Laser-induced disruption of systemically administered liposomes for targeted drug delivery

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Abstract. Liposomal formulations of drugs have been shown to enhance drug efficacy by prolonging circulation time, increasing local concentration and reducing off-target effects. Controlled release from these formulations would increase their utility, and hyperthermia has been explored as a stimulus for targeted delivery of encapsulated drugs. Use of lasers as a thermal source could provide improved control over the release of the drug from the liposomes with minimal collateral tissue damage. Appropriate methods for assessing local release after systemic delivery would aid in testing and development of better formulations. We use *in vivo* bioluminescence imaging to investigate the spatiotemporal distribution of luciferin, used as a model small molecule, and demonstrate laser-induced release from liposomes in animal models after systemic delivery. These liposomes were tested for luciferin release between 37 and 45 °C in PBS and serum using bioluminescence measurements. *In vivo* studies were performed on transgenic reporter mice that express luciferase constitutively throughout the body, thus providing a noninvasive readout for controlled release following systemic delivery. An Nd:YLF laser was used (527 nm) to heat tissues and induce rupture of the intravenously delivered liposomes in target tissues. These data demonstrate laser-mediated control of small molecule delivery using thermally sensitive liposomal formulations.
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1 Introduction

Despite significant advances in the development of molecular therapeutics, effective delivery of these compounds to tissue targets can present a significant challenge. Delivery strategies based on liposomes, nanoparticles, and polymeric complexes have emerged as promising approaches that offer the potential for improved circulation time, targeted delivery, and controlled release.[1,2] Effective development of micro-and nanocarrier systems with triggered release would reduce off-target effects and potential toxicities leading to more effective therapies. Liposomes have gained acceptance as a carrier for chemotherapeutics for cancer therapy, but their effective use has suffered from rapid clearance from the circulation via cells of

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the reticulo-endothelial system (RES), including Kupffer cells in the liver and fixed macrophages in the spleen.[3] Liposomes containing polyethylene glycol (PEG; i.e., pegylated STEALTH liposomes), demonstrate reduced uptake by the RES and extended circulation time *in vivo*.[1,4] An example of such a liposomal formulation that is approved for medical use is pegylated liposomal doxorubicin (doxorubicin HCL liposome; Doxil or Caelyx). This structure allows for a pharmacokinetic profile characterized by an extended circulation time and a reduced volume of distribution, which then promotes the uptake by tumors. The associated unique reduction of the volume of distribution of doxorubicin is due to the stable liposomal encapsulation, which allows for an improved safety profile.

Pegylated strategies have, to a large extent, addressed the challenges of limited circulation times, leading to a need for controlled and/or targeted release. Strategies for directed delivery of liposomal contents include the use of targeting ligands or antibodies on the surface or the use of sheddable PEG coatings.[5] Although PEG prevents uptake of the liposomes by the RES, it also prevents uptake by target cells. This can be overcome by the use of ligands that mediate cell-surface attachment and promote endocytosis, or by removal of PEG at the target site.[5] Liposome formulations have been reported that release drug contents at temperatures slightly above the normal 37 °C body temperature (42 to 45 °C).[6–13] Directed heating can induce phase changes in the liposomes for targeted release, offering control over time and location of drug delivery.[6,7,9,10,12–14] Potential noninvasive methods for heating the liposomes include laser irradiation, microwave or radio frequency energy, or phased array ultrasound.[6,10,15–29] Here, we have investigated laser-mediated tissue heating and determined that lipid-based carriers released contents at slightly elevated, clinically attainable temperatures (39 to 42 °C). Laser activation may enable tissue-specific, controlled release.

The temperature-sensitive liposomes that release drug contents in a clinically obtainable mild hyperthermia range (39 to 42 °C) have been previously described.[7,9,10] By incorporating water-soluble lysolipid, monopalmitoylphosphatidylcholine (MPPC; 10 mol%) into dipalmitoylphophatidylcholine (DPPC) liposomes, the phase transition temperature of the liposomes is slightly decreased from 41.9 to ~40.5 °C (Ref. 10). It has been shown that heating liposomes that have accumulated at tumor sites released the drug contents predominantly in the tumor.[10] Studies of the liposomes for treating human tumor xenograft in mice, in combination with local hyperthermia (42 °C), have shown excellent local controlled release.[7]

An ideal temperature-sensitive liposome vesicle would be one that maintains stability at 37 °C during distribution throughout the body. This can be accomplished by the addition of distearoyl-phosphatidylcholine-poly(ethylene glycol-2000) [DSPE-PEG(2000)]. This would be followed by rapid, triggered release via thermal activation at the appropriate temperature (45 °C) at the targeted tissue site. Liposomes comprised of DPPC:MPPC:DSPE-PEG(2000) with an 86:10:4 mol% exhibited stability at body temperature and released the encapsulated drug rapidly, on the order of >80% within tens of seconds after thermal activation.[7–9]

The encapsulation and leakage of the fluorescent dye carboxyfluorescein (6-CF and 5,6-CF) from liposomes is one of the commonly used methods to investigate stability and release.[3,10,12,16–20,28,30–32] To provide such similar readout or a *signal switch* in living cells and tissues, we used the luciferin–luciferase reaction with luciferin as a model small molecule. Since this reaction is limited to the cytoplasm, the bioluminescent signal indicates release of luciferin from the liposomes and cellular uptake.

Transgenic reporter mice, which were engineered to ubiquitously and constitutively express firefly luciferase in all tissues,[33,34] were used to noninvasively reveal spatiotemporal biodistribution of luciferin using *in vivo* bioluminescent imaging (BLI). BLI has been shown to be extremely sensitive, and the use of the luciferin–luciferase reaction provides a signal switch for intracellular delivery. Signal to noise is relatively high with BLI due to extremely low background, which offers advantages over the use of fluorescent reporters.[35,36] In this manner, BLI can be used for monitoring the effectiveness of drug treatment and provides a broad dynamic range for quantification with acceptable resolution for tissue localization.[37]

Use of luciferin as a model drug allows for the quantification of its release from the liposome after laser activation. The photons emitted from the transgenic mouse provide an improved method of analysis based on BLI. Studies reported by Kim et al. have demonstrated thermal release of luciferin after local delivery.[38] However, the purpose for thermal activation is to enable local release after systemic delivery. The use of DSPE-PEG(2000)-PDP in this study likely limited this liposome preparation to local delivery, since this lipid has a sulfhydryl group for the purpose of functionalization of the lipid surface.[38] By not utilizing this sulfhydryl for functionalization, or otherwise reducing it, this group would remain available for forming disulfide linkages between liposomes, cells, and the endothelial surface, thus limiting its use to local injection. Since the purpose of thermal activation is to locally release systemically delivered drug, our objective was to use a liposome formulation that would allow systemic delivery and local release.[39] By using a transgenic mouse model and luciferin encapsulated in a liposome vesicle, we were able to assess the extent and location of release of liposomal contents in real time at a desired location after intravenous delivery. The capability for real-time *in vivo* assessment of delivery of the liposome payload provides an effective tool for optimizing liposome composition and assessing effective delivery.

2 Materials and Methods

2.1 *Liposome Preparation*

DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; MPPC, 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine; and DSPE-PEG(2000), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] were purchased from Avanti Polar Lipids (Alabaster, Alabama). D-luciferin thermosensitive liposome (TSL) preparation was adapted from the method described by Anyarambhatla and Needham.[9] DPPC: MPPC: DSPE-PEG(2000) in a (86:10:4) molar ratio, were dissolved in chloroform and deposited on a flat-bottomed flask. A lipid film was obtained by slow evaporation under nitrogen followed by overnight

vacuum drying. The dried lipids were hydrated in phosphate buffered saline (PBS, Gibco, Carlsbad, California) containing 10 mg/ml D-luciferin in PBS (Biosynth, Staad, Switzerland) via vortexing to create the combined drug.

Once the liposomes were made containing the D-luciferin, they were homogenized by sonication for 10 min at 45 °C to achieve a 76 ± 3 nanometer diameter for IV injection. The fluid temperature was measured continuously during sonication and was found to be increased up to ~48 °C by the end of the 10 min process. The liposome size was determined by dynamic laser light scattering using unimodal and differential size distribution processor analysis (Zeta Pals, Brookhaven Instruments Corporation, Holtsville, (New York). The results are given by the mean ± the standard error of the mean (SEM) of three measurements. Size determination was followed by 12-h dialysis in PBS to remove unencapsulated luciferin. After dialysis and size determination, the liposomes were tested prior to mouse injection to determine the amount of encapsulated luciferin as well as their release at 45 °C in both PBS and serum over time. Since luciferin is also a fluorophore; the amount of luciferin encapsulation was first measured by fluorescence. The fluorescence measurements in photons were compared with a standard calibration curve to determine the absolute amount of luciferin/μl of liposome solution. These measurements were confirmed using bioluminescence measurements (see next section).

2.2 *Liposomal Luciferin–Luciferase Assays*

The amount of D-luciferin contained within the liposomes was quantified by fluorescence measurements using a microplate fluorescence reader (Spectra Max GeminiEM, Molecular Devices, Sunnyvale, (California Ex-330 nm, Em-525 nm) in a 96-well black-walled plate (Corning, Inc., Corning, New York) done at $n=3$ for comparison. For bioluminescence measurements, purified luciferase (Luc) was obtained from Promega Corp (Madison, Wisconsin). The luciferase was prepared in a bioluminescence buffer (40 mM Tris Acetate, 1 mM EDTA, 1 mM DTT, 3.45 mM ATP, 0.2 M NaCl, 5.7 mM $MgSO_4$, and 0.76 mM Coenzyme A, pH 7.6; all chemicals obtained from Sigma-Aldrich Corp., St. Louis, Missouri). Liposomal formulations that contained D-luciferin (Biosynth, Staad, Switzerland) were serially diluted in PBS, and 50 μl of each liposome dilution was added to 50 μl of the preceding Luc enzyme solution (2 ng luciferase/μl). All of the assays were carried out in triplicate. The photon emission was measured in a Berthold Detection Systems Sirius Luminometer. The liposomes were subjected to heat treatment at either 37 or 45 °C for 5 min, and luciferin release was analyzed in either PBS alone or with serum. The assessment of free luciferin in the liposome preparation performed using the bioluminescence assay kit was extremely sensitive relative to the fluorescence assays. The luciferin available to the luciferase enzyme was considered to be not contained with in the liposome and we observed an increase in bioluminescent signals over the course of the dialysis procedure (at 0, 4, and 12 h) and after heating. A twofold increase was seen following 12 h of dialysis, suggesting that the luciferin is slowly released from the liposomes at room temperature (data not shown).

2.3 *In Vivo Thermosensitive Liposome Release*

All animal studies were approved by the Administrative Panel for Laboratory Animal Care (APLAC) at Stanford University. A luciferase transgenic mouse, L2G85, was used for these experiments that ubiquitously and constitutively expresses modified firefly luciferase (from the pGL3 vector from Promega Corp.). The luciferase-GFP fusion is expressed in this animal by a modified β-actin promoter with the immediate early promoter from cytomegalovirus (CMV) and is known as the CAGS promoter.[34] The transgenic animal provided a platform for obtaining a bioluminescent readout for the biodistribution of free luciferin wherever present, and available for the cytoplasmic luciferase. Thus, upon injection of liposomes into the animal, the biodistribution profile of free luciferin was obtained by BLI measurements. The release of luciferin from the liposomes after thermal activation was compared to signals without heating to provide an internal control. The liposomes were disrupted either by thermal heating with a water bath or a laser source (527 nm). The right rear foot of the mouse was chosen as a target for investigating liposomal release of luciferin after heat treatment. The thermosensitive liposomes were designed to release their contents at or above 42 °C. To achieve an optimum luciferin release from the liposomes across the foot, a temperature of 45 °C was used with the water bath and the laser experiments. The animals did not show any signs or symptoms of inflammation or discomfort throughout the course of the experiments. The mice were anesthetized by an intraperitoneal injection of Avertin (100 μl/10 g body weight) prior to the intravenous injection of 100 μl of liposome per mouse—the total luciferin content of the liposomes was ~500 μg/100 μl.

2.4 *Water Bath*

Five 20-gram female mice, at three months of age, were used for the water bath heating experiments. The mice were imaged after injection of the liposomes in an IVIS200 imaging system (Caliper, Inc., New York) to determine the background signal from free luciferin. It was determined that the background signal had decayed by 40 min post-injection. The right rear foot of each mouse was then immersed in a 45 °C water bath (Isotemp202, Fisher Scientific, Pittsburgh, Pennsylvania) for 2 min. The mouse was immediately placed in the IVIS200 for imaging post–water heating. All data were analyzed using LivingImage 2.50.1 software (Caliper, Inc.). Analysis was performed around the lower-left and lower-right quadrant of each mouse to compare the increased signal due to heating. The right foot versus left foot analysis was normalized to the signal of the whole mouse. The mice were also reheated and imaged three days after the injection to determine if liposomes containing luciferin were still circulating at that time.

2.5 *Nd:YLF Laser*

Liposomes were injected into four 20-gram three-month-old female mice as described above. Mice were imaged with an IVIS50 imaging system (Caliper, Inc.) at 90 min post–liposome injection prior to laser heating of the right foot. Each mouse was imaged and heated one at a time. The right foot was heated for 2 min by a Nd:YLF (Merlin, Spectra Physics/Newport, Mountain View, (California) at 527 nm

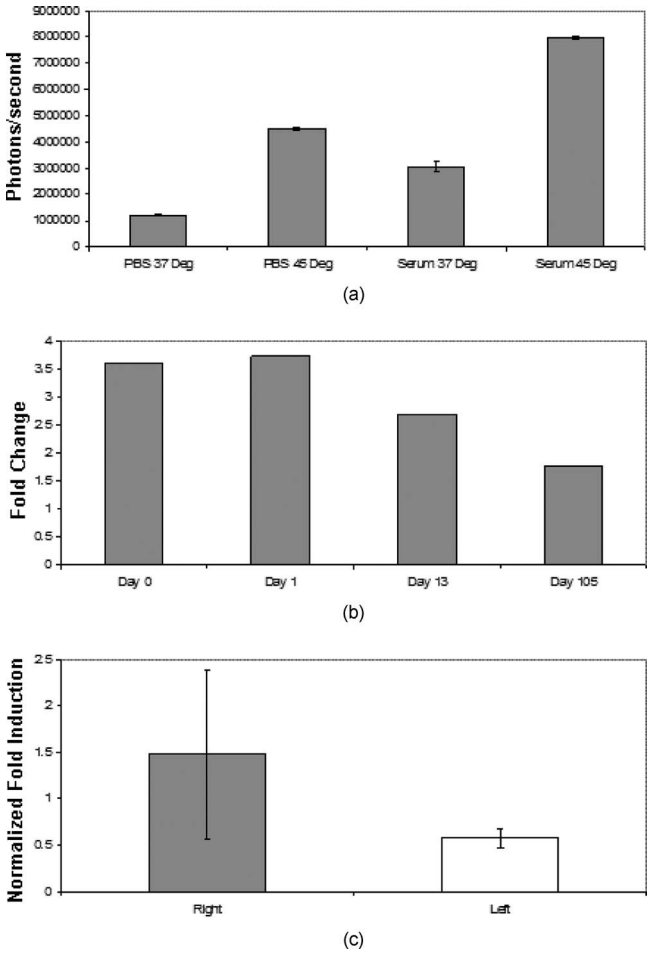

Fig. 1 Liposome stability *in vitro*. (a) The average photons/second ($N=3$) of luminescence from the designed 86:10:4 molecular weight percent of the MPPC:DPPC:DSPE-PEG(2000) liposome containing D-luciferin is shown *ex vivo* for analysis. The data is shown for a 1:10 dilution of the liposome by adding 10 μl of liposome to 90 μl of PBS or serum. The liposome was thermally heated to either 37 or 45 °C for comparison. (b) The average fold change ($N=3$) of bioluminescence from the D-luciferin containing liposome from a 1:10 dilution in PBS between 37 and 45 °C at 0, 1, 13, and 105 days after formation to determine the stability of the liposome over time. (c) The average fold increase ($N=4$) for bioluminescence after intravenous injection of 100 μl of D-luciferin containing liposomes following 2-min water bath heating at 45 °C on the heated right paw and the unheated left paw. The data was normalized to the bioluminescence of the whole body. The thermal release and imaging was performed 40 min after intravenous injection. Imaging was performed with 1-min integration. The error bars show ± the standard deviation of the normalized signal.

with 650 mW of energy at 240 Hz. The temperature of the foot was measured with a FLIR A20M infrared thermal camera (Boston, Massachusetts). It took 1 min for the laser to heat the foot to 45 °C, and the temperature was maintained at 45 °C ± 1 °C by adjusting the laser energy for 1 min by a Uniblitz CS45S3TO electronic shutter (Vincent Associates, Rochester, New York) for a total exposure of 2 min. Mice were imaged following the laser heating using the IVIS50 imaging system—the time between heating and BLI measurements was approximately 90 s, and the FLIR camera revealed tissue returning to normal temperature in less than 5 s. Analyses were performed as described above. Following the thermal release of liposomes in the foot, the Nd:YLF laser was used to test the results of thermal release in the right ear with the same 2-min laser heating as described for the foot.

3 Results and Discussion

In this study, we used D-luciferin as a model drug. This compound, when used with transgenic reporter mice, allows for imaging the location of targeted delivery of model drug and can be used to assess laser-mediated thermal release from liposomes. The luciferin-containing liposomes in the tissues were heated using a Nd: YLF (527 nm) laser source, and the *in vivo* spatiotemporal biodistribution of luciferin was imaged using BLI. In addition, the onset of release for the entrapped contents (in this case D-luciferin) occurs more rapidly than for the pure DPPC lipid at temperatures just above 39 °C. At high concentrations (greater than 30 mM), the traditionally used carboxyfluorescein dye is self-quenched and emits a very low fluorescent signal. When released from the liposomes, the fluorescence signal can be measured with time due to dilution into a larger, external volume. Although useful for assessing liposome stability, this approach cannot be used to indicate when the liposomal contents have been released and have crossed the cellular membrane into the cytoplasm of the target cell. As far as we know, this is the first report that shows the controlled release of D-luciferin from a liposome at the intended target after intravenous injection. As the L2G85 transgenic mouse model constitutively expresses luciferase, we can effectively quantify the release of the luciferin at the heated site, and any off-target tissues, using BLI measurements. In this study, we used the MPPC:DPPC:DSPE-PEG(2000) liposome which is reported[7,10–13] to have both a reasonable *in vivo* half-life due to the protection from the RES, and good thermal release kinetics at ~42 °C. In addition, we were able to show that a Nd: YLF laser operating at 527 nm in wavelength was able to release the IV injected small molecule, luciferin, within the target tissue by transiently heating the region to 45 °C at the skin surface.

Luciferin release from the liposomes was tested at temperatures of 37 and 45 °C in both PBS and serum using bioluminescence measurements with the luminometer as described in the methods. The liposomes combined with PBS were heated from 37 to 45 °C for 5 min on the day that they were made. The release of luciferin is reported as fold increase in bioluminescence, which was calculated by dividing the average bioluminescence at 45 °C by the average bioluminescence at 37 °C. A 3.5-fold increase of bioluminescence was seen for the liposomes in PBS, and a 2.5-fold increase was seen for the liposomes in serum. The results are shown as an average in photons/sec for day zero between PBS and serum at the two different temperatures in Fig. 1(a).

The next experiment compared the release of luciferase from the liposomes after storage. Bioluminescent signals were measured at 37 °C and then heat treated (45 °C) in PBS on days 0, 1, 13, and 105 to determine the long-term stability of the liposome prior to injection. Results are reported as fold change in luciferase activity measured at 37 °C and 42 °C. A 3.7-fold increase in signal was noted at day 1, and a 2.7-fold increase was seen at day 13 following formulation. By day 105, a 1.7-fold increase of bioluminescence for liposomes/

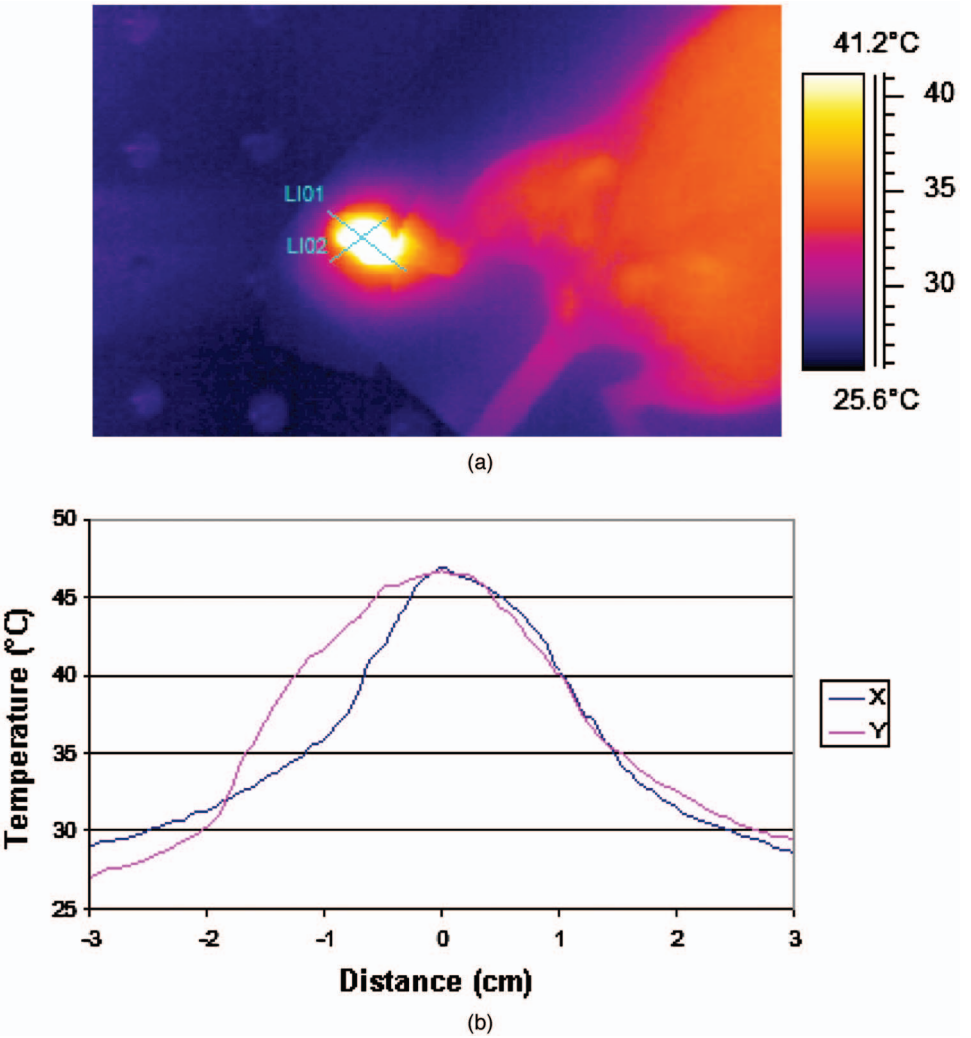

Fig. 2 Thermal analysis of liposome laser release. (a) Thermal imaging of the right paw during Nd:YLF laser irradiation at 650 mW, 240 Hz, with a 1-cm elliptical laser spot is shown from the FLIR mid-infrared thermal imaging system. LI01 shows the Y line, and LI02 shows the X line for analysis of the temperature across the elliptical laser spot on the mouse paw. The laser energy raised the temperature of the paw to 45 °C within 1 min and was maintained at 45° ±1 °C with a shutter maintaining the temperature by continually blocking the laser energy during one additional minute. The scale bar shows the temperature relating to each color throughout the image. (b) The temperature in degrees Celsius between the X and Y lines at the end of the first minute after the 45 °C temperature was achieved on the right paw.

PBS was observed at the two temperatures (Fig. 1). In addition to these long-term results, *Needham* et al. have shown the ability of PEG containing liposomes to have a greater stability due to the hydrophilic properties of PEG, which should translate to long term stability *in vivo*.[7,8,40] While luciferin is maintained inside these liposomes at room temperature (25 °C) and body temperature (37 °C), increased temperatures release the liposomal contents, and once luciferin is released, it permeabilizes through nearby cell membranes due to the amphipathic structure of this molecule.[41–44]

For *in vivo* studies, 100 μl of liposome solution was injected intravenously into transgenic reporter mice. These mice express luciferase constitutively throughout the body and provide an excellent platform for investigating the biodistribution of luciferin and directed delivery. The liposomal release was first tested 40 min post–IV injection by placing the right paw of the transgenic mouse into a 45 °C water bath for 2 min. Some background bioluminescence was observed throughout the mouse after IV injection as a result of luciferin that had either leaked out or was not encapsulated. The thermal activation studies were performed 40 min after the IV injection of the liposomes, which was determined to be a time when the background signals were low. Each mouse was imaged in the IVIS200 (Caliper/Xenogen) immediately after heating. Analyses of the *in vivo* images were performed using LivingImage 2.5 (Caliper). An $n=4$ was used for comparison of mice. The results showed a 1.5-fold increase in signal on the right paw after heating and a 0.6-fold increase in the left paw when normalized to the whole body signal [Fig. 1(c)].

A Nd:YLF laser was used at 527 nm to heat the right foot of mice 90 min after injection of the luciferin-liposomes. The 50-min difference between this experiment and the water bath experiment was due to the location of the laser relative to the imaging system. The laser was some distance from the imaging system and required transfer of the mice and the 50-min

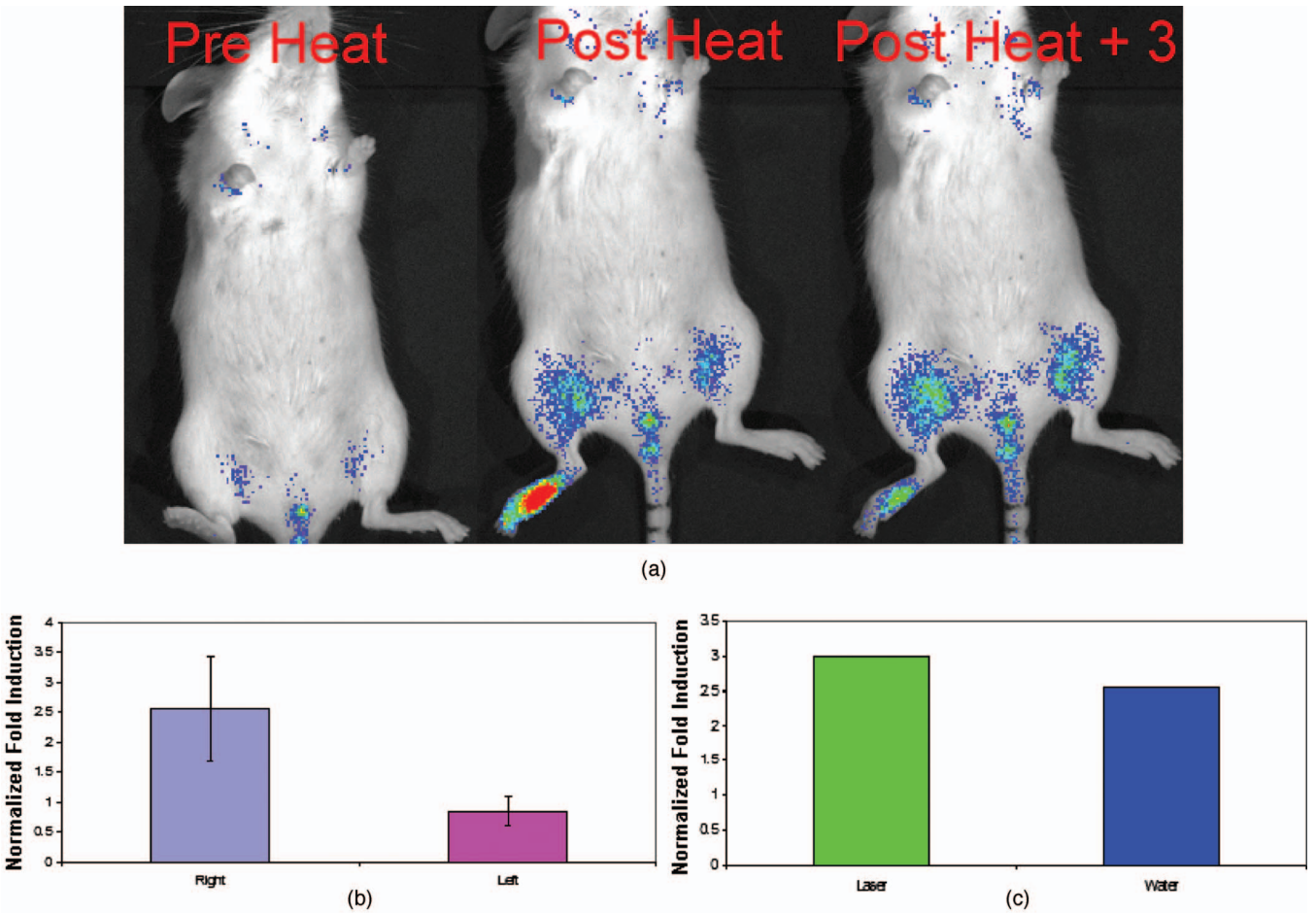

Fig. 3 *In vivo* imaging of the release of liposome contents. (a) Image of one of the laser-irradiated mice. This imaging was done with an IVIS50 imaging system (Caliper/Xenogen, Inc.) with a 1-min integration time. The imaging was done 1 min prior to laser heating, immediately after laser heating, and 3 min after laser heating. The image shows a clear difference between the bioluminescence signal seen in the laser heated right paw and the unheated left paw. (b) The average fold increase (N=4) in bioluminescence signal after intravenous injection of 100 μl of D-luciferin containing liposomes following 2-min laser heating to 45 °C on the heated right paw and the unheated left paw. The data was normalized to the bioluminescence of the whole body. The thermal release and imaging was performed 90 min after intravenous injection. Imaging was performed with 1-min integration. The error bars show ± the standard deviation of the normalized signal. (c) A comparison between the average fold induction between the water bath and the Nd:YLF laser system is shown. The data was normalized to the whole body region of interest and the left paw in each mouse imaged. The final result shows a 3-fold increase in bioluminescence signal related to the laser heating compared to a 2.5-fold increase related to the water bath heating.

delay. Given that the 40-min background data indicated that the background had reached a plateau, the delay in imaging likely did not affect background signals but may have reduced signals from luciferin released from liposomes. The right hind feet of the mice were heated to 45 °C for 2 min as determined by a FLIR camera [FLIR System, Boston, MA, Fig. 2(a)]. The first minute of laser irradiation raised the skin temperature from 35 to 45 °C, while the temperature was maintained at 45 °C ± 1 °C for an additional minute. The laser heat treatment was appropriately guided with the use of a shutter (Vincent Associates) and the FLIR camera. Figure 2(b) shows the average temperature of the right paw in the x and y direction at the end of the first minute of laser heating. The background signal had dissipated by 90 min post-injection [Figure 3(a); Pre-Heat]; therefore, laser heating and imaging was accomplished at 90 min post-injection for each of the four mice used for this experiment.

An IVIS50 imaging system (Caliper LifeSciences, New York) was used to image the mice. Differences in luciferase activity have been noted between the temperatures of 25 and 37 °C (Ref. 45). Over this 12-degree temperature range, there is only a 0.2-fold increase in activity. Since the FLIR camera indicated that the tissue return to normal temperature by the time the animals are imaged, and the modest differences in activity that may be possible at elevated temperatures, it is evident that increases in activity after heating are due to thermal release. In this study, the normal, 37 °C, temperature was achieved within a few seconds after heating and well before the imaging began.[46,47] The image of one laser-heated mouse is shown in Fig. 3(a). The mouse is shown prior to laser heating at 90 min post-injection of liposomes (IV) and immediately after laser heating, as well as at 3 min after laser heating. A 2.6-fold increase in bioluminescence in the heated right paw was seen compared to a 0.8-fold increase in

the left paw [Fig. 3(b)]. Figure 3(c) shows a normalization between the heating of the liposome for 2 min with the water bath and the Nd:YLF laser. This data was normalized to the whole body region of interest and the left paw region of interest for each mouse prior to averaging with an $N=4$ for each experiment. The laser heating showed a normalized 3-fold increase in bioluminescence compared to a 2.5-fold increase due to water bath heating.

4 Conclusions

We have tested stable, temperature-sensitive liposomes that release their drug contents upon heating for the ability to administer the liposomes systemically and activate them locally in a target tissue. The technique was characterized and optimized by employing luciferin as a method for *in vitro* and *in vivo* tracking and analysis. The tested formulations have shown encouraging results with regards to stability and release profile upon heating *in vitro* in PBS and serum. We were able to thermally release the liposome contents with both a water bath and after heating with a Nd:YLF 527 nm laser. The laser showed a 3-fold increase in bioluminescence compared to a 2.5-fold increase following heating in a water bath. We have shown that using D-luciferin as a model molecule and employing the transgenic reporter mouse as an indicator of drug release by BLI is a useful strategy for developing and optimizing a range of liposomes and other drug delivery methods.[38,48,49] Such a system will allow us to develop better drug delivery tools for improved control of drug release in target tissues. While every drug has unique properties, it is possible to use this system for analyzing delivery tools that may normalize these differences.

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