of interest to accurately measure temperature during NIR laser-induced PTA [21, 22]; in the clinic, magnetic resonance imaging (MRI) is used to monitor temperature during laser treatment. However, MRI is expensive and sensitive to motion. A potential alternative to MRI in this setting is photoacoustic imaging (PAI), an emerging non-invasive technique for quantifying acoustic signals generated after laser irradiation of tissue containing plasmonic materials such as gold [23]. The short laser pulse irradiation generates an acoustic wave with pressure amplitude that is directly proportional to the dimensionless Grueneisen parameter. The Grueneisen parameter is temperature dependent due to its dependence on the volume expansion coefficient and the speed of sound. Thus, both the Grueneisen parameter and photoacoustic signal are directly related to temperature [24, 25]. Because it can be used to visualize temperature distribution and nanoparticle contrast agents, PAI is a promising approach to monitoring both temperature during PTA and Dox release and localization [26–28]. However, no study has investigated the use of PAI to monitor temperature during PTA and Dox release for localization.

In the present study, we investigated the use of fluorescence optical imaging and PAI to monitor the release of Dox from Dox@PEG-HAuNS and the tumor temperature following NIR laser irradiation, under different Dox@PEG-HAuNS injection modes (intratumoral or

intravenous), and different NIR laser treatment modes (surface or interstitial). We performed histological analysis to evaluate the efficacy of the treatment.

2. Materials and methods

Dox was obtained from Ochem Incorporation (Des Plaines, IL). Methoxy-polyethylene glycol (PEG-SH, MW 5000), gold(III) chloride trihydrate, and phosphate-buffered saline (pH 7.4) were obtained from Sigma-Aldrich (St. Louis, MO). Sodium citrate dihydrate, trisodium citrate dihydrate (>99%), cobalt(II) chloride hexahydrate (99.99%), and sodium borohydride (99%) were obtained from Fisher Scientific (Pittsburgh, PA). American Chemical Society-grade methylene chloride was obtained from Baxter Healthcare Corp (Deerfield, IL).

Murine mammary carcinoma 4T1 cells were obtained from American Type Culture Collection (Manassas, VA) and maintained in Dulbecco's modified Eagle's medium and 10% fetal bovine serum at 37°C in a humidified 5% CO₂ atmosphere.

2.2. Synthesis of HAuNS, PEG-HAuNS, and Dox@PEG-HAuNS

HAuNS, PEG-HAuNS, and Dox@PEG-HAuNS (1:3:1 wt/wt, 1.32×10^{12} particles/mL) were synthesized as described previously. [17]. Briefly, we added 4.5 mL of sodium borohydride (1 M), 2.8 mL of sodium citrate (0.1 M), and 1.0 mL of cobalt chloride (0.4 M) to pre-deoxygenated deionized water with stirring to synthesize cobalt nanoparticles. We then added chlorauric acid to the suspension of cobalt nanoparticles formed *in situ*, which reduced the gold ions to gold nanoparticles on the surface of the cobalt nanoparticles and oxidized cobalt to cobalt(II) ions. Any remaining cobalt was oxidized by air, resulting in the final product, HAuNS. The size of the HAuNS was determined using dynamic light scattering at a 90° scatter angle on a particle size analyzer (Brookhaven Instruments, Holtsville, NY). The amount of gold in the HAuNS was quantified using inductively coupled plasma mass spectroscopy (Galbraith, Knoxville, TN) [29].

To conjugate PEG to the HAuNS, we added PEG-SH to the HAuNS suspension under argon protection at room temperature. Excess PEG-SH was removed by subjecting the suspension to centrifugation and washing the residue with 1.5 mM sodium citrate (pH 6.5–7) twice.

To load Dox onto the PEG-HAuNS, we slowly added Dox to the PEG-HAuNS suspension under stirring at room temperature. After the mixture was stirred for 24 h, excess Dox was removed by subjecting the mixture to centrifugation and washing the supernatant with sodium citrate (1.5 mM) repeatedly until the supernatant became colorless. The amount of excess Dox in the supernatant was determined by spectrophotometry at 480 nm.

2.3. Imaging

2.3.1. Fluorescence optical imaging—We used the IVIS Imaging System with filter sets (emission = 575–650 nm, excitation = 500–550 nm) to perform optical imaging studies. Scanning parameter include: excitation wavelength = 500 nm, emission wavelength = 600 nm, field of view = 12.5 cm, fluency rate = 2 mW/cm². The camera was set to maximum gain, a binning factor of 4, and a luminescent exposure time of 9 s.

2.3.2. Photoacoustic imaging—We used the Vevo LAZR-2100 PAUS imaging system (VisualSonics Inc., Toronto, Canada) to perform PAI. For laser irradiation for photoacoustic imaging, we coupled a 21-MHz, 24 mm-wide linear acoustic array transducer to the LAZR system. Mice were imaged at a laser wavelength of 800 nm.

To determine the relationship between measured photoacoustic signal amplitude and temperature change, we first constructed a PAI phantom by filling 3 plastic tubes with Dox@PEG-HAuNS samples (3.3×10^{10} particles/mL, 6.6×10^{10} particles/mL, and 3.3×10^{11} particles/mL), sealing the tube ends with epoxy, and suspending the tubes in a water-filled plastic tub. The phantom tubes consisted of silicone catheter with 0.5 mm inner diameter and 0.9 mm outer diameter. We aligned the 3 tubes so they would be at the same depth during imaging. The water in the phantom was heated from 21 °C to 70 °C, and the phantom was imaged at laser wavelengths of 680, 750, 808, 900, and 970 nm. We used the PAI system to monitor the photoacoustic signal and a Fluoroptic temperature probe coupled to an m3300 Fluoroptic thermometer (Luxtron) to monitor temperature. The increase in photoacoustic signal with respect to time was plotted, and the equation of the line was used to convert the photoacoustic signal to temperature.

2.4. Dox release from Dox@PEG-HAuNS

We used an 808-nm NIR laser (Diomed 15 Plus, Cambridge, UK) with varying output powers for all Dox release studies. All Dox release studies were performed at room temperature.

2.4.1. *In vitro* release studies—We used two approaches to analyze Dox release *in vitro*. In the first approach, we placed Dox@PEG-HAuNS (1 OD, 6.6×10^{10} particles/mL at 808 nm) in a 5-mm cuvette; irradiated them with an NIR laser (2-mm spot diameter) at an output power of 0 W (0 W/mm²), 1.5 W (0.48 W/mm²), or 3 W (0.95 W/mm²) for 1 min; and subjected them to spectrofluorometry (550–650 nm) immediately after irradiation with a Spex Fluorolog spectrofluorometer (Jobin Yvon, Inc., Edison, NJ) using 470 nm as the excitation wavelength. In the second approach, we placed Dox@PEG-HAuNS in solution into a 96-well plate (200 µL per well); irradiated them with an NIR laser (2-mm spot diameter) at an output power of 0 W (0 W/mm²), 1.5 W (0.48 W/mm²), 3 W (0.95 W/mm²), or 6 W (1.9 W/mm²) for 1 min; and subjected them to optical imaging immediately after irradiation with an IVIS Imaging System 200 Series (Xenogen Corp., Alameda, CA).

To determine the maximum temperatures different laser powers could achieve in identical concentrations of Dox@PEG-HAuNS, we placed 500 µL of Dox@PEG-HAuNS (5 OD, 3.3×10^{11} particles/mL) into a quartz cuvette and irradiated them with an NIR laser (2-mm spot diameter) at a power output of 0 W (0 W/mm²), 1.5 W (0.48 W/mm²), 3 W (0.95 W/mm²), or 6 W (1.9 W/mm²) for 1 min. We used a Fluoroptic temperature probe coupled to a m3300 Fluoroptic thermometer (Luxtron, Santa Clara, CA) to monitor temperature.

To determine the optimal Dox@PEG-HAuNS concentration for fluorescence imaging *in vivo*, we measured the fluorescence released by different Dox@PEG-HAuNS concentrations immediately after treatment with an NIR laser. We placed Dox@PEG-HAuNS at Dox concentrations ranging from 0.17 µg/mL to 350 µg/mL into a 96-well plate (200 µL per well) and irradiated them with an NIR laser (2-mm spot diameter) at an output power of 3 W (0.95 W/mm²). Non-laser-treated samples of Dox@PEG-HAuNS at Dox concentrations ranging from 0.17 µg/mL to 350 µg/mL served as controls.

2.4.2. *In vivo* release studies and *ex vivo* analysis—All animal studies were carried out in the Small Animal Imaging Facility at The University of Texas MD Anderson Cancer Center under Institutional Animal Care and Use Committee–approved protocols.

To create the murine tumor model, we injected breast 4T1 cells (5×10^5 cells, 100 µL subcutaneously into each flank of female Sprague-Dawley nude mice (Charles River Laboratories). Experiments were conducted after the tumors grew to 0.6–0.8 cm in diameter.

We assessed the release of Dox from intravenously injected Dox@PEG-HAuNS and from intratumorally injected Dox@PEG-HAuNS after surface laser treatment. We randomly divided 6 4T1 tumor-bearing nude mice into 2 groups of 3 mice each; one group received intravenous injections of Dox@PEG-HAuNS (3.3×10^{12} particles/mL, 200 μ L), and the other received intratumoral injections of Dox@PEG-HAuNS (1.32×10^{12} particles/mL, 50 μ L). We placed an NIR laser (5-mm spot diameter) over one tumor per mouse and irradiated the tumors at an output power of 3 W (0.15 W/mm^2) for 1 min; the unirradiated tumors were the controls. All tumors were optically imaged immediately after Dox@PEG-HAuNS injection ($t=0$ min), immediately after laser treatment ($t=5$ min), and 0.5 h, 1 h, 2 h, 4 h, and 24 h after laser treatment.

Because laser treatment is done interstitially in the clinic, we also assessed the release of Dox after the placement of the laser probe inside the tumors and compared this release to that elicited by a surface laser treatment. We intravenously injected 200 μ L of Dox@PEG-HAuNS (3.3×10^{12} particles/mL) into 3 4T1 breast tumor-bearing nude mice 24 h before laser treatment to provide sufficient time for the agent to selectively accumulate in the tumors. We then placed an NIR laser fiber (6 W) interstitially into one tumor per mouse and irradiated the tumors at 6 W for 1 min; the unirradiated tumors were the controls. Given a diffusing fiber length of 1 cm and a fiber applicator diameter of 1.6 mm, we used the equation $\text{laser power}/(\pi \times \text{diameter} \times \text{length})$ to calculate a power density of 0.12 W/mm^2 , which was approximately equivalent to that of surface laser treatment at 3 W for 1 min (0.15 W/mm^2). (This calculation assumed perfect transmission of the fiber and negligible optical coupling and diffusing fiber losses.) All tumors were optically imaged at the same times as those in the surface laser experiment groups. Animals were killed after 24 h.

For the *ex vivo* analysis, we intratumorally injected 50 μ L of Dox@PEG-HAuNS (20 OD, 1.33×10^{12} particles/mL) into 3 4T1 tumor-bearing mice. We then placed an 808-nm surface laser probe (2-mm spot diameter) over one tumor per mouse and irradiated the tumors at 6 W (1.9 W/mm^2) for 1 min; the unirradiated tumors were the controls. The mice were killed after 24 h, and all tumors were removed and optically imaged.

In a separate experiment, we subjected 2 4T1 tumor-bearing mice to PAI to assess tumor temperature before and after intratumoral Dox@PEG-HAuNS injection and after surface laser treatment.

2.5. Histological analysis

At the end of each imaging experiment, mice were killed by cervical dislocation, and tumors were collected and fixed in formalin. Tumors were cut into 5- μ m sections and stained with hematoxylin and eosin. Two to four slices of each tumor were analyzed to determine the percentage of tumor necrosis. Image J software (Imageworks, Inc.) was used to calculate the percentage of tumor necrosis, which was defined as $(\text{area of necrosis}/\text{area of entire tumor}) \times 100$.

Statistical Analysis

Results were analyzed using a two-tailed unpaired Student's *t* test or one-way ANOVA, with $p < 0.05$ considered statistically significant.

3. Results

3.1. Synthesis of Dox@PEG-HAuNS

The structure of Dox@PEG-HAuNS is shown in Fig. 1A. The Dox loading efficiency was >98%. PEG-HAuNS had an average diameter of 42.5 nm and a maximum absorbance at 808 nm (Fig. 1B), which is optimal for NIR laser experiments.

The concentration of Dox loaded onto 1.32×10^{12} particles/mL PEG-HAuNS was 0.35 mg/mL. Free Dox had maximum absorbance at 495 nm, whereas PEG-HAuNS had a maximum absorbance at 808 nm (Fig. 1B); thus, Dox@PEG-HAuNS showed absorbance at both 495 nm and 808 nm. The normalized absorption and emission spectra for Dox@PEG-HAuNS, Dox, HAuNS and polyethylene glycol are shown in Figure 1C. The excitation and emission bandwidths for the filter sets used in the Xenogen optical imaging system are also indicated in the figure to show that there is no spectral overlap. Dox@PEG-HAuNS concentrations at which the Dox signal was initially quenched were used for all release studies. Dox conjugation did not significantly change the size of the PEG-HAuNS.

3.2. Dox release from Dox@PEG-HAuNS

3.2.1. *In vitro* release with surface laser treatment—In our *in vitro* studies, we used lasers of different powers to irradiate Dox@PEG-HAuNS to determine an effective treatment condition. As measured using a fluorescence spectrometer, the fluorescence intensity achieved with an output power of 1.5 W and that achieved with an output power of 0 W did not differ significantly. However, the fluorescence intensity achieved with an output power of 3 W laser was 10 times higher than that achieved with an output power of 0 W (Fig. 2A). This higher fluorescence intensity indicated the release of Dox from Dox@PEG-HAuNS. Because the *in vivo* experiments were to be performed using the commercially available optical imaging system, we repeated the fluorescence release experiments using this instrument and found that the data were reproducible using both the fluorescence spectrometer and optical imaging systems (Fig. 2A–B).

To determine whether the laser treatment would result in ablative temperatures, we assessed the maximum temperatures reached under laser powers of 0, 1.5, 3, and 6 W (Fig. 2C). Similar to the findings of the fluorescence intensity study, a laser power of 1.5 W did not result in ablative temperatures, but treatment at 3 W and 6 W resulted in temperatures greater than 50°C.

We then determined the fluorescence intensity of Dox concentrations ranging from 0.17 µg/mL to 350 µg/mL under a laser power of 3 W (Fig. 2D). Dox concentrations <0.68 µg/mL had a limited increase in fluorescence intensity, suggesting that a sufficient amount of Dox must be present to be detected by the optical imaging system. Dox concentrations >10.94 µg/mL had quenched fluorescence, as has been shown in previous studies [17, 30].

3.2.2. *In vivo* release—Once we confirmed that an NIR laser could release the Dox from Dox@PEG-HAuNS *in vitro*, we investigated whether a surface laser probe could release Dox from intratumorally injected or intravenously injected Dox@PEG-HAuNS. After intravenously or intratumorally injecting Dox@PEG-HAuNS into mice, we treated the tumors with a 3-W laser or left them untreated and used a commercially available optical imaging system to assess fluorescence intensity in the tumors over 24 h. In both the intratumoral and intravenous injection groups, the fluorescence intensity in the laser-treated tumors was significantly higher than that in the untreated tumors ($p=0.015$ and 0.008 , respectively) (Fig. 3A,B). In addition, between 4 h and 24 h after injection, the fluorescence intensity increased 83% in the intratumoral injection group and 85% in the intravenous

injection group, indicating a delayed release of a substantial amount of Dox from the Dox@PEG-HAuNS ($p=0.055$ and 0.011 , respectively). The fluorescence activity of the intratumoral and intravenous injection groups at different times did not differ significantly ($p=0.319$ – 0.482). In both injection groups, fluorescence was present 5 min after Dox@PEG-HAuNS injection and laser treatment, indicating an almost immediate release of Dox from Dox@PEG-HAuNS.

We found no significant difference in fluorescence intensity between the surface and interstitial laser treatment groups immediately after Dox@PEG-HAuNS injection, immediately after laser treatment ($p=0.817$), or 0.5 h ($p=0.480$), 1 h ($p=0.980$), 2 h ($p=0.984$), 4 h ($p=0.569$), or 24 h ($p=0.703$) after laser treatment (Fig. 3C). Similar to our findings in the surface laser treatment experiments, the fluorescence intensity observed in the mice that received interstitial laser treatment was significantly higher than that of the untreated mice 24 h after treatment ($p=0.002$). Fluorescence intensity increased 56% between 4 h and 24 h after interstitial laser treatment, indicating a significant level of delayed Dox release ($p<0.05$).

Ex vivo analysis revealed that the fluorescence intensity of tumors treated with a 6-W surface laser was twice as high as that of untreated tumors ($p=0.009$) (Fig. 4B).

A separate experiment in which we used PAI to assess temperature before and after Dox@PEG-HAuNS injection and after laser treatment revealed that the mean photoacoustic signal amplitude during laser treatment was 20% higher than that before treatment, indicating a significant increase in temperature ($p<0.0001$) (Fig. 5).

3.3. Histological analysis

Histological analysis revealed that the necrosis percentage of excised tumors treated with a surface laser probe was significantly higher (64%) than that of untreated tumors (7%, $p<0.001$) (Fig. 6).

4. Discussion

The present study's findings indicate that fluorescence optical imaging and PAI can be used to monitor Dox release and tumor temperature in real time. Results show that intravenous or intratumoral injection of Dox@PEG-HAuNS followed by surface or interstitial NIR laser irradiation at equivalent doses result in similar antitumor activity.

Like previous studies, the present study revealed that Dox fluorescence is quenched when Dox is loaded to PEG-HAuNS, indicating that Dox is tightly bound to PEG-HAuNS [17, 30]. In addition, Dox is self-quenching at higher concentrations [30]. Because of its initial quenched state, Dox release can be readily monitored after laser irradiation.

In mice that received intravenous Dox@PEG-HAuNS, fluorescence was observed 5 min after laser treatment, which suggests that laser treatment causes the gold nanoshell to collapse, thereby releasing Dox. However, the fluorescence intensity observed 24 h after laser treatment was significantly higher than that observed 5 min after laser treatment, which suggests that another mechanism causes further Dox release after the initial mechanical breakdown of Dox@PEG-HAuNS. One factor that may have contributed to this delayed release is the tumor environment. Previous studies have shown that the release of Dox from Dox@PEG-HAuNS is pH-dependent and that an acidic environment could trigger Dox release [17]. Tumor cells have an intracellular lysosome environment with a pH of approximately 5.0, which could induce further Dox release after NIR laser irradiation [31]. The results of our comparison of the intratumoral and intravenous Dox@PEG-HAuNS

injection groups suggest that tumor pH is Dox-dependent. In both groups, the fluorescence intensity 24 h after laser treatment was significantly higher than that 5 min after laser treatment, and this increase may have been due to changes in tumor pH. In addition to pH changes within the tumor, another reason for the increased fluorescence observed at 24h could be the ablation of blood vessels within the tumor. Our *in vitro* studies revealed that temperatures $>54^{\circ}\text{C}$ were ablative; therefore, laser treatment may have ablated blood vessels in the treatment zone, causing them to release Dox, which then became entrapped within the tumor.

We also found significant differences between laser-treated and -untreated tumors in mice that received intravenous injections of Dox@PEG-HAuNS. This was true for both surface and interstitial laser treatment conditions. The 24 h following the intravenous injection of Dox@PEG-HAuNS may have been sufficient time for the tumor's acidic environment to induce Dox release before NIR laser irradiation. However, the effect of pH on Dox release for the tumors with Dox@PEG-HAuNS that were not treated with laser appears to be much less significant compared to laser-induced Dox release. This finding suggests that intravenous Dox@PEG-HAuNS has potential for localized and controlled NIR laser irradiation-triggered Dox release. Meaning, when the Dox@PEG-HAuNS is injected, it accumulates into the region of interest, such as the tumor, release of Dox would be minimal since the effect to tumor acidity would be less compared to when it was treated with laser. In previous studies, Dox@PEG-HAuNS was intravenously injected only 6 h before NIR laser irradiation [10]. Also, mice that received intravenous injections and mice that received intratumoral injections had similar increases in fluorescence intensity 24 h after treatment, which suggests that a substantial amount of the Dox@PEG-HAuNS that was intravenously injected successfully localized to the tumor, likely owing to its enhanced permeability and retention in the tumor tissue [32].

Histological analysis of the excised tumors to determine the percentage of tumor necrosis confirmed optical imaging findings indicating that laser treatment induces tumor necrosis, thus confirming the efficacy of gold nanoshell-mediated laser-induced thermal therapy.

In the present study, we used PAI, an emerging imaging modality, to monitor not only the localization of Dox@PEG-HAuNS but also temperature before, during, and after NIR laser irradiation. PAI enabled us to monitor temperature noninvasively, without the use of thermocouples inserted into the tumor. Compared with the pre-treatment photoacoustic signal amplitude, the photoacoustic signal amplitude during laser treatment was about 20% higher on average. This higher amplitude was equivalent to a temperature of $54\text{--}55^{\circ}\text{C}$. Histological analysis studies have shown that within-tumor temperatures of $54\text{--}55^{\circ}\text{C}$ are sufficient to cause cell death [33, 34]. These findings indicate that PAI may be used to plan thermal ablation procedures and predict their outcomes.

Our work showed that Dox release could be monitored in real-time using the inherent fluorescence of Dox using optical imaging, however, we found that the use of fluorescence to directly quantify Dox release was limited because the fluorescence was quenched at high concentrations of Dox. The detailed quantification of the amount of Dox released, which requires the use of standard techniques, such as the use of absorbance at 490 nm and flow cytometry of homogenized/processed tissue, exceed the scope of current study, are currently under way. Also, since PAI is a relatively new technique, a side-by-side comparison of temperature measurement *in vivo* with standard MRI thermography is currently being investigated. Although with the above-mentioned limitations, the study showed a proof-of-concept of the *in vivo*, real-time monitoring of Dox release and temperature achieved after NIR laser treatment mediated by Dox@PEG-HAuNS.

Conclusion

In summary, we found that NIR laser-induced PTA with Dox@PEG-HAuNS can be monitored using both fluorescence optical imaging and PAI. This approach enabled us to monitor temperature as well as the release of Dox from Dox@PEG-HAuNS under different injection and laser treatment conditions. Dox release, which depends on the power of NIR laser irradiation and tumor pH, has delayed enhanced chemotherapeutic effects and allows for a localized, controlled anticancer therapy. Significant tumor necrosis was achieved through intratumoral Dox@PEG-HAuNS injection, and surface and interstitial laser treatment elicited equivalent results. Treatment with intravenous injection of Dox@PEG-HAuNS followed by interstitial NIR laser irradiation of the tumor monitored noninvasively with fluorescence optical imaging and PAI is a promising approach to PTA in the clinic.

Acknowledgments

The authors thank Joe Munch in MD Anderson's Department of Scientific Publications for editing the manuscript. This work was supported in part by a grant from the John S. Dunn Foundation (to C.L.) and by the National Institutes of Health through MD Anderson's Cancer Center Support Grant CA016672 for the small animal imaging and veterinary pathology core facilities.

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Fig. 1. (A) Schema of Dox@PEG-HAuNS. (B) Ultraviolet to visible absorbance spectrum of free Dox, PEG-HAuNS, and Dox@PEG-HAuNS. (C) Fluorescence excitation and emission spectra for Dox, Dox@PEG-HAuNS, HAuNS and polyethylene glycol. The bandwidths of the filter sets used in the Xenogen optical imaging system are also shown.

Fig. 2.

In vitro fluorescence intensity studies. Effect of surface laser power (0, 1.5, 3, and 6 W) on the release of Dox from Dox@PEG-HAuNS detected using (A) a fluorescence spectrometer and (B) a fluorescence optical imaging system. (C) Effect of surface laser power (0, 1.5, 3, and 6 W) on maximum temperature at identical Dox concentrations (3.3×10^{11} particles/mL). (D) Effect of Dox concentration (0.17–350 $\mu\text{g/mL}$) on the release of Dox from Dox@PEG-HAuNS following identical laser treatments at room temperature.

Fig. 3.

(A) *In vivo* Dox release following intratumoral injection of Dox@PEG-HAuNS (1.32×10^{12} particles/mL) and treatment with a 3-W surface laser for 1 min. (B) *In vivo* Dox release following intravenous injection of Dox@PEG-HAuNS (3.3×10^{12} particles/mL) and treatment with a 6-W surface laser for 1 min. (C) Comparison of *in vivo* Dox release following intravenous injection of Dox@PEG-HAuNS (3.3×10^{12} particles/mL) and treatment with a 3-W surface laser probe or with a 6-W interstitial laser probe. Fluorescence intensity was measured using a fluorescence optical imaging system.

Fig. 4. *In vivo* fluorescence intensity studies. (A) Representative *in vivo* fluorescence optical imaging of Dox release following the intratumoral injection of Dox@PEG-HAuNS at $t = 24$ h. (B) Ex vivo mean fluorescence intensity of the laser-treated tumor was significantly higher than that of the untreated tumor ($p=0.009$).

Fig. 5.

Photoacoustic imaging of Dox release *in vivo* following intratumoral injection of Dox@PEG-HAuNS (1.32×10^{12} particles/mL) and treatment with a 6-W surface laser. (A) Ultrasonogram of tumor xenographs obtained in B mode, (B) photoacoustic image of tumor injected with Dox@PEG-HAuNS, and (C) overlaid photoacoustic and B-mode images. (D) Plot of photoacoustic (PA) signal amplitude over time. A baseline photoacoustic signal was recorded for 40 s and then laser treatment was delivered for 3 min. The first vertical line indicates the start of laser treatment, and the second vertical line indicates the end of laser treatment. (E) The photoacoustic signal increased linearly with temperature; this was determined using a phantom in a heated water bath. (F) Conversion of photoacoustic signal to temperature using the equation derived from the line in (E). The first vertical line indicates the start of laser treatment, and the second vertical line indicates the end of laser treatment.

Fig. 6. Histological analysis. The percentage of tumor necrosis in laser-treated mice (64%) was significantly higher than that in untreated mice (7%). Inset, representative hematoxylin and eosin-stained slides of 4T1 tumors injected intratumorally with Dox@PEG-HAuNS with and without NIR surface laser treatment (0.15 W/mm^2 for 1 min).