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Rapid, Reversible Release from Thermosensitive Liposomes Triggered by Near-Infra-Red Light

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Abstract

We present a novel drug carrier consisting of plasmonic hollow gold nanoshells (HGN) chemically tethered to liposomes made temperature sensitive with lysolipids (LTSL). Continuous-wave irradiation by physiologically friendly near infra-red light at 800 nm for 2.5 minutes at laser intensities an order of magnitude below that known to damage skin generates heating localized to the liposome membrane. The heating increases the liposome permeability in an irradiation dose-dependent, but reversible manner, resulting in rapid release of small molecules such as the self-quenching dye carboxyfluorescein or the chemotherapeutic doxorubicin, without raising the bulk temperature. The local rise in nanoshell temperature under laser irradiation was inferred by comparing dye release rates from the LTSL via bulk heating to that induced by irradiation. Laser-irradiation of LTSL enables precise control of contents release with low temperature gradients confined to areas irradiated by the laser focus. The combined effects of rapid local release and localized hyperthermia provide a synergistic effect as shown by a near doubling of androgen resistant PPC-1 prostate cancer cell toxicity compared to the same concentration of free doxorubicin.

Keywords

Doxorubicin; Plasmon-resonant gold nanoshells; laser light; prostate cancer

1. Introduction

One of the biggest challenges facing liposomal drug delivery is simultaneously optimizing drug retention and drug release. It is difficult to switch from drug retention to varied levels of drug release, or switch from fast release back to drug retention within the constraints of a single bilayer.^[1–9] For example, encapsulating doxorubicin within PEGylated liposomes significantly reduces cardiotoxicity and increases the half-life in the systemic circulation due to optimized doxorubicin retention in the liposome. However, efficacy did not improve as expected from the increased liposomal doxorubicin accumulation at the tumor site^[10] because of slow drug release. Cisplatin incorporated into PEGylated liposomes extends

systemic half-life to 40 – 55 hrs,^[11] in comparison to the 15 – 20 minute clearance of free cisplatin. However, the formulation failed in human trials because cisplatin was not released at a therapeutic rate from the liposomes, even though 5- to 10-fold greater liposome encapsulated cisplatin accumulated in the tumors. This highlights the conflict between “retain” and “release” – factors that enhance drug retention usually work against an optimal rate of drug release.

“Thermosensitive liposomes” (TSL) and other stimuli-sensitive liposomes were developed to resolve this conflict.^[3–5,12] TSL have a membrane composition designed to induce a step-change in permeability in response to modest changes in temperature above 37 °C.^[4,13–19] Needham, Dewhirst and coworkers^[15–18] pioneered Lyoslipid-TSL formulations (LTSL), which in addition to dipalmitoylphosphatidylcholine (DPPC), contain lysolipid and lipid-conjugated PEG²⁰⁰⁰ (DSPE-PEG²⁰⁰⁰); experimental and computer simulations suggest that the permeability transition is due to the formation of lysolipid-stabilized transient pores that occur at the phase transition temperature of DPPC (Figure 1).^[18,20–24] LTSL have shown improved efficacy with a number of cancer cell lines *in vitro* and *in vivo*, and clinical trials are ongoing for treatment of liver and breast cancers with combined hyperthermia and LTSL.^[18,20] Release from LTSL is typically done via bulk heating with water baths, radiofrequency (RF) or microwave (MW) radiation, etc.^[16–19,25] Controlling the temperature profile and extent of heating are difficult, especially in highly perfused tissues. Here, we demonstrate a non-invasive method to initiate, control, and even stop drug release via membrane-localized temperature control. Plasmonic hollow gold nanoshells (HGN)^[26–29] are tethered to LTSL via thiol-PEG-lipids to create a hybrid nanocarrier (Figure 1). The LTSL/HGN are irradiated with continuous wave near infra-red (NIR) laser light at 800 nm at intensities an order of magnitude less than that known to damage skin, causing a sufficient temperature rise near the liposome membrane to induce transient membrane pores that rapidly release the liposome contents (Figure 1).^[16,17,20,30,31] The great advantage of using NIR light to induce release is that tissue, blood, etc. are relatively transparent to 650–950 nm wavelength light, allowing NIR transmission in soft tissues at depths up to several cm.^[19,32,33] Laser heating induces a near instantaneous response, allowing the liposome contents to be released in seconds. Continuous laser irradiation of gold nanoparticles only generates small temperature increases ($\Delta T \sim 2\text{--}4\text{ }^{\circ}\text{C}$) above the average solution temperature.^[34–36] As a consequence, the liposome temperature reverts to ambient quickly when NIR irradiation stops, allowing the liposomes to re-seal which stops drug release. Only HGN/LTSL complexes irradiated by the laser release drug, providing a potential targeting mechanism.

NIR-induced doxorubicin release from HGN/LTSL results in irradiation-dose dependent killing of PPC-1 androgen resistant prostate cancer cells *in vitro*. Only three minutes of NIR irradiation is required to release the doxorubicin; surprisingly, even this short irradiation time provides a synergistic heating and concentration effect that roughly doubles the killing efficiency of free doxorubicin. Passive permeation from the liposomes required nearly two orders of magnitude higher doxorubicin concentration to effect similar toxicity, showing the benefits of rapid drug release and localized hyperthermia.

2. RESULTS

LTSL were formed by self-assembly from dipalmitoylphosphatidylcholine (DPPC) with 4 mol% DSPE-PEG²⁰⁰⁰ with various fractions of the lysolipid, monopalmitoylphosphatidylcholine (MPPC). Figure 2A shows that CF release after 2.5 minutes is strongly dependent on the sample temperature and can be modified by changes in liposome composition. Over 2.5 minutes, DPPC with 4 mol% DSPE-PEG²⁰⁰⁰ has a minimal change in CF release at the phase transition temperature of 41°C.^[29] Adding 15 mol% MPPC increased CF release rates by orders of magnitude for temperatures $\geq 39^\circ\text{C}$; however, release was undetectable at 37°C.^[20] Further increases in temperature to 41°C increased liposome permeability sufficiently so 100% of the encapsulated CF was released within 2.5 minutes (Fig. 2A). The permeability transition can be shifted to higher temperature by adding DSPC to the liposome membrane. DPPC has a gel to liquid crystal temperature of 41°C, while DSPC has a transition temperature of 54°C.^[20,37] Adding 30 mol% DSPC and 15 mol% MPPC to DPPC liposomes increased the permeability transition temperature from 39–40°C to 41–42°C (Fig. 1a) which is also the increase in the gel to liquid crystal transition.^[37]

Fig. 2B shows cryo-TEM images of DPPC liposomes with 4 mol% DSPE-PEG²⁰⁰⁰, which have the typical, spheroidal shape of gel phase liposomes. Adding 10 mol% MPPC does not alter the liposome appearance. However, liposomes with 15 mol% MPPC are polygonal with sharper edges between the facets. This suggests that the MPPC is segregated to the high curvature regions at the grain boundaries.^[21] Increasing MPPC to 20 mol% destabilized the liposomes and led to coexistence between bilayer discs (arrows) and intact liposomes^[21,23].

The discs are likely stabilized by the segregation of the MPPC to the disc perimeters.^[21,23,24] Components like MPPC and DPSE-PEG²⁰⁰⁰ that prefer high-curvature, micellar structures act as “edge-actants,” and lower the energy associated with the bilayer edge. When this edge energy is less than the curvature energy associated with bending a gel-phase bilayer to form a closed vesicle, discs are stabilized.^[21,23,24] Mixtures of DPPC with diheptanoylphosphatidylcholine, the seven carbon-long alkane version of DPPC, also form discs with the shorter-tail lipids stabilizing the edges, which are known in the literature as bicelles.^[21,38] Molecular simulations show that stable and unstable bilayer pores form spontaneously in bilayers with edge-actant concentrations less than that needed^[21] to break up the liposomes into discs. Such transient pores are likely the route through which small molecules are released.^[20] Such a mechanism would also allow for the pores to be re-sealed by lowering the temperature, which decreases the mobility of the lysolipid in the gel-phase membrane.

2.1 Local vs. Collective Heating

To tether the HGN, the DPSE-PEG²⁰⁰⁰ on the liposome exterior were modified with a sulfhydryl group allowing for thiol-gold bonds to chemically link the liposomes and HGN (Figure 3).^[27–29] Tethering reduces the laser power density needed to induce CF release from LTSL compared to untethered HGN at a HGN concentration of 10^{11} HGN/ml (Fig. 3C). For the tethered LTSL/HGN, dye release begins at $> 0.6\text{ W/cm}^2$ laser power density, while 1.2 W/cm^2 was required to initiate dye release with the free HGN. Dye release for

the untethered nanoshells was due primarily to an increase in the bulk solution temperature, as in Fig. 2A. No dye release was observed over this time scale in LTSL without nanoshells and NIR irradiation. The sample temperature reached after irradiation for extended times (> 10 minutes) was the same for the tethered and untethered samples, which confirms that the total HGN concentrations were the same in both samples (Eqn. 1).

Fig. 4A shows the temperature change induced by irradiating various concentrations of HGN in saline from an initial temperature of 37 °C for 2.5 minutes as a function of laser power density. Saline without HGN did not increase in temperature significantly at any laser power. The increase in temperature from some initial value, $T-T_0$, of a volume V of heat capacity, C_p (4.2 J/gr-K for water), and density ρ (1 gr/cm³ for water), due to the heating supplied by n nanoparticles of average energy input per time, Q , is given by:

$$T-T_0 = \frac{nQ}{V\rho C_p} t = \frac{NQ}{\rho C_p} t \quad (1)$$

$n/V = N$, the nanoparticle concentration^[34,35]. Eqn. 1 shows that the temperature increase should be linear in the applied laser power. For each concentration, the correlation coefficient, R , is > 0.96, confirming a statistically significant linear fit ($p < 0.01$). The temperature increase at a given power density also increases with increasing concentration, N . Fig. 4A shows that HGN concentrations of below 10¹¹ HGN/ml would not provide a sufficient bulk temperature increase (> 39 °C, Fig. 2A) to induce the permeability transition at any of the power densities tested.

Fig. 4B shows that the laser-irradiated, tethered LTSL/HGN release their contents at a bulk temperature ~ 2–3 °C less than LTSL/HGN that are not irradiated. For the non-irradiated LTSL/HGN, an external heat source was applied to rapidly increase the bulk temperature of the sample to a particular value (open squares, Fig. 4B) and the fractional CF release was determined after 2.5 minutes. The fractional CF release is a reproducible function of the LTSL temperature; the standard deviation is less than the size of the open squares; this is not surprising as the permeability transition is associated with the DPPC gel to liquid crystalline transition temperature.^[15–18] This reproducible behavior led us to postulate that a given CF release rate implies a particular membrane temperature, so that we could use CF release as a “membrane thermometer”. For the irradiated samples, the sample temperature was fixed at the beginning of irradiation at a particular value along the dotted line in Fig. 4B. The sample was then exposed to 800 nm NIR light of given intensity for 2.5 minutes (1.2 W/cm² for the data in Fig. 4B; other laser intensities not shown), and the final average sample temperature and fractional CF release were determined for a given initial bulk temperature (closed squares). The change in the bulk temperature in the irradiated sample was due to the collective heating effect of the irradiated HGN (See Eqn. 1 and Fig. 4A). However, as can be seen in Fig. 4B, the equivalent CF release from the LTSL/HGN occurs at a lower bulk temperature (closed squares) than the non-irradiated LTSL/HGN (open squares).

This implies that the irradiated LTSL/HGN membrane temperature is greater than the bulk temperature by an amount given by the red arrow. The black arrow in Fig. 4B shows the

increase in bulk sample temperature for 50% CF release. However, the increase in bulk sample temperature is not sufficient to release CF. The LTSL membrane needs to reach an effective temperature of 39.3 ± 0.2 °C to release 50% CF, which requires a net temperature increase of 3.6 ± 0.3 °C above the initial temperature of 35.7 ± 0.2 °C (dotted line). Of the 3.6 °C increase needed to release the dye, 1.3 ± 0.3 °C is due to the increase in the average bulk temperature (hatched), and 2.3 ± 0.3 °C is due to the local temperature difference between the LTSL membrane and the bulk solution (grey) (Fig. 4C). At lower laser power density, the initial sample temperature must be increased (data not shown) for the effective membrane temperature to reach the 39.3 ± 0.2 °C necessary to release 50% CF (Fig. 4C). At 0.4 W/cm^2 irradiation intensity, the LTSL membrane experiences an effective combined temperature increase of 1.6 ± 0.3 °C (initial temperature of 37.7 ± 0.2 °C), while for 0.8 W/cm^2 , the net temperature increase is 2.6 ± 0.3 °C (initial temperature of 36.7 ± 0.2 °C). As for the 1.2 W/cm^2 data, about one third of the temperature increase is due to bulk heating, and two thirds due to the local heating. The magnitude of the local heating is consistent with other experimental estimates of the temperature differences around continuously irradiated gold^[39–41] and RF-heated magnetic nanoparticles.^[34,35]

The localized heating is the origin of the differing power intensities required to release CF for the tethered and untethered HGN (Fig. 3C). The threshold laser power density is the lowest laser intensity that will ensure that the localized HGN heating (Fig. 4C) is sufficient to raise the liposome membrane temperature > 39 °C. This will enable contents release irrespective of the concentration at which the LTST/HGN have accumulated at the treatment site, as the effects of the collective heating depend on HGN concentration (Fig. 4A), this laser threshold power density is 1.2 W/cm^2 . At this laser power density, the localized nanoshell heating is sufficient to raise the liposome membrane temperature to 2.3°C above physiological temperature, 37 °C, which is enough to increase membrane permeability and trigger release. This power intensity is an order of magnitude below the 12 W/cm^2 threshold observed in previous studies that caused skin irritation in animals, which is the maximum power that could be used *in vivo*.^[42–44]

Localized heating also means a rapid temperature response on both starting and stopping irradiation. The steady state temperature distribution around a HGN of radius R on irradiation is established after $t_{ss} \sim 10R^2/\alpha_w$,^[45] in which $\alpha_w = .14 \times 10^{-6} \text{ m}^2/\text{s}$ is the thermal diffusivity of water. For a 40 nm diameter HGN, $\tau_{ss} < 0.1 \mu\text{s}$. Fig. 5A shows that for 2.4 W/cm^2 light intensity, dye release begins almost instantly after irradiation starts and is 90% complete within 30 seconds for a sample of 10^{10} HGN/ml (Figure 5A). Without the effects of localized heating, the entire sample volume would need to increase in temperature to 39 °C to reach the permeability transition before any CF release would begin; extrapolating from Fig. 4A for 10^{10} HGN concentrations, it would take ~ 5 minutes to reach 39 °C.

The rapid response of the HGN to irradiation also means that the local temperature increase dissipates rapidly after irradiation stops. Fig. 5B shows that staged release of LTSL/HGN contents is possible. LTSL/HGN encapsulating CF were held at 37°C for 22 hours, followed by 30 seconds of irradiation at an intensity of 1.6 W/cm^2 . Prior to irradiation, the LTSL/HGN effectively retained the encapsulated dye; $13 \pm 3\%$ of the dye was lost due to

passive leakage, which is similar to values in the literature for other LTSL.^[16,17,20] On irradiation, $35 \pm 3\%$ of the encapsulated dye was released during a 30-second irradiation (total release after 24 hr is $48 \pm 3\%$). The LTSL/HGN were not irreversibly altered by the irradiation or the CF release; the remaining CF was retained within the LTSL/HGN for the following 24 hours ($49 \pm 3\%$ after 48 hours, $p = 0.05$). In addition to CF release starting immediately on irradiation, Fig. 5B shows that dye release stops equally quickly when irradiation stopped. This is consistent with the release mechanism being the formation of transient, but reversible pores near the phase transition temperature (Fig. 1)^[20] initiated by the heating effects of the HGN. Irradiation does not disrupt LTSL integrity; the LTSL is just as impermeable to CF release after the first irradiation as before as indicated by the retention of the remaining encapsulated dye for the 24 hours between the first and second irradiation. Suggestions in the literature that the lysolipid is ejected from the LTSL during release^[20] are likely not correct as an additional $35 \pm 3\%$ of CF (total CF release of $85 \pm 3\%$) was released by a second 60 second pulse of 1.6 W/cm^2 intensity after 46 hours at 37°C . Had the lysolipid been ejected from the LTSL, drug release may have stopped after the first irradiation, but would not have resumed on the second irradiation, as pure DPPC liposomes do not show a significant increase in CF release with temperature (Fig. 2A).

Figure 6 shows cryo-TEM images of doxorubicin-containing LTSL/HGN before and after irradiation. Continuous wave NIR irradiation at 1.2 W/cm^2 power density does not affect the LTSL or HGN structure; the only difference is that the doxorubicin precipitates present before irradiation disappear after irradiation. The liposomes remain as closed spherical structures and the nanoshells retained their structure of a thin shell with a hollow core. The HGN still strongly absorb NIR light after the CF is released, which means that subsequent irradiation can be used to induce a bulk temperature increase. This is consistent with a small difference in temperature between the HGN and the surroundings. In our previous work, we found that the same average light intensity from femtosecond pulsed lasers, which establish large temperature gradients, rupture the liposomes via formation of nanobubbles, while annealing the hollow nanoshells into solid gold nanoparticles.^[26–28,46]

2.2 Doxorubicin Release in an *In Vitro* Prostate Cancer Cell Model

Figure 7A compares PPC-1 prostate cancer cell killing by free doxorubicin (solid squares) to doxorubicin encapsulated within lysolipid-containing temperature sensitive liposomes (TSL, open circles) after incubation for 24 hours. The slow dox release from the TSL requires about an order of magnitude higher dox concentration ($p = 0.01$ for all dox concentrations except 250 nM where $p = 0.05$) to reach equivalent PPC-1 cell toxicity, which shows the difficulty reconciling good doxorubicin retention and therapeutic levels of drug release. The better doxorubicin is retained in the liposome, the harder it is to achieve a toxic level of active doxorubicin, even when the doxorubicin is allowed to accumulate within the confines of the small volume of the cell culture well. It is likely that this difficulty would be exacerbated *in vivo*, as it is difficult to confine the doxorubicin once it is released in a tumor environment.^[30]

Fig. 7B shows that 3 minutes of irradiation of LTSL/HGN with 250 nM total doxorubicin with 0.8 (triangle) or 1.2 W/cm^2 (diamond) of 800 nm light showed a significant ($p < 0.001$),

light-dose dependent increase in toxicity after 48 hours compared to the un-irradiated LTSL/HGN. At 0.8 W/cm², 55 ± 6% of the PPC-1 cells were killed; 84 ± 6% of the PPC-1 cells were killed when irradiated at 1.2 W/cm², compared to 16 ± 9% cell killing with no irradiation. About 50 times the dox concentration in non-irradiated LTSL/HGN was required to reach the levels of cell toxicity as the LTSL/HGN irradiated at 1.2 W/cm².

Fig. 7C shows the time evolution of cell killing for 250 nM free dox, LTSL/HGN without irradiation, or LTSL/HGN with NIR irradiation with 0.8 or 1.2 W/cm² power density for 3 minutes, all with a total of 250 nM dox concentration. Without NIR irradiation, after 48 hours, 16 ± 9% cell killing occurs by non-irradiated LTSL/HGN and 46 ± 10% toxicity was observed for cells treated with the same 250 nM concentration of free doxorubicin. LTSL/HGN irradiated with 0.8 W/cm² laser power showed equivalent cell killing as free doxorubicin after 48 hours (55 ± 6% vs 46 ± 10%), and a small, but significant increase in cell killing at 24 hours (35 ± 5% vs 25 ± 5%, $p = 0.05$). However, NIR-induced dox release from LTSL/HGN irradiated for 3 minutes at 1.2 W/cm² power density provided a near doubling of cell killing compared to free doxorubicin at 24 (55 ± 5% vs 25 ± 5%, $p = 0.001$) and 48 hours (84 ± 6% vs 46 ± 10%, $p = 0.001$).

Fig. 7D summarizes the comparisons for cell killing after 48 hours with and without irradiation for LTSL/HGN/Dox, LTSL/Dox (no HGN, similar to conventional thermosensitive liposomes), free doxorubicin, LTSL and HGN without doxorubicin, and untreated control cells 48 hours after initiation of treatment. There were minimal differences (*, $p < 0.01$) between any of the samples on irradiation except for LTSL/HGN/Dox, which showed an increase in cell killing from 16 ± 9% to 84 ± 6% ($p < 0.001$). This shows the value of rapid, NIR-light triggered drug release compared to the slow release due to simple permeation through the unperturbed membrane (compare to Fig. 7A,B). In the absence of HGN, there were minimal effects; irradiation of untreated PPC-1 cells for 10 minutes had no significant impact on cell viability or well temperature. LTSL/HGN/Dox showed equivalent cell toxicity ($p = 0.05$) without radiation (16 ± 9%) as LTSL/Dox both without (16 ± 5%) and with radiation (23 ± 7%).

What was surprising was the apparent synergism between irradiation and doxorubicin toxicity. Free doxorubicin at 250 nM killed 46 ± 10% in irradiated samples and 56 ± 5% without irradiation, which was not a statistically significant difference ($p = 0.05$). However, the equivalent amount of doxorubicin released by irradiation from the LTSL/HGN/Dox construct reproducibly (N=12) induced a higher toxicity of 84 ± 6% ($p = 0.001$, ** in Fig. 7D). During irradiation, the sample temperature gradually increased to 41°C, and returned to 37 °C within minutes after irradiation ended, so drug release was accompanied by short period of mild hyperthermia. Park et al. have shown that the toxicity of free doxorubicin increases when cells are heated to 42 °C for five minutes compared to cells held at 37 °C^[47]. Previous work with thermosensitive liposomes has shown that cell membrane permeability to doxorubicin increases with increasing temperature, and this increased membrane permeability leads to enhanced uptake of doxorubicin at 40–43°C.^[48–51] Mild hyperthermia is also known to reduce the drug resistance of tumor cells.^[52,53]

3. DISCUSSION

The challenge still facing liposomal drug carriers is initiating and controlling drug *release*, without compromising drug *retention*. Tethering HGN to LTSL provides a new method of photothermally triggering local drug release using low intensity, physiologically-friendly NIR laser light. An LTSL composition with 15 mol% MPPC, 4 mol% DPSE-PEG2000 and 81 mol% DPPC has low permeability to small, charged molecules at 37 °C, which provides excellent CF and doxorubicin retention for 48 hours, similar to other TSL formulations.^[20] What differentiates this new construct from existing thermosensitive liposomes is that the LTSL/HGN carries with it a novel mechanism of NIR-light induced rapid contents *release*, that does not compromise drug *retention*. Irradiating the LTSL/HGN at light intensities an order of magnitude less than that previously shown to damage skin^[42,44] provides a sufficient increase in the LTSL membrane temperature that drugs can be released without the need to alter the “bulk” temperature. This feature makes the rate of drug release less dependent on the LTSL/HGN concentration; the release rate does not depend on the collective heating of the solution, which is dependent on the HGN concentration as well as the laser intensity (Fig. 4).^[19,44] The great advantage of using NIR light to activate LTSL/HGN drug release is that tissue, blood, etc. are relatively transparent to 650–950 nm wavelength light, allowing NIR transmission in soft tissues at depths up to 10 cm.^[19,32,44,54,55]

A uniform, elevated temperature distribution in a tumor is difficult to control due to variable thermal conductivities of different tissues, blood and bone, as well as the natural convective losses due to blood and fluid flow. The temperature rise in off-target tissues must be limited to prevent thermal damage as well as off-target drug release.^[5] In all previous applications of TSL, hyperthermia has been externally applied via bulk heating with water baths, radiofrequency (RF) or microwave (MW) radiation, lasers, and ultrasound.^[16–18,25] Maintaining constant, regional hyperthermia throughout the volume to be dosed is no longer required for drug release from the LTSL/HGN. The rapid inter-conversion of light energy into heat along with the nanometer scale of the HGN makes the temperature response extremely rapid, on the order of seconds or less. This makes it feasible to rapidly turn drug release on and off from the LTSL (Fig. 5), without damaging the ability of the LTSL to retain or release drugs (Fig. 6). This allows for the possibility of multiple doses delivered from the same liposomal carriers. The total energy supplied by the laser over the 1–5 minutes necessary for drug release does not result in significant bulk heating; it is possible for the HGN to heat only the LTSL membrane to initiate drug release,^[56,57] and release only occurs as long as the irradiation continues.

This is because continuous-wave irradiation at physiologically acceptable power densities does not result in large temperature differences between the “hot” nanoparticles and the surrounding liquid medium,^[34–36] as does femto- to pico-second pulsed irradiation.^[26,27,46,58–60] Continuous, low-power energy input, either from NIR lasers or from RF heating of magnetic particles, results in temperature differences between the nanoparticles and the surrounding fluid of order 10 °C which decay over 0.01–0.1 μm distances.^[34–36] For pulsed laser irradiation, the time for power input and conversion from light energy to thermal energy is faster (picoseconds) than the time for heat dissipation to the

surrounding liquid (nanoseconds), and all the energy input is confined to heating the nanoparticle.^[26,27,46,58–60] The HGN reach sufficiently high temperatures that vapor bubbles are formed, which rapidly expand and collapse, similar to the cavitation bubbles induced by ultrasound. These nanobubbles rupture the liposome membranes, causing burst release.^[27,28] For continuous wave heating, the time for power input (seconds) is much longer than that for heat equilibration with the surrounding fluid (microseconds); the nanoparticle temperature does not vary significantly from the solution temperature and quickly achieves a steady state.^[34,35]

The steady state temperature profile around a single nanoparticle in an infinite pool of liquid of thermal conductivity, k , with energy input per nanoparticle of Q , at a distance r from the nanoparticle, is $T = Q/4\pi kr$.^[34,35] The energy input per HNG, $Q \sim 10^{-12}$ W, giving a temperature increase for an isolated nanoparticle of $\sim 10^{-4}$ °C at $r \sim 1$ nm for water with $k = 0.6$ W/m-K. Hence, relying on the temperature gradient supplied by a single HGN is not likely to provide even the 3–4 °C change in temperature that that we require to initiate drug release from LTSL.

However, as shown in Huang et al.,^[34] a high *local* concentration of continuously heated nanoparticles can cause the temperature in the vicinity of the LTSL membrane to increase the necessary amount. The TEM images in Figs. 3A and 6, show that there are, on average, 2 or more HGN per 200 nm diameter liposome. If 10^{10} HGN/ml were distributed randomly in the solution, their average separation would be ~ 4500 nm. Tethering the HGN to the membrane increases the local HGN concentration, N , by decreasing the local separation around individual liposomes to ~ 200 nm.

There have been a limited number of indirect measurements of nanoparticle temperatures heated by light or RF^[34,35,41]. For iron oxide nanoparticles, Huang et al.^[34] used the temperature dependence of the fluorescence intensity of a streptavidin-coupled dye to show

$T \sim 10$ °C following RF heating. However, gold nanoparticles quench dye fluorescence in a distance dependent fashion, so similar measurements cannot be done for HGN.^[60] We estimate the local T (Fig. 4C) by the change in CF dye release from LTSL; CF release is an indirect measure of the average LTSL membrane temperature. Fig. 4C shows that $T \sim 2$ –3 °C at the light power densities used in this study. In order to take advantage of this small

T , we need to tether multiple HGN to a bilayer with a permeability transition close to 37 °C. The membrane composition needs to be such that there is a step-change in permeability at ~ 39 –41 °C, so that we can reach the permeability transition using laser powers (1.2 W/cm²) significantly less than those that induce skin damage (> 12 W/cm²).^[42–44]

Without laser-induced fast doxorubicin release, 50 times the liposomal doxorubicin concentration is required to induce equivalent cell toxicity over 48 hours (Fig. 7B). This illustrates the quandary of liposomal delivery: improving drug retention works against efficacy. For LTSL/HGN with 250 nM doxorubicin, toxicity increased from $16 \pm 9\%$ without irradiation to $55 \pm 6\%$ with 0.8 W/cm² and $84 \pm 6\%$ with 1.2 W/cm² NIR light exposure for 3 minutes. By separating the mechanism of drug release from that of drug retention, we can

retain the important encapsulation properties of the LTSL, while increasing the efficacy of the drug carrier.

Surprisingly, rapid drug release from the LTSL/HGN with laser heating offers the potential for more effective cell killing than free doxorubicin or mild heating separately.^[20,47] About 84% of PPC-1 cells were killed by doxorubicin released from LTSL/HGN irradiated at 1.2 W/cm² for 3 minutes compared to about 50% killed by the same concentration of free doxorubicin with or without irradiation (Fig. 7C, D). During irradiation, the sample temperature gradually increased to 41 °C at the end of 3 minutes of irradiation, and returned to 37 °C within minutes after irradiation ended. Doxorubicin membrane permeability increases with increasing temperature leading to enhanced uptake of doxorubicin during irradiation.^[48–51] Hyperthermia is also known to reduce the drug resistance of tumor cells.^[52,53] Clinical trials of soft-tissue sarcoma showed increased patient response rate when chemotherapy was delivered in combination with hyperthermia at 43 °C.^[61] Park et al. have shown that the toxicity of free doxorubicin increases when cells are heated to 42 °C for five minutes compared to cells held at 37 °C.^[47] We find that even short exposures to mild hyperthermia in combination with doxorubicin leads to significant increases in PPC-1 cell toxicity, which shows the potential synergism of doxorubicin delivered by LTSL/HGN with laser-triggering via NIR light.

4. CONCLUSIONS

We present a novel drug delivery carrier consisting of plasmonic hollow gold nanoshells (HGN) chemically tethered to liposomes made temperature sensitive with lysolipids (LTSL). Continuous-wave irradiation by physiologically friendly near infra-red light at 800 nm for 2.5 minutes at laser intensities an order of magnitude below that known to damage skin generates heating localized to the liposome membrane. The heating increases the liposome permeability via a transient pore mechanism in an irradiation dose-dependent, but reversible manner, resulting in rapid release of small molecules such as the self-quenching dye carboxyfluorescein or the chemotherapeutic doxorubicin. This enables precise spatial and temporal control of contents release with low temperature gradients confined to areas irradiated by the laser focus. The LTSL/HGN separates the mechanism of drug retention from drug release, making it possible to optimize retention and release simultaneously. The LTSL/HGN shows an interesting synergistic effect of local hyperthermia resulting in a near doubling of androgen resistant PPC-1 prostate cancer cell toxicity compared to the same concentration of free doxorubicin.

5. Experimental Section

Thermosensitive Liposome Preparation

DPPC, DSPE-PEG²⁰⁰⁰-NH₂, DSPE, and MPPC (Avanti Polar Lipids; Alabaster, Al) were dissolved in chloroform in glass vials and the solvent removed by evaporation. Carboxyfluorescein 5,6 (CF) was dissolved in NaOH in 65 mM PBS at pH 7.4 buffer to achieve a final 50mM CF solution. Doxorubicin (Fisher Scientific; Pittsburgh, Pa) was dissolved at 10 mg/ml in a 10% sucrose solution. The dried lipids were hydrated overnight at 55 °C with either 50 mM CF or 300 mM ammonium sulfate, and extruded using an Avanti

Mini-Extruder (Alabaster, AI) with Watson 200 nm polycarbonate filters. Terminal amine groups were converted to thiol moieties by adding 2-iminothiolane (Traut's Reagent) at a 1:1 molar ratio of DSPE-PEG²⁰⁰⁰-NH₂:Traut's Reagent and incubating for 1 hour at 55 °C. For doxorubicin-containing liposomes, a MicroSpinTM G-50 column (GE Healthcare) was used to exchange external ammonium sulfate for PBS at pH 7.4 prior to thiolation and remove excess Traut's Reagent following the reaction. Doxorubicin was added at a 0.05 drug:lipid ratio and incubated overnight at 36°C to achieve > 90% doxorubicin loading. Unencapsulated CF or doxorubicin was removed by size exclusion chromatography using a PD MiniTrapTM G-25 column (GE Healthcare) equilibrated with PBS at pH 7.4. Doxorubicin encapsulation was calculated by measuring fluorescence intensity of eluted fractions following liposomal lysis with Triton X-100 using a Varian Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies). The lipophilic carbocyanine tracer DiD (Invitrogen) at 0.05 mol% was used to identify the liposome-containing fractions, and the doxorubicin fluorescence emission intensity of these fractions was integrated to determine the final doxorubicin concentration.

Hollow Gold Nanoshell Synthesis and Tethering

Hollow gold nanoshells (HGN) were synthesized using a galvanic replacement reaction as described previously.³⁰ Silver and gold concentrations were adjusted to center the nanoshell absorbance peak maxima at 800 nm as measured on a Jasco V-530 UV/vis spectrometer. HGN diameter and shell thickness was determined via cryo-TEM (FEI Technai Sphera G2). HGN concentration and size distributions were measured using single particle tracking with a Nanosight NTA 2.3 particle-tracking device. HGN were stabilized against aggregation in high ionic strength media by adding a surface coating of thiol-terminated methoxypolyethylene glycol (mPEG⁷⁵⁰-SH). The ratio of gold to mPEG⁷⁵⁰-NH₂ was optimized to give good stability against aggregation in PBS, while still leaving enough free gold surface to bind to the thiols on the liposomes. The HGN were washed three times by centrifugation at 12,000×g for 10 min and re-dispersed in PBS buffered to pH 7.4. Nanoshells were tethered to the exterior liposome membrane by incubating PEGylated nanoshells with the liposomes overnight at a 4:1 ratio of nanoshells to liposomes.

About 2 µl of sample at 1 to 2 mg/ml lipid was spread as a thin film onto lacey carbon TEM grids (Electron Microscopy Sciences) at > 95% humidity and 25°C within a Vitrobot (FEI) sample freezing apparatus, and then rapidly frozen by rapid plunging into liquid ethane.^[62,63] Cryo-TEM imaging was performed on an FEI Technai Sphera G2 microscope operated at 200 kV with a Gatan liquid nitrogen specimen cryo-holder using low-dose conditions.

Laser Induced Dye Release

CF release was measured in semi-micro optical glass cuvettes (Starna) within a custom, temperature-controlled fluorescence spectrometer that was coupled to a continuous wave laser diode for irradiation. 200 µl of sample was placed within a cuvette of 4 mm width and 10 mm path length. The temperature was controlled using a qpod 2e® Peltier sample compartment (Quantum Northwest). Irradiation was performed with a continuous wave laser diode with 797 nm wavelength (Coherent F6 Series) to match the resonance peak maxima of

the nanoshells, and the beam diameter was adjusted to 5 mm to ensure that the entire sample volume was irradiated. Incident laser power was varied using an ITC4000 controller (ThorLabs) and calibrated using a PM30 Optical Power Meter (ThorLabs). Sample heating was measured with a thermocouple in solution linked to an Omegaette H306 digital thermometer (Omega). At discrete intervals, fluorescence was measured by exciting the sample with a 475 nm LED source (Ocean Optics, LS-475 Mikropack) and measuring the emission spectra with a Maya2000 Pro Spectrometer (Ocean Optics). Fluorescence emission spectra were integrated over the range of 510 to 530 nm, and dye release was calculated as

$$\% \text{Release} = \frac{I(t) - I_0}{I_{\text{Lysis}} - I_0}$$
 where $I(t)$ was the intensity at a given time, I_0 was the intensity prior to heating or irradiation, and I_{Lysis} was the intensity accompanying complete release following liposomal lysis with Triton X-100.

Doxorubicin Treatment of Cancer Cells

The androgen resistant human prostate cancer cells (PPC-1) were a generous gift from Erkki Rouslahti (Sanford-Burnham Medical Research Institute, University of California, Santa Barbara, CA). They were grown in DMEM/high glucose medium with phenol red (Invitrogen), supplemented with 10% FBS (Invitrogen). Cells were incubated at 37 °C in 5% CO₂ atmosphere. Cells were plated in 96-well plates (BD Falcon) at 8000 cells per well in 90 µL of medium. After 24 hours, cells were treated with the LTSL/HGN, control LTSL without HGN, and HGN alone and immediately irradiated with the laser. The Coherent F6 Series continuous wave laser diode was collimated to a beam diameter of 8 mm to irradiate the plate well and incident laser power was adjusted using an ITC4000 controller (ThorLabs). Irradiation was performed within a temperature controlled chamber to ensure a sample temperature of 37°C. Cell viability was quantified at 5, 24, and 48 hours following irradiation using a resazurin-based assay by adding 10 µl PrestoBlue® (Invitrogen) to each well, incubating for 1 hour, and measuring the fluorescence signal on a Tecan Infinite 2000 Pro plate reader. Each treatment was performed 3 times with at least 4 replicates per treatment, and the results were averaged and normalized with respect to the cell-only control. Calibration curves for the assay showed a linear response at all cell densities used.

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Figure 1. Schematic diagram of a lysolipid temperature sensitive liposome/hollow gold nanoshell (LTST/HGN). The HGN are coupled via a thiol-PEG linker to the LTSL membrane. At 37 °C, the LTSL membrane is in the solid phase, with low permeability to hydrophilic drugs. The HGN adsorb NIR light and convert the light energy into heat, which raises the membrane temperature, initiating lysolipid-stabilized pores at membrane solid-fluid coexistence from 39–41 °C, which dramatically increase the membrane permeability.

Figure 2.

(A) Carboxyfluorescein (CF) release after 2.5 minutes at a given bulk temperature from DPPC:4 mol% DSPE-PEG²⁰⁰⁰ liposomes with: 0 mol% MPPC and DSPC (squares); 15 mol % MPPC and 0 mol% DSPC (triangles), 15 mol% MPPC and 30 mol% DSPC (circles). Standard deviations are within the size of the data points. (B) Representative cryo-TEM images of extruded liposomes formed from DPPC:4 mol% DSPE-PEG²⁰⁰⁰ on addition of MPPC. For 0 and 10 mol% MPPC, liposomes are smooth with some faceting. For 15 mol% MPPC, the liposomes are more faceted than smooth. 20 mol% MPPC destabilizes the liposome structure by reducing the energy of the bilayer edge and stabilizing discs (shown by arrows).^[20,22,23]

Figure 3.

Cryo-TEM images showing nanoshells either (A) tethered to the exterior membrane or (B) freely suspended in solution. (C) LTSL with tethered or suspended HGN at 10^{11} HGN/ml were irradiated for 2.5 min in a chamber controlled at 37 °C. Release was observed at lower laser power when the HGN were tethered to the membrane than when they were freely suspended in solution, and minimal release was observed in the absence of HGN. Standard deviations are within the size of the data points.

Figure 4.

(A) Samples of different HGN concentration were held at 37 °C, irradiated for 2.5 minutes with various laser powers and the final bulk temperature was measured. The bulk temperature change at a given HGN concentration increases linearly with power density as expected from Eqn. 1. Increasing the HGN concentration also increases the bulk temperature change at a given power density, also consistent with Eqn. 1 (B) CF release profiles and final sample temperatures for LTSL/HGN initially held at the temperature given by the dotted line (at a given CF release) after irradiation with 1.2 W/cm² of laser power (filled squares) for 2.5 minutes. Open squares show CF release profiles for LTSL/HGN without laser irradiation at different bulk sample temperatures (open squares). Irradiated LTSL/HGN release their contents at lower bulk temperatures than a non-irradiated sample, which gives an estimate of the effects of local heating on release (red arrow) relative to the collective effect of the HGN on raising the sample temperature (black arrow). Standard deviations are within the size of the data points. (C) Total temperature increase measured by CF dye release at a given power density divided into bulk heating (hatched region, corresponds to black arrow in Fig. 4B) and local heating (gray region, corresponds to red arrow in Fig. 4B) provided by laser irradiation at a given power density. The bulk heating is dependent on the LTSL/HGN concentration; the localized heating does not depend on LTSL/HGN concentration. ** signifies $p < .01$ regarding difference between total heating and local heating, * signifies $p < 0.05$.

Figure 5.

(A) The rate of release from LTSL/HGN depends on laser power density, with more rapid release at higher power density and more gradual release at lower power density. (B) LTSL/HGN encapsulating CF held at 37°C for 22 hours, $13 \pm 4\%$ of the dye was lost due to passive leakage. On irradiation for 30 sec at 1.6 W/cm^2 laser power density, $35 \pm 5\%$ of the encapsulated dye was released (total release after 24 hr is $48 \pm 5\%$). The remaining CF was retained within the LTSL/HGN for the following 24 hours ($49 \pm 5\%$ after 48 hours, $p = 0.05$). An additional $35 \pm 5\%$ of CF (total CF release of $85 \pm 5\%$) was released by a second 60 second pulse of 1.6 W/cm^2 intensity after 46 hours at 37 °C. Standard deviations are within the size of the data points.

Figure 6.

Cryo-TEM images of (A,B) doxorubicin containing LTSL/HGN prior to irradiation and contents release. Multiple HGN are tethered to each LTSL. The arrows point to doxorubicin precipitates. Compare to Figs. 3A,B. (C, D) After 3 minutes of 1.2 W/cm² laser irradiation with 800 nm NIR light, the LTSL remain sealed and there is no difference in the tethering of the HGN to the LTSL. The HGN retain their hollow shape and ~ 40 nm diameter. Only the doxorubicin precipitates have disappeared, consistent with release during radiation. These images are consistent with only minor changes in temperature around the HGN during

irradiation and a permeability transition due to transient pores opening up and closing with temperature.

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Figure 7.

(A) PPC-1 prostate cancer cell killing by free doxorubicin (solid squares) compared to doxorubicin encapsulated within lysolipid-containing temperature sensitive liposomes (TSL, open circles) after incubation for 48 hours. The slow dox release from the TSL requires about an order of magnitude higher dox concentration to reach equivalent PPC-1 cell toxicity. This shows the excellent retention of doxorubicin in the LTSL, as well as demonstrating the difficulty reconciling good doxorubicin retention and therapeutic levels of drug release. (B) Cell death measured 48 hours after introduction of LTSL/HGN to PPC-1

prostate cancer cells at different liposomal doxorubicin concentrations (squares); and after 3 minutes of laser irradiation at 0.8 (triangle) and 1.2 W/cm² (diamond) laser intensity for 250 nM LTSL/HGN doxorubicin. More than 50 times the liposomal doxorubicin concentration is required to affect the same level of cell toxicity over 48 hours as the laser-released doxorubicin. (C) Cell death measured 5, 24, or 48 hours following irradiation. Cells were treated with 250 nM doxorubicin encapsulated within LTSL/HGN and irradiated for 3 minutes at 0.8 (triangles) or 1.2 W/cm² (diamonds); with 250 nM free doxorubicin without irradiation (circles) or with 250 nM doxorubicin in LTSL/HGN without irradiation (squares). The combination of 1.2 W/cm² irradiation and 250 nM doxorubicin resulted in roughly twice the cell killing than the 250 nM free doxorubicin at 24 and 48 hours ($p < 0.01$). (D) Total cell killing after 48 hours for all samples and controls with and without irradiation. NIR irradiation for 3 minutes increased cell killing from 16% to 84% for TSL-HGN-Dox ($p < 0.001$). Irradiation of the LTSL/HGN/doxorubicin construct provided a significantly greater ~ 84% cell killing compared to ~ 50% cell killing for the same concentration of free doxorubicin (** $p < 0.01$). No significant differences between cell killing were found for irradiated or non-irradiated free dox, TSL-Dox, HGN, TSL-HGN, TSL or cell only control (* $p > 0.01$). No statistical differences were found between cell killing for HGN, blank TSL-HGN, TSL and cell only control, with or without irradiation. Doxorubicin was required to induce significant cell killing, either in free or liposome form. (Overall statistical analysis by one-way ANOVA with $p < 0.01$; individual pairs of irradiated vs non-irradiated samples were compared by two-tailed t-test; comparison of free dox to TSL-HGN dox and TSL-HGN-Dox with and without irradiation by two-tailed t-test.)