

Cooperative Nanoparticles for Tumor Detection and Photothermally Triggered Drug Delivery

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The ability of one structural type to perform multiple medical diagnostic or therapeutic functions is often cited as an advantageous characteristic of nanomaterials that cannot be achieved with organic small molecules.^[1–3] Although there are now many examples of nanosystems that integrate multiple functions into a single structure, the designs can reduce the efficacy of the individual functions due to space and surface-chemistry limitations in the tiny platforms. For example, magnetic nanoparticles and drug molecules can be co-encapsulated in liposomes to simultaneously perform multiple functions, such as magnetic resonance imaging, magnetic drug delivery and hyperthermia,^[4] but the loading capacity and the stability are typically compromised relative to a single-component liposome. There has been some effort to develop intrinsically multifunctional nanomaterials such as magnetic nanocapsules and luminescent porous silicon nanoparticles to overcome such problems,^[5,6] although these more complicated structures may lose versatility in terms of the types of payloads they can carry. Access to the payloads can also be limited, reducing the ability to control their release. Separating functions into two or more nanoparticle formulations is one means to simplify the problem. If two separate nanomaterials can be engineered to synergistically cooperate in their diagnostic or therapeutic functions, it is possible that the overall dosage can be reduced, minimizing side effects and providing a safer transition to the clinic.

Combination therapies are commonly employed in a wide range of cancer treatments. In particular, hyperthermia can increase the concentration of an administered therapeutic

nanoparticle in a tumoral region by increasing blood flow and vessel permeability.^[7,8] Hyperthermia can also enhance drug toxicity in cancer cells that are otherwise resistant to chemotherapeutics.^[9] Furthermore, local hyperthermia can improve the accumulation of a drug, which is encapsulated in a thermosensitive carrier.^[10,11] The combination of hyperthermia and chemotherapeutics can, therefore, be employed synergistically to treat high-risk tumors with a goal of total tumor eradication. From a clinical perspective, precise and site-specific heat transfer to a diseased site would improve the safety and efficacy of thermal cancer therapies.

Recent advances in the field of plasmonic nanomaterials have presented new opportunities for localized hyperthermia therapy. Plasmonic nanomaterials are metallic structures that efficiently convert optical radiation into heat by coupling into one or more plasmon modes.^[12,13] Of particular interest are gold nanorods (GNRs) due to their large optical coefficients in the NIR region of the spectrum, where living tissue is highly transparent.^[14,15] Previously, we and other groups demonstrated that GNRs can be modified to circulate for long periods of time in the blood stream and passively accumulate in tumors *in vivo*, where they can be heated with localized NIR radiation to selectively destroy malignant tissue regions.^[16–18] In addition, the optical properties of GNRs or gold-based nanoparticles have been harnessed to image targeted tissues *in vivo*.^[19–21] In this Communication, we hypothesized that GNRs could be used to detect a diseased site and act as tumor-specific triggers to amplify the therapeutic function of a circulating drug carrier (Fig. 1a). We find that GNR-mediated photothermal heating is highly localized in tumors and significantly improves the selectivity and efficacy of cancer treatment with thermosensitive drug carriers.

Cetyltrimethylammonium bromide (CTAB)-coated GNRs were coated with a mixed monolayer of poly(ethylene glycol) (PEG) and surface-enhanced Raman scattering (SERS)-active reporter molecules to increase circulation times, reduce *in vivo* toxicity, and provide remote imaging as described previously.^[18,22] The gold cores appear as dark rods in the transmission electron microscopy (TEM) image (Fig. 1b), with an average width of ~13 nm and length of ~47 nm. The PEG coating is observed as a faint halo around the metal cores. The wavelength of maximum optical absorption of the GNR is 800 nm (Supporting Information, Fig. S1). As observed previously, intravenously injected PEG-coated GNRs were observed to circulate in the blood stream with a half-life of >17 h in mice, enabling passive accumulation in a xenografted MDA-MB-435 human melanoma tumor through the porous vascular structures.^[18] Passive accumulation in tumors allowed tunable photothermal heating selectively in the tumor region by excitation with an 810 nm laser

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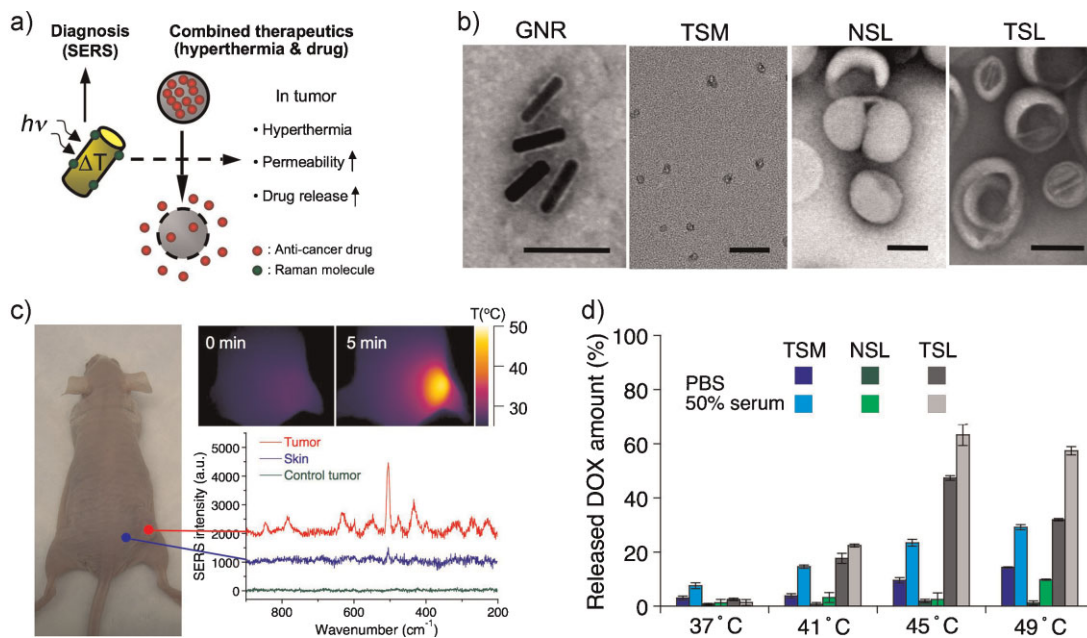


Figure 1. Characterization of the two components of a cooperative nanosystem. a) Schematic diagram depicting the cooperative nanomaterials system. b) TEM images of PEG-coated GNR, TSM, NSL, and TSL. The scale bars are all 100 nm. c) Optical image, thermal images, and Raman spectra of a mouse depicting the multifunctionality of the GNRs. The GNRs act as thermal transducers or as SERS contrast agents when irradiated with a laser of the appropriate wavelength and intensity. The mouse, bearing a MDA-MB-435 tumor, was injected intravenously with GNRs (10 mg Au kg^{-1}). The IR thermographic maps shown were obtained 24 h post-injection; the left image was acquired immediately before and the right image 5 min after onset of irradiation with a diode laser ($\lambda = 810 \text{ nm}$, 0.75 W cm^{-2}). Raman spectra (5 acquisitions of 60 s each) were acquired from the tumoral region (red trace) and from skin near the tumor (blue trace). The GNRs in this experiment were labeled with a cyanine dye and the peaks in the trace correspond to the SERS spectrum cyanine dye (verified by in vitro control). d) Amount of DOX released in vitro from the liposome and micelle formulations used in this study, as a function of temperature. The samples were incubated for 10 min at the indicated temperatures and the amount of free DOX was quantified by fluorescence spectroscopy, relative to a standard curve. Values represent the mean and the error bars indicate standard deviation.

(Fig. 1c and Fig. S2). As has been observed with SERS-tagged gold nanoparticles,^[20,22] the SERS spectrum of a surface-embedded cyanine dye on the GNRs was evident in Raman spectra obtained from the tumor in vivo (Fig. 1c and Fig. S3). Thus, the GNR formulation used in this study is an effective hyperthermia agent that can be observed in vivo with spectroscopic imaging methods.

A second nanostructure that could deliver a drug in response to the thermal stimulus from the GNRs was synthesized using a modification of published preparations.^[23,24] The formulation contains thermally responsive lipid constituents chosen, in part, for their potential for translation to clinical studies. Three preparations were studied: a thermally sensitive liposome (TSL, $132.4 \pm 7.2 \text{ nm}$ hydrodynamic size), a control liposome that is not thermally sensitive (NSL, $137.1 \pm 4.9 \text{ nm}$ hydrodynamic size), and a thermally sensitive micelle (TSM, $25.9 \pm 2.1 \text{ nm}$ hydrodynamic size). All three sample types were prepared with the chemotherapeutic agent doxorubicin (DOX) in this study. The TSL displayed similar thermal properties to the published formulation.^[23,25] The TSLs released $\sim 50\text{--}60\%$ of their drug contents in a short period of time ($\sim 30 \text{ s}$) when heated to 45°C , while the nonthermally sensitive liposomes showed no significant drug release (Fig. 1d and Fig. S4). The micellar TSMs exhibited thermal sensitivity similar to that of the TSL, but they released their DOX payload more slowly at 45°C .

We next investigated if the thermally sensitive drug carriers could deliver DOX into cancer cells at increased temperature in vitro (Fig. 2a). In the absence of drug, the cells were observed to tolerate temperatures of $\sim 45^\circ\text{C}$ without any significant indications of toxicity. In the presence of the drug, free DOX molecules rapidly internalized into cells and bound to nuclei either with or without heating, although more DOX was observed in the nuclei of the cells heated to 45°C . Of the three types of nanoparticles, TSLs delivered the largest quantity of DOX to cells when heated, while TSMs exhibited much lower DOX delivery. As expected, NSLs showed no temperature-induced DOX release, consistent with the lack of thermally responsive lipids in their structure. None of the three nanoparticle types displayed significant internalization of DOX into cells at 37°C .

GNR-mediated photothermal heating significantly increased DOX-related toxicity towards cancer cells compared with control samples heated in an incubator (Fig. 2b and Fig. S5). In agreement with previous results,^[9] free DOX molecules, as well as the TSMs and the TSLs, displayed slightly greater cytotoxicity at the higher temperature (45°C) while NSLs showed no toxic effect on the timescale of the experiments. Importantly, the cytotoxicity of free DOX and TSLs was significantly enhanced when exposed to controlled GNR-mediated photothermal heating (45°C , $P < 0.05$; Fig. 2b). The result suggests that photothermal heating by GNRs not only enhances cytotoxicity of DOX molecules, but it

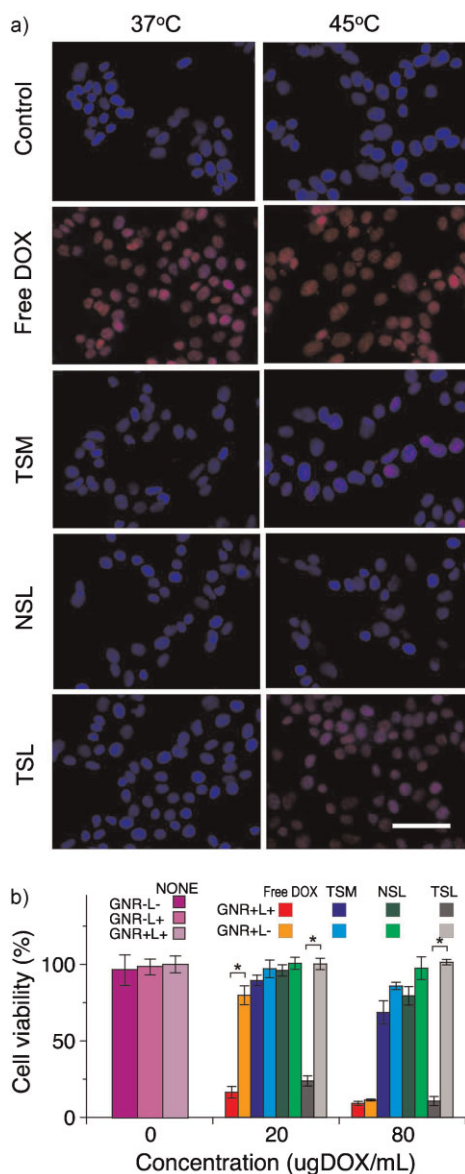


Figure 2. Temperature-induced intracellular drug delivery and cytotoxicity of the cooperative nanosystem. a) Images showing the intracellular delivery of DOX (red) from various therapeutic nanoparticle formulations to MDA-MB-435 human melanoma cells, at two different temperatures. The cells were incubated with free DOX, TSM, NSL, and TSL for 15 min at the indicated temperatures in a cell incubator. All formulations contain $80 \mu\text{g mL}^{-1}$ of DOX. Samples were rinsed three times with cell medium, then incubated for an additional 1 h at 37°C and imaged. The nuclei were stained with 4'-6-diamidino-2-phenylindole (DAPI, blue). The scale bar indicates $100 \mu\text{m}$. b) Cytotoxicity of various photothermally activated therapeutic nanoparticle formulations to MDA-MB-435 human melanoma cells, quantified by MTT assay ($*P < 0.05$). The cells were incubated with free DOX, TSMs, NSLs, and TSLs at the indicated concentrations. GNR+ and GNR- indicate the presence or absence of GNRs ($7 \mu\text{g Au mL}^{-1}$) in the mixture, respectively. L+ and L- indicate the presence or absence of NIR irradiation (810 nm , 0.75 W cm^{-2} for 15 min), respectively. After irradiation (or 15 min in the dark for L- samples), the cells were rinsed three times with the cell-culture medium and incubated for an additional 48 h at 37°C before administration of the MTT assay. Values represent the mean and the error bars indicate standard deviation.

also induces DOX release from the heat-sensitive drug carriers, thereby, enhancing the therapeutic effect.

Consistent with the in vitro results, we found that heating of thermosensitive nanoparticles also improves drug accumulation in tumors in vivo. We first tested the ability of the thermosensitive nanoparticles to release their contents (NIR fluorescent dyes) in vivo by heating the tumoral region of the mouse with an external water bath at 45°C (Fig. 3a and Fig. S6). Significant fluorescence intensity was observed in the heated tumors relative to the unheated control tumors. We attribute the increased nanoparticle uptake to heat-induced dilation of the vascular pores feeding the tumors.^[8,10,11] Additionally, bladders from the mice injected with TSM or TSL containing a surface-attached or encapsulated fluorescent dye (TSM-VT750 and VT750@TSL, respectively) displayed strong fluorescence signals relative to the other formulations, suggesting that these fluorescent payloads are released into the blood stream selectively upon heating. However, the data indicate that TSMs are readily dissociated into free lipids in vivo at either temperature studied (Fig. S6).

Having established that application of external heat can trigger drug release in a tumor, we next tested the ability of injected GNRs to improve nanoparticle drug delivery using the laser-induced photothermal effect. The biodistribution of NSL and TSL upon photothermal heating was similar to that of other nanomaterials, with a tendency to undergo significant clearance into the mononuclear phagocyte system (MPS)-related organs (liver and spleen, Fig. 3b).^[6,20,26] However, we found that the quantity of DOX accumulated in the GNR-heated tumors was comparable to that observed in the MPS-related organs, particularly for the TSM and TSL formulations. Strikingly, the TSLs combined with GNR-mediated heating resulted in a $\sim 12 \times$ larger quantity of DOX accumulating in the heated tumor relative to an unheated tumor. Such a significant excess is difficult to achieve with actively targeted nanomaterials (i.e., materials containing molecular species that bind specifically to tumors or tumor-related tissues).^[20,26–28] The excess can be attributed to a combination of two factors: an increase in uptake of nanoparticles through heat-dilated vessel pores and an increase in diffusion of drug out of the nanocarriers. Once free of the carrier, DOX molecules are known to bind efficiently to cancer cells in vivo.^[29] TSM and NSL formulations also showed greater accumulation of DOX in GNR-heated tumors, although the quantities are statistically lower than observed for TSLs ($P < 0.05$). Free DOX molecules did not accumulate in tumors, even with GNR-mediated heating, presumably due to their short blood half-life ($\sim 5 \text{ min}$) and small molecular size relative to the nanoparticle carriers. The blood half-life of the liposomal and micellar formulations is much greater ($\sim 3 \text{ h}$), and the data illustrate the significant advantage of using encapsulants for delivery of short-lived or toxic therapeutics. Microscopic histological analysis of the tumor tissues supports the macroscopic quantitative DOX biodistribution data. Upon photothermal heating, significant quantities of DOX were observed in the extravascular region of the tumor for the TSL formulations, while DOX delivery to the tumor was somewhat limited for NSL and TSM samples (Fig. 3c).

Finally, we investigated longer-term therapeutic efficacy of the cooperative nanosystem for treatment of a human xenograft tumor in the mouse model (Fig. 4a–c). We found that the

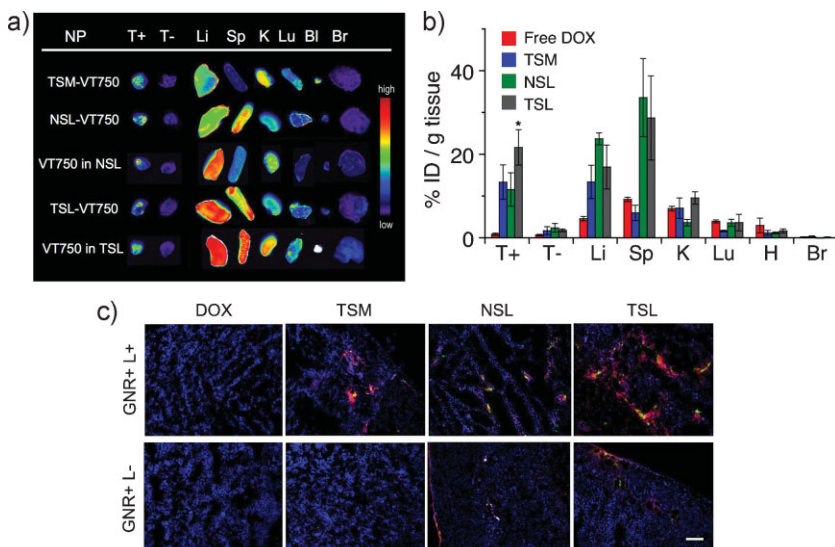


Figure 3. Fate of thermally responsive nanostructures in vivo. a) Fluorescence images of organs from mice, where one tumor was heated at 45 °C for 30 min immediately after injection of various VT750-labeled nanoparticle formulations. The NIR-fluorescent molecular probes VT750 (750 nm excitation, 780 nm emission) were either attached to the nanoparticle surface (TSM-VT750, NSL-VT750, and TSL-VT750) or encapsulated in the internal space of nanoparticles (VT750@NSL and VT750@TSL). Mice bearing bilateral MDA-MB-435 tumors were intravenously injected with various VT750-labeled nanoparticle formulations and one tumor was immediately heated at 45 °C for 30 min in a temperature-controlled water bath. At 2 h post-injection, the tissues were collected from the mice and imaged with a NIR fluorescence imaging system. b) Biodistribution of DOX for various therapeutic nanoparticle formulations after GNR-mediated photothermal heating. Mice bearing bilateral MDA-MB-435 tumors were injected with GNRs (10 mg Au kg⁻¹). At 72 h post-injection, the indicated free DOX or therapeutic nanoparticle formulation was administered and one tumor was immediately irradiated (810 nm, ~0.75 W cm⁻²) for 30 min while maintaining an average tumor surface temperature of ~45 °C under IR thermographic surveillance. 24 h post-injection of the therapeutic nanoparticles, the tissues were collected from the mice and the native fluorescence intensity from DOX was quantified as a percentage of injected dose per tissue mass. Values represent the mean and the error bars indicate standard deviation. The differences in DOX concentration released from TSL in the heated tumor, relative to the other formulations (TSM and NSL), are significant (**P* < 0.05). c) Histological analysis of DOX distribution in MDA-MB-435 tumors treated with free DOX or the indicated therapeutic nanoparticle formulations and then subjected to GNR-mediated photothermal heating. The tissues were collected for histological analysis 6 h post-injection of the therapeutic nanoparticles (red: DOX, blue: DAPI nuclear stain, green: Alexa Fluor 488 labeled on the therapeutic nanoparticles). Abbreviations: NP, nanoparticle; T+, tumor heated to 45 °C; T-, tumor maintained at ambient temperature; Li, liver; Sp, spleen; K, kidney; Lu, lung; Bl, blood; Br, brain; H, heart. The scale bar indicates 200 μm.

combination of GNR-mediated heating and the presence of the thermosensitive therapeutic nanoparticles significantly inhibits tumor growth without displaying significant systemic toxicity. Controls using the individual components or combinations using non- or weakly thermosensitive therapeutic nanoparticles, with or without laser irradiation, proved to be less effective.

In summary, we demonstrate that a pair of synthetic nanoparticles can work together to detect a diseased site and more effectively deliver chemotherapeutics to the site than individual nanoparticle treatments. This system relies on GNR transduction of an external optical signal into a tumor-specific thermal signal that enhances recruitment of circulating drug carriers into the tumor and triggers drug release from the carriers. GNRs localized in tumors can be identified in vivo by their intense SERS signals, making it possible to use this technology in both diagnostic and therapeutic applications. It is

important to point out that non-specific uptake of the drug carriers in the MPS-related organs still remains a problem with this cooperative nanosystem, although it is significantly reduced relative to conventional therapies using single targeted drug carriers. The data presented here show that use of cooperative nanomaterials, in which each of the components has a dedicated function, can be effective at treating diseased tissues. Furthermore, it illustrates the potential advantage of dual therapeutic nanomaterials in the precision treatment of more drug-resistant cancers.

Experimental

GNR Preparation: GNRs containing a CTAB coating and displaying a peak plasmon resonance at 800 nm were purchased from Nanopartz, inc. and their CTAB coating was replaced with PEG (5kDa) molecules. To identify particles SERS, 3,3'-Diethylthiatriacarbocyanine iodide molecules were trapped into the PEG coating.

Therapeutic Nanoparticle Preparation: Nonthermally sensitive DOX liposomes, thermally sensitive DOX liposomes, and DOX-loaded TSMs were prepared using a modification of published preparations [23,24]. For fluorophore conjugation (Cy7 or Alexa Fluor 488), the amine-terminated liposomes and micelles were also prepared. The prepared nanoparticles were intravenously injected in vivo to ensure that TSM, NSL, and TSL exhibited all similar circulation times (blood half-lives for all: ~3 h).

Nanoparticle Characterization: TEM images of GNRs, TSMs, NSLs, and TSLs were obtained using a Hitachi H-600A transmission electron microscope. The hydrodynamic size of GNRs, TSMs, NSLs, and TSLs was obtained using a Zetasizer ZS90 dynamic-light-scattering machine (Malvern Instruments, Worcestershire, UK).

SERS Spectral Acquisition: A Horiba Jobin Yvon Labram HR800 spectrometer was used for recording SERS spectra. Spectra were acquired with a 785-nm diode laser. In vitro SERS spectra of the cyanine-dye-tagged PEG GNRs were acquired between 100 and 1900 cm⁻¹ of the Raman shift. For in vivo SERS

spectra of cyanine dye-tagged PEG GNRs, 24 h after intravenous injection, in vivo Raman spectra of the anesthetized mouse were obtained in the Raman shift range 100 to 1900 cm⁻¹, using acquisition times of 30 s (liver and spleen) or 60 s (tumors and skin). The Raman laser was focused on the anatomical regions corresponding to the tumor, liver, spleen, and the skin-near tumor.

In vitro Imaging and Cytotoxicity: For fluorescence microscopy, MDA-MB-435 human melanoma cells were incubated with 80 μg DOX/mL of free DOX, TSMs, NSLs, and TSLs per well for 15 min at 37 °C or 45 °C and rinsed three times with cell medium. The cells were then incubated for an additional 1 hr at 37 °C in the presence of 10% fetal bovine serum (FBS), fixed with 4% paraformaldehyde, stained, and observed with a fluorescence microscope (Nikon, Tokyo, Japan). Cytotoxicity of the in vitro cooperative nanosystem was performed using MDA-MB-435 human melanoma cells. Cells were incubated with free DOX, TSMs, NSLs, or TSLs at different concentrations and GNRs at 7 μg Au mL⁻¹ for 15 min at 37 °C or 45 °C in a cell incubator. For laser-induced (photothermal) heating, samples were irradiated for 15 min with ~0.75 W cm⁻² of 810-nm laser light, either in the

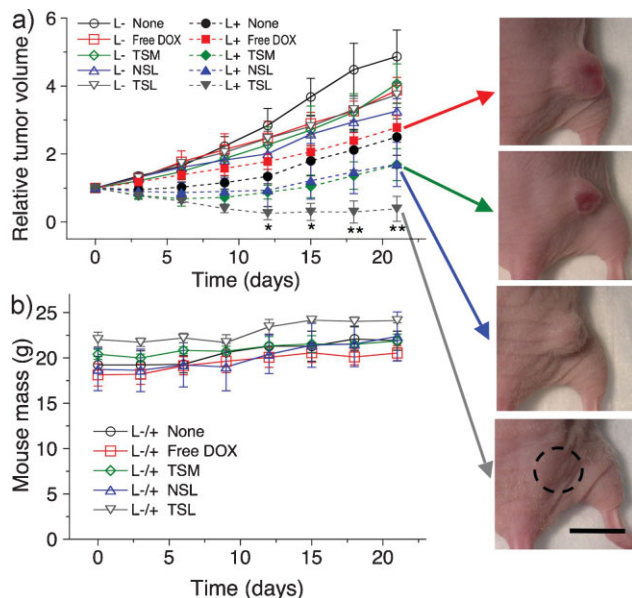


Figure 4. Tumor therapy using cooperative nanosystem. a) Relative tumor volume in different treatment groups ($n \approx 5-7$ mice per trace) for MDA-MB-435 human melanoma tumors. Mice bearing bilateral MDA-MB-435 tumors were injected with GNRs (10 mg Au kg^{-1}). After 72 h, a single dose of the indicated saline, free DOX, or various therapeutic nanoparticle formulations (3 mg DOX kg^{-1}) was administered through tail vein injection and one tumor was irradiated with a NIR laser (810 nm , $\sim 0.75 \text{ W cm}^{-2}$) for 30 min, while maintaining an average tumor surface temperature of $\sim 45^\circ \text{C}$ (monitored by IR thermographic surveillance). Tumor volumes were quantified every 3 d post-irradiation. The differences between TSL plus irradiation and all other groups were significant ($*P < 0.05$ for 12 and 15 days and $**P < 0.02$ for 18 and 21 days) in the tumor volume change curves. b) Representative images of treated tumors in the live animals for the indicated treatments (21 days post-treatment). Scale bar indicates 1 cm. c) Mouse mass as a function of days post-treatment for the indicated treatment groups. L+ and L- indicate animals whose tumors received NIR irradiation, or no irradiation, respectively, 72 h after GNR injection. Normal growth of all groups is observed for 3 weeks post-treatment. TSL, TSM, and NSL are the different nanoparticle formulations, defined in Figure 2.

presence or absence (control) of GNRs, while maintaining an average solution temperature of $\sim 45^\circ \text{C}$ (monitored by IR thermographic surveillance). After irradiation, the cells were rinsed three times with cell medium. The cells were then incubated for an additional 48 h at 37°C . The cytotoxicity of free DOX, TSMs, NSLs, and TSLs was evaluated using an MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Invitrogen).

In vivo Behavior of Thermosensitive Nanoparticles (Laser Photothermal Heating): First, GNRs were intravenously injected into mice bearing bilateral MDA-MB-435 human melanoma tumors (10 mg Au kg^{-1}). At 72 h post-injection, when the GNRs were completely cleared from the blood stream, therapeutic molecules or nanoparticles (5 mg DOX kg^{-1}) were systemically administered and either the right or the left flank (area where the tumor is located) was immediately irradiated with near-infrared (NIR)-light ($\sim 0.75 \text{ W cm}^{-2}$ and 810 nm) for 30 min, maintaining the average tumor surface temperature at $\sim 45^\circ \text{C}$ (monitored by IR thermographic observation). At 24 h post-injection of the therapeutic nanoparticles, the tissues were harvested, weighed, and homogenized to release DOX from the tissues. Following homogenization, the samples were centrifuged and supernatants of samples were analyzed for DOX

fluorescence using a fluorescence microplate reader (Molecular Devices, SpectraMax GeminiEM).

In vivo Therapeutic Studies: At 72 h post-injection of GNR (10 mg Au kg^{-1}), saline, therapeutic molecules or nanoparticles (3 mg DOX kg^{-1}) were systemically administered and one flank of the mouse (the area where the tumor is located) was either not irradiated or immediately irradiated with NIR-light ($\sim 0.75 \text{ W cm}^{-2}$ and 810 nm) for 30 min, maintaining an average tumor surface temperature of $\sim 45^\circ \text{C}$ (monitored by IR thermographic observation). Each therapeutic cohort included $\sim 5-7$ mice. After the single treatment, tumors were measured and mice were weighed at 3 day intervals over a period of 3 weeks. All animal work was performed in accordance with the institutional animal protocol guidelines in place at the Massachusetts Institute of Technology, and it was reviewed and approved by the Institute's Animal Research Committee. Student's t-test was used for statistical analysis of the results.

A full description of the methods is available as Supporting Information.

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- [1] J.-H. Park, G. von Maltzahn, E. Ruoslahti, S. N. Bhatia, M. J. Sailor, *Angew. Chem. Int. Ed.* **2008**, *47*, 7284.
- [2] K. C. Weng, C. O. Noble, B. Papahadjopoulos-Sternberg, F. F. Chen, D. C. Drummond, D. B. Kirpotin, D. H. Wang, Y. K. Hom, B. Hann, J. W. Park, *Nano Lett.* **2008**, *8*, 2851.
- [3] G. H. Wu, A. Milkhailevsky, H. A. Khant, C. Fu, W. Chiu, J. A. Zasadzinski, *J. Am. Chem. Soc.* **2008**, *130*, 8175.
- [4] L.-A. Tai, P.-J. Tsai, Y.-C. Wang, Y.-J. Wang, L.-W. Lo, C.-S. Yang, *Nanotechnology* **2009**, *20*, 135101.
- [5] Y. Piao, J. Kim, H. Bin Na, D. Kim, J. S. Baek, M. K. Ko, J. H. Lee, M. Shokouhimehr, T. Hyeon, *Nat. Mater.* **2008**, *7*, 242.
- [6] J.-H. Park, L. Gu, G. von Maltzahn, E. Ruoslahti, S. N. Bhatia, M. J. Sailor, *Nat. Mater.* **2009**, *8*, 331.
- [7] S. K. Huang, P. R. Stauffer, K. L. Hong, J. W. H. Guo, T. L. Phillips, A. Huang, D. Papahadjopoulos, *Cancer Res.* **1994**, *54*, 2186.
- [8] G. Kong, M. W. Dewhirst, *Int. J. Hyperthermia* **1999**, *15*, 345.
- [9] T. S. Herman, *Cancer Res.* **1983**, *43*, 517.
- [10] M. H. Gaber, N. Z. Wu, K. Hong, S. K. Huang, M. W. Dewhirst, D. Papahadjopoulos, *Int. J. Radiat. Oncol. Biol. Phys.* **1996**, *36*, 1177.
- [11] G. Kong, G. Anyambhatla, W. P. Petros, R. D. Braun, O. M. Colvin, D. Needham, M. W. Dewhirst, *Cancer Res.* **2000**, *60*, 6950.
- [12] L. R. Hirsch, R. J. Stafford, J. A. Bankson, S. R. Sershen, B. Rivera, R. E. Price, J. D. Hazle, N. J. Halas, J. L. West, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 13549.
- [13] M. Hu, J. Y. Chen, Z. Y. Li, L. Au, G. V. Hartland, X. D. Li, M. Marquez, Y. N. Xia, *Chem. Soc. Rev.* **2006**, *35*, 1084.
- [14] P. K. Jain, K. S. Lee, I. H. El-Sayed, M. A. El-Sayed, *J. Phys. Chem. B* **2006**, *110*, 7238.

- [15] R. Weissleder, *Nat. Biotechnol.* **2001**, *19*, 316.
- [16] T. Niidome, M. Yamagata, Y. Okamoto, Y. Akiyama, H. Takahashi, T. Kawano, Y. Katayama, Y. Niidome, *J. Controlled Release* **2006**, *114*, 343.
- [17] E. B. Dickerson, E. C. Dreaden, X. Huang, I. H. El-Sayed, H. Chu, S. Pushpanketh, J. F. McDonald, M. A. El-Sayed, *Cancer Lett.* **2008**, *269*, 57.
- [18] G. von Maltzahn, J.-H. Park, A. Agrawal, N. K. Bandaru, S. K. Das, M. J. Sailor, S. N. Bhatia, *Cancer Res.* **2009**, *69*, 3892.
- [19] H. F. Wang, T. B. Huff, D. A. Zweifel, W. He, P. S. Low, A. Wei, J. X. Cheng, *Proc. Natl Acad. Sci. USA* **2005**, *102*, 15752.
- [20] X. Qian, X.-H. Peng, D. O. Ansari, Q. Yin-Goen, G. Z. Chen, D. M. Shin, L. Yang, A. N. Young, M. D. Wang, S. Nie, *Nat. Biotechnol.* **2008**, *26*, 83.
- [21] K. H. Song, C. H. Kim, C. M. Cobley, Y. N. Xia, L. V. Wang, *Nano Lett.* **2009**, *9*, 183.
- [22] G. von Maltzahn, A. Centrone, J.-H. Park, R. Ramanathan, M. J. Sailor, T. A. Hatton, S. N. Bhatia, *Adv. Mater.* **2009**, *21*, 3175.
- [23] M. H. Gaber, K. Hong, S. K. Huang, D. Papahadjopoulos, *Pharm. Res.* **1995**, *12*, 1407.
- [24] N. Tang, G. J. Du, N. Wang, C. C. Liu, H. Y. Hang, W. Liang, *J. Natl. Cancer Inst.* **2007**, *99*, 1004.
- [25] D. Needham, G. Anyarambhatla, G. Kong, M. W. Dewhirst, *Cancer Res.* **2000**, *60*, 1197.
- [26] Z. Liu, W. B. Cai, L. N. He, N. Nakayama, K. Chen, X. M. Sun, X. Y. Chen, H. J. Dai, *Nat. Nanotech.* **2007**, *2*, 47.
- [27] D. B. Kirpotin, D. C. Drummond, Y. Shao, M. R. Shalaby, K. Hong, U. B. Nielsen, J. D. Marks, C. C. Benz, J. W. Park, *Cancer Res.* **2006**, *66*, 6732.
- [28] R. Weissleder, K. Kelly, E. Y. Sun, T. Shtatland, L. Josephson, *Nat. Biotechnol.* **2005**, *23*, 1418.
- [29] J. Cummings, C. S. McArdle, *Br. J. Cancer* **1986**, *53*, 835.

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SUPPLEMENTARY METHODS

Gold nanorod (GNR) preparation. GNRs containing a cetyltrimethylammonium bromide (CTAB) coating and displaying a peak plasmon resonance at 800 nm were purchased from Nanopartz, inc. To replace the CTAB-monolayers with polyethylene glycol (PEG) molecules, solutions of CTAB-GNRs were made 100 μ M in 5 kDa thiol-PEG (Nektar) and dialyzed for 24-48 hrs in a 5 kDa cutoff cellulose ester membrane (SpectraPor). After dialysis, the PEG-GNRs were purified with multiple rounds of centrifugation using molecular weight cutoff centrifugal filters (100 kDa cutoff, Millipore) and stored at 4 °C. For the experiments to identify particles by surface-enhanced Raman scattering (SERS), the thiol-PEG solution contained between 1-5 μ M of 3,3'-Diethyl-thia-tricarbo-cyanine iodide.

Therapeutic nanoparticle preparation. Doxorubicin-loaded liposome and micelle formulations were synthesized from hydrogenated soy *sn*-glycero-3-phosphocholine (HSPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), cholesterol, 1,2-Distearoyl-*sn*-Glycero-3-Phosphoethanolamine-N-[Amino(Polyethylene Glycol)2000] [DSPE-PEG-NH₂(2k)] and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-(polyethylene glycol 2000) [DSPE-PEG(2k)], purchased from Avanti Polar Lipids (Alabaster, AL). Doxorubicin (DOX) and triethylamine (TEA) was purchased from Sigma Chemical Co. (St.Louis, MO). Non-thermally sensitive DOX liposomes (NSLs) were prepared from HSPC, cholesterol, and DSPE-PEG(2k) in the molar ratio of 75:50:2 by lipid film hydration and membrane (100 nm) extrusion method^[1]. Thermally sensitive DOX liposomes (TSLs) were prepared from DPPC, HSPC, cholesterol, and

DSPE-PEG(2k) in the molar ratio of 90:60:30:3 by a lipid film hydration and membrane (100 nm) extrusion method. Encapsulation of DOX into the liposomes was then carried out using a pH gradient-driven loading protocol [2]. Free doxorubicin was removed by gel filtration on Sephadex G-50. For DOX-loaded thermally sensitive micelles (TSMs), DOX was dissolved in chloroform at room temperature (molar ratio of DOX:TEA = 1:2) and then mixed with DSPE-PEG(2k) in chloroform (molar ratio of DOX:DSPE-PEG(2k) = 1:2). The chloroform was removed using a rotary evaporator to form the drug-containing lipid film. To form DOX micelles, the lipid film was hydrated with 10 mM HEPES-buffered saline (HBS, pH 7.4) at 37 °C for 30 minutes. The DOX micelles were filtered with a membrane of pore size 100 nm and purified on a desalting column (GE healthcare) to remove free DOX molecules. For fluorophore conjugation (Cy7 or Alexa Fluor 488), the amine-terminated phospholipid derivative [DSPE-PEG-NH₂(2k)] was included at 1 mol % of total lipid. The prepared nanoparticles were intravenously injected *in vivo* to ensure that TSM, NSL, and TSL exhibited all similar circulation times (blood half-lives for all: ~ 3 hrs).

Nanoparticle characterization. For transmission electron microscope (TEM) imaging, an aliquot of GNRs, TSMs, NSLs, or TSLs dispersed in water was dropped onto the carbon film covering a 300-mesh copper minigrid (Ted Pella, Inc., CA, USA), which was then gently wiped off after approximately 1 min and air-dried. For negative staining, the grid was incubated with 1.3% phosphotungstic acid for an additional 1 min. TEM images were obtained using a Hitachi H-600A transmission electron microscope. Hydrodynamic

size of GNRs, TSMs, NSLs, and TSLs was obtained using a Zetasizer ZS90 dynamic light scattering machine (Malvern Instruments, Worcestershire, UK).

Raman (SERS) Spectral Acquisition. A Horiba Jobin Yvon Labram HR800 spectrometer was used for recording the SERS spectra. Spectra were acquired with a 785 nm diode laser using 600 grooves/mm grating and a 10x long working distance microscope objective. *In vitro* SERS spectra of the cyanine dye-tagged PEG-GNRs were acquired between 100 and 1900 cm^{-1} of Raman shift using 50 mW of laser power. For *in vivo* SERS spectra of cyanine dye-tagged PEG-GNRs, MDA-MB-435 human melanoma cells were injected in the flank of athymic (nu/nu) mice ($\sim 2 \times 10^6$ cells/tumor). Three weeks following implantation, tumors of $\sim 150 \text{ mm}^3$ had formed. Cyanine dye-tagged PEG-GNRs were intravenously injected into the mouse (10 mgAu/kg). Twenty-four hrs after injection, *in vivo* Raman spectra of the anesthetized mouse (tumors, skin, liver and spleen) were obtained in the Raman shift range 100 to 1900 cm^{-1} , using acquisition times of 30s (liver and spleen) or 60 s (tumors and skin) and 50 mW of laser power. The Raman laser was focused on the anatomical regions corresponding to the tumor, liver, and spleen and the skin near tumor. The spectra of the organs and of the tumor were obtained through the skin, in a completely non-contact and non-invasive manner. Background correction was applied to the spectra for clarity.

Photothermal treatments were conducted under the guidance of infrared thermography (FLIR S60 camera). A custom-built 30W, variable output 810 nm diode laser source was utilized for thermographic experiments. All *in vitro* and *in vivo* experiments were

conducted using a 0.8 cm diameter and 0.75 W/cm² intensity beam. For photothermal tumor heating experiments, MDA-MB-435 human melanoma cells were injected in the flank of athymic (nu/nu) mice (~2E6 cells/tumor). Photothermal experiments were performed 3 weeks following tumor implantation, at which point tumors of ~150mm³ had formed. SERS-coded PEG-GNRs were intravenously injected into the mouse (10mgAu/kg). The *in vivo* photothermal heating thermographs of mouse tumors were obtained under anesthesia 24 hrs after injection.

The temperature-induced release of DOX encapsulated in micelles and liposomes at various temperatures was quantified by measuring the amount of encapsulated DOX that was released from a given sample of micelles and liposomes in PBS or 50% bovine serum (in PBS) after incubation for 0.5, 1, 3, 5 min (supplementary figure 4) or for 10 min (figure 1d) at a given temperature. The fluorescence intensity of the incubated sample was measured with a fluorescence spectrophotometer (Molecular Devices). The relative percentage of fluorescence intensity after incubation at different temperatures was calculated by comparison with the total fluorescence intensity obtained after disrupting the liposomes by adding 0.3 M HCl-50% ethanol to the samples.

***In vitro* imaging and cytotoxicity.** MDA-MB-435 human melanoma cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 µg/ml penicillin-streptomycin. For fluorescence microscopy, the cells (3000 cells per well) were seeded into 8-well chamber slides (Lab-Tek) overnight. The cells were incubated with 80 µg DOX/mL of free DOX, TSMs,

NSLs, and TSLs per well for 15 min at 37 °C or 45 °C in the presence of 10% FBS and rinsed three times with cell medium. The cells were then incubated for an additional 1 hr at 37 °C in the presence of 10% FBS, fixed with 4% paraformaldehyde for 20 min, mounted in Vectashield Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA), and observed with a fluorescence microscope (Nikon, Tokyo, Japan). The nuclei stained with DAPI were observed in the blue channel (excitation at 360 nm/emission at 460 nm) and the fluorescence intensity from DOX was observed in the Cy3 channel (excitation at 540 nm/emission at 580 nm).

Cytotoxicity of the *in vitro* cooperative nanoystem was performed using MDA-MB-435 human melanoma cells. Cells were incubated with free DOX, TSMs, NSLs, or TSLs at different concentrations and GNRs at 7 ugAu/mL for 15 min at 37 °C or 45 °C in a cell incubator. For laser-induced (photothermal) heating, samples were irradiated for 15 min with ~ 0.75 W/cm² of 810 nm laser light, either in the presence or absence (control) of GNRs, in a 10% FBS (Fetal Bovine Serum) medium while maintaining an average solution temperature of ~ 45 °C (monitored by infrared thermographic surveillance). After irradiation, the cells were rinsed three times with cell medium. The cells were then incubated for an additional 48 hrs at 37 °C in a medium containing 10% FBS. The cytotoxicity of free DOX, TSMs, NSLs, and TSLs was evaluated using an MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Invitrogen). Cell viability is expressed as the percentage of viable cells compared with controls (cells treated with PBS).

***In vivo* behavior of thermosensitive nanoparticles (heated with an external water bath, not a laser).** The NIR fluorophore, VT750-NHS (750 nm excitation/780 nm emission, Visen) was attached to the surface (TM-VT750, NL-VT750, and TL-VT750), or VT750-COOH (750 nm excitation/780 nm emission, Visen) was encapsulated in the internal space (VT750@NL and VT750@TL) of nanoparticles. Mice bearing bilateral MDA-MB-435 human melanoma tumors or control mice without tumors were intravenously injected with VT750-labeled nanoparticles and one flank (area where the tumor is located) of each mouse was either not heated or heated at 45 °C for 30 min in a temperature-controlled water bath. The tissues were then harvested at 2 hrs post-injection of nanoparticles (particularly, the bladder was carefully harvested before the mice urinated) and imaged in the Cy7 channel (750 nm excitation/800 nm emission) with a NIR fluorescence imaging system (LI-COR Odyssey Infrared Imaging System).

***In vivo* behavior of thermosensitive nanoparticles (laser photothermal heating).** We first intravenously injected GNR into mice bearing bilateral MDA-MB-435 human melanoma tumors (10 mgAu/kg). At 72 hrs post-injection when the GNR were completely cleared from the blood stream, therapeutic molecules or nanoparticles (5 mgDOX/kg) were systemically administered and either the right or the left flank (area where the tumor is located) was immediately irradiated with NIR-light (~ 0.75 W/cm² and 810 nm) for 30 min, maintaining the average tumor surface temperature at ~ 45 °C (monitored by infrared thermographic observation). At 24 hrs post-injection of the therapeutic nanoparticles, the tissues were harvested, weighed, incubated with 500 μ l of 70% ethanol, 0.3 N HCl, and homogenized (Tissue Tearor, Biospec Products) to release

doxorubicin from the tissues. Following homogenization, another 1 ml of 70% ethanol, 0.3 N HCl, was added to the samples and they were centrifuged. Supernatants of samples were analyzed for doxorubicin fluorescence using a fluorescence microplate reader (Molecular Devices, SpectraMax GeminiEM) and quantified using standard curves.

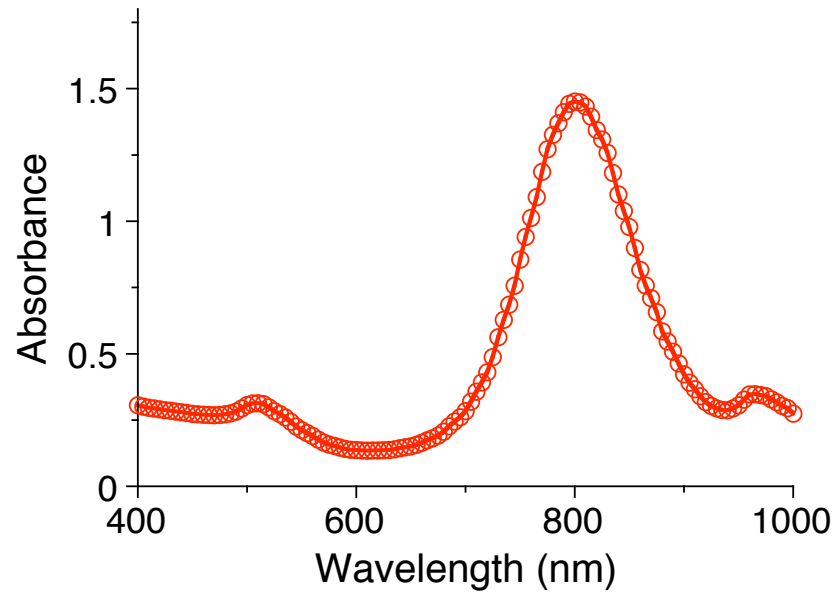
DOX quantification and distribution in tissues. For histologic analysis, therapeutic nanoparticles (TSM, NSL, and TSL) were labeled with Alexa Fluor 488. Frozen sections of tumors were prepared 6 h after treatment with the combination GNR-heating and therapeutic molecule or nanoparticle injection. The sections were fixed with 4% paraformaldehyde and stained with 4',6-diamidino-2-phenylindole (DAPI). At least three images from representative microscopic fields were analyzed for each tumor sample.

***In vivo* therapeutic studies.** We first intravenously injected GNR or saline into mice bearing bilateral MDA-MB-435 human melanoma tumors (10 mgAu/kg). At 72 hrs post-injection of GNR, saline, therapeutic molecules or nanoparticles (3 mgDOX/kg) were systemically administered and one flank of the mouse (the area where the tumor is located) was either not irradiated or immediately irradiated with NIR-light ($\sim 0.75 \text{ W/cm}^2$ and 810 nm) for 30 min, maintaining an average tumor surface temperature at $\sim 45 \text{ }^\circ\text{C}$ (monitored by infrared thermographic observation). Each therapeutic cohort included 5~7 mice. After the single treatment, tumors were measured and mice were weighed at 3 day intervals over a period of 3 weeks.

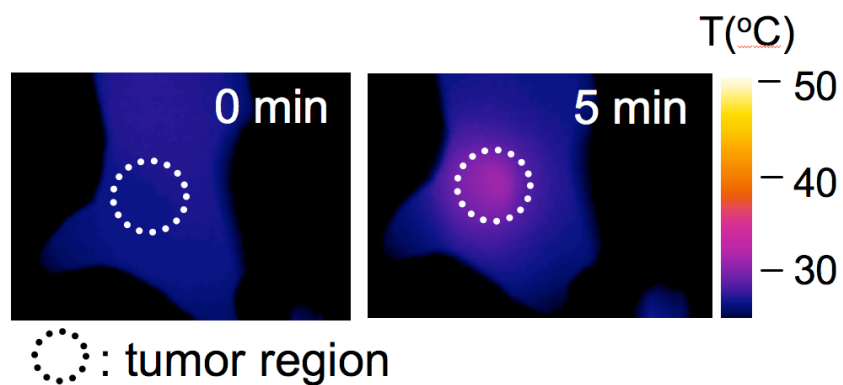
All animal work was performed in accordance with the institutional animal protocol guidelines in place at the Massachusetts Institute of Technology, and it was reviewed and approved by the Institute's Animal Research Committee. Student's t-test was used for statistical analysis of the results.

References

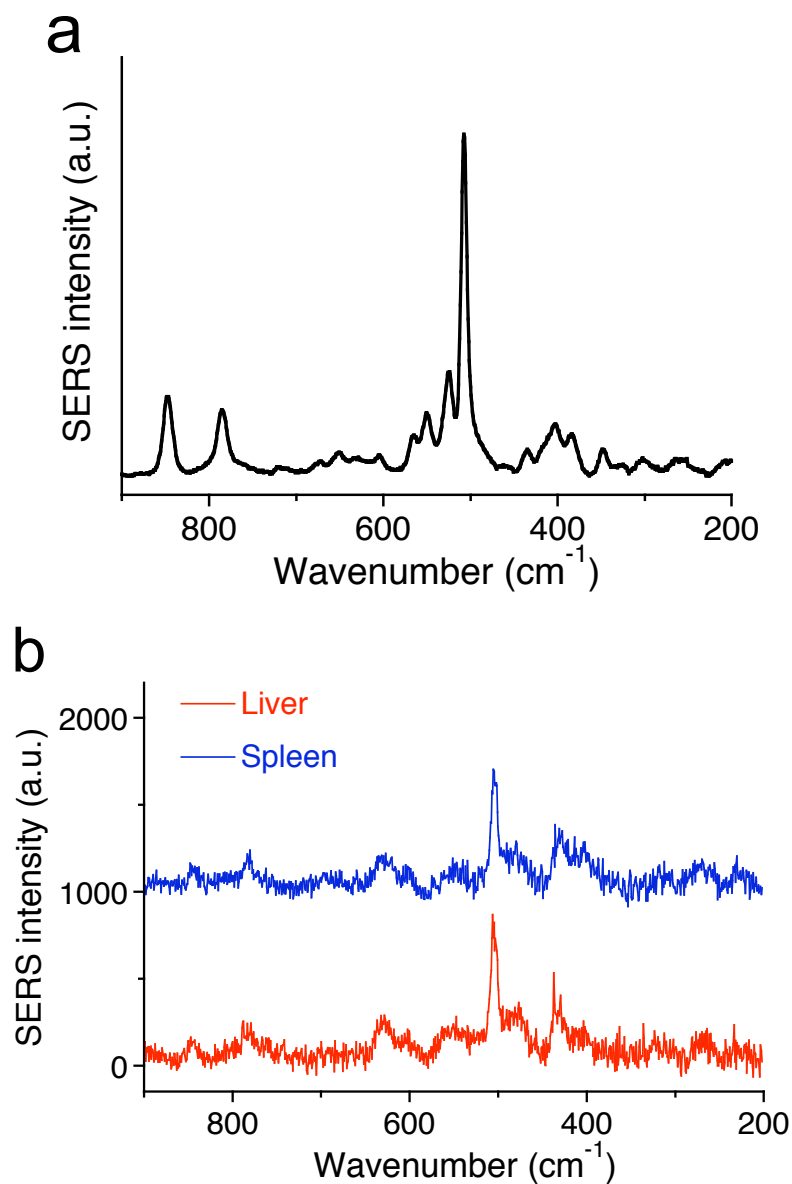
- [1] M. J. Hope, M. B. Bally, G. Webb, P. R. Cullis, *Biochimica Et Biophysica Acta* **1985**, *812*, 55.
- [2] L. D. Mayer, M. B. Bally, M. J. Hope, P. R. Cullis, *Biochimica Et Biophysica Acta* **1985**, *816*, 294.

SUPPLEMENTARY FIGURES

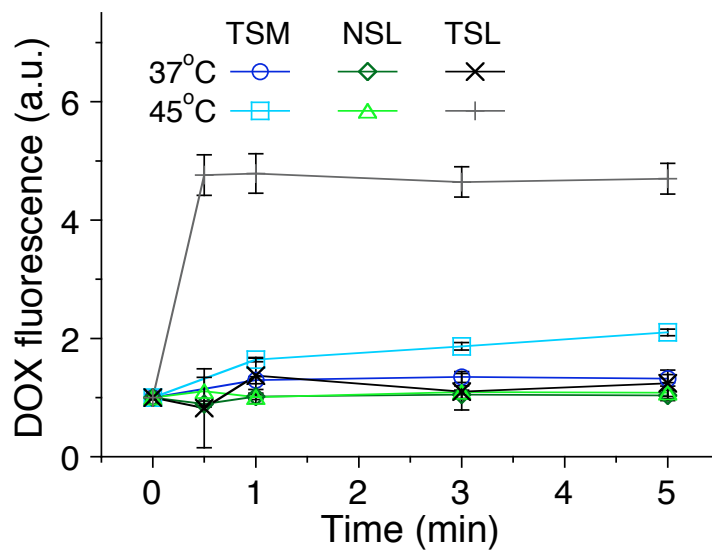
Supplementary Figure 1. Optical absorption spectrum of PEG-coated gold nanorods.



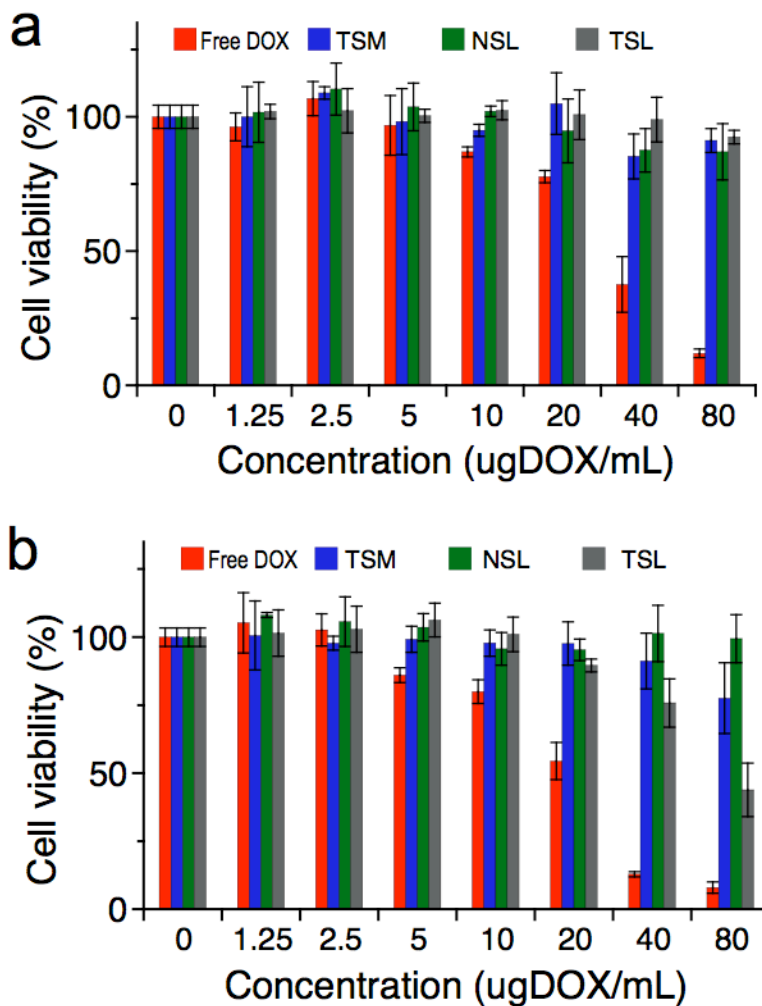
Supplementary Figure 2. NIR laser irradiation effect on the tumor without GNR injection. Infrared thermographic maps of tumor surface temperature of mice bearing lateral MDA-MB-435 tumors were obtained before and 5 min after onset of irradiation with the diode laser (810nm, 0.75 W/cm²).



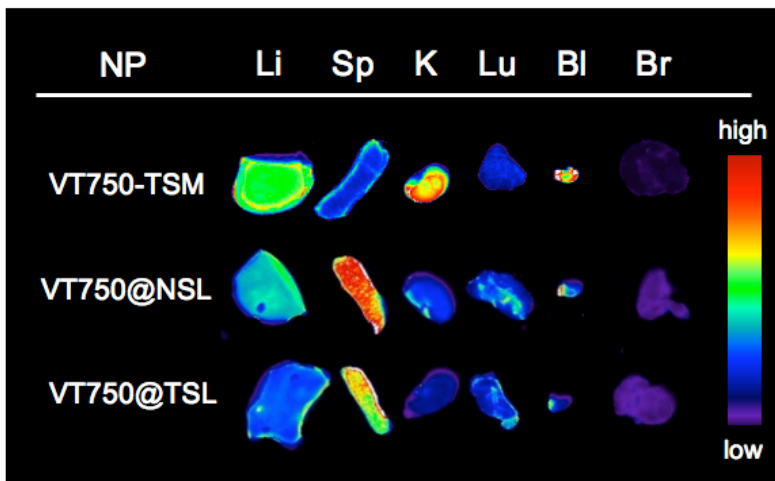
Supplementary Figure 3. (a) Raman spectra of SERS-coded GNRs used for *in vivo* experiments in figure 1c. (b) *In vivo* Raman spectra of SERS-coded GNRs of liver and spleen from the mouse in Figure 1c. Athymic (nu/nu) mouse bearing lateral MDA-MB-435 tumors were injected intravenously with SERS-coded GNRs (10 mgAu/kg). At 24 h post-injection, *in vivo* Raman spectra of SERS-coded GNRs (10 acquisitions of 30 seconds each) were acquired in the liver and the spleen.



Supplementary Figure 4. Relative fluorescence intensity attributed to free doxorubicin (DOX) as a function of time, demonstrating the release kinetics from various therapeutic nanoparticles at different temperatures (20 ugDOX/mL, in PBS buffer solution). Fluorescence of doxorubicin is less intense when inside the nanoparticle, due to concentration self-quenching. Error bars indicate standard deviation.



Supplementary Figure 5. *In vitro* cytotoxicity (by MTT assay) of various therapeutic nanoparticle formulations towards MDA-MB-435 human melanoma cells maintained at two different temperatures. The cells were incubated with free DOX, TSMs, NSLs, and TSLs at the indicated concentrations (based on free or contained DOX) and GNRs (7 ugAu/mL) at 37 °C (a) or 45 °C (b) in a cell incubator for 15 min and rinsed three times with cell medium. The cells were then incubated for an additional 48 hrs at 37 °C and evaluated for cytotoxicity. Error bars indicate standard deviation.



Supplementary Figure 6. Fluorescence images of organs from mice after injection of various VT750-labeled nanoparticle formulations (no heating). The NIR fluorophore VT750 (750 nm excitation/780 nm emission) was either attached to the lipids of micelles (TSM-VT750) or encapsulated in the internal space of liposomes (VT750@NSL and VT750@TSL). Mice bearing bilateral MDA-MB-435 tumors were intravenously injected with the indicated VT750-labeled nanoparticle formulations. At 2 hrs post-injection of nanoparticles, the tissues were collected from the mice and the fluorescence intensity (780 nm) was quantified using a NIR fluorescence imaging system.