Cooperative nanomaterial system to sensitize, target, and treat tumors

Ji-Ho Park*†, Geoffrey von Maltzahn‡, Mary Jue Xu§, Valentina Fogal¶, Venkata Ramana Kotamraju**, Erkki Ruoslahti††, Sangeeta N. Bhatai*‡§, and Michael J. Sailor*∥†¶

*Materials Science and Engineering Program, ‡Department of Chemistry and Biochemistry, and ¶Department of Bioengineering, University of California, San Diego, La Jolla, Ca 92093, and ∥Harvard-MIT Division of Health Sciences and Technology, and †Electrical Engineering and Computer Science, Massachusetts Institute of Technology, Cambridge, Ma 02139, and ‡Division of Medicine, Brigham and Women’s Hospital, Boston, Ma 02115, and **Cancer Research Center, Burnham Institute for Medical Research, 10901 North Torrey Pines Road, La Jolla, Ca 92037, and ∥Vascular Mapping Center, Burnham Institute for Medical Research at UCSD, University of California, Santa Barbara, Santa Barbara, Ca 93106

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A significant barrier to the clinical translation of systemically administered therapeutic nanoparticles is their tendency to be removed from circulation by the mononuclear phagocyte system. The addition of a targeting ligand that selectively interacts with cancer cells can improve the therapeutic efficacy of nanomaterials, although these systems have met with only limited success. Here, we present a cooperative nanosystem consisting of two discrete nanomaterials. The first component is gold nanorod (NR) "activators" that populate the porous tumor vessels and act as photothermal antennas to specify tumor heating via remote near-infrared laser irradiation. We find that local tumor heating accelerates the recruitment of the second component: a targeted nanoparticle consisting of either magnetic nanoworms (NW) or doxorubicin-loaded liposomes (LP). The targeting species employed in this work is a cyclic nine-amino acid peptide LyP-1 (Cys-Gly-Asn-Lys-Arg-Thr-Arg-Gly-Cys) , referred to as LyP-1, which binds to the stress-related protein, p32, which we find to be upregulated on the surface of tumor-associated cells upon thermal treatment. Mice containing xenografted MDA-MB-435 tumors that are treated with the combined NR/LyP-1LP therapeutic system display significant reductions in tumor volume compared with individual nanoparticles or untargeted cooperative system.

In the past few decades, nanomaterials have played a propitious role in delivering therapeutic molecules effectively to diseased sites. In addition to their role as effective carriers of conventional therapeutic drugs, nanoscale materials can be harnessed to damage or destroy malignant tissues by converting external electromagnetic energy into heat (1–6). Furthermore, most nanomaterial surfaces can be decorated with targeting ligands, enhancing their ability to home to diseased tissues through multivalent interactions with tissue-specific receptors (7). Targeted liposomes (8, 9), micelles (10, 11) and dendrimers (12, 13) incorporated with therapeutic molecules have displayed impressive anticancer effects in animal studies, and these nanomaterials are considered to be close to clinical translation due to their biocompatibility. In spite of these merits, nanotechnology-based cancer therapies have been slow to reach the clinic compared to conventional cancer therapies such as small molecule drugs, whole-body or local hyperthermia, and radiation.

Tumorigenesis is a multistep process that requires expression of tumor-associated proteins and suppression of proteins controlling normal cell growth (14). Many of the identified tumor-specific proteins have been exploited to develop powerful antibody, aptamer, peptide, and small molecule-based ligands for targeting of diagnostic or therapeutic agents (15). Ligand-directed targeting of therapeutic nanomaterials has been widely pursued to improve therapeutic efficacy, although limitations imposed by the tumor microenvironment, such as restricted trans-vascular transport and receptor accessibility, have prevented realization of their full capabilities. Although the porous microstructure of tumor blood vessels is favorable for nonspecific infiltration of circulating nanomaterials into the extravascular region of the tumor (16), extravasated nanomaterials are generally deposited close to the vessels, resulting in a highly heterogeneous distribution of therapeutic agents in the tumor.

Hyperthermia has been reported to not only improve nanoparticle extravasation in tumors, (17) but it also can selectively damage neoplastic cells to activate immunological processes and induce expression of particular proteins (18). Widely used in the clinical setting in concert with chemotherapy and radiotherapy (19, 20), tumor-specific hyperthermia would be a powerful tool to manipulate tumor microenvironments in order to enhance the interactions between cancerous tissues and therapeutic agents. However, hyperthermia methods in clinical practice lack intrinsic specificity for tumor tissues, requiring complex implementation strategies and frequently resulting in exposure of large volumes of normal tissues to hyperthermic temperatures alongside tumors. We hypothesized that gold nanorods (NRs), passively accumulated in tumors via their fenestrated blood vessels, could be used to precisely heat tumor tissues by amplifying their absorption of otherwise benign near-infrared energy (2, 6) and allow the recruitment and more effective penetration of a second, specifically targeted nanoparticle. Thus, in this work, we demonstrate a cooperative nanosystems, wherein NRs accumulated in a tumor photothermally activate the local microenvironment to amplify the targeting efficacy of two types of targeted, circulating nanoparticles: magnetic nanoworms (NWs) and liposomes (LPs) loaded with the anticancer drug doxorubicin (DOX) (Fig. 1A).

Results

The first stage of the cooperative nanoparticle system, the photothermally heated gold nanorods, has already been demonstrated by our group (6). Polyethylene glycol (PEG)-coated NRs with a maximum optical absorption of 800 nm are found to accumulate passively in a MDA-MB-435 xenograft tumor. Effective in vivo photothermal heating of the tumor is achieved by application of NIR irradiation (810 nm, ∼0.75 W/cm²) from a diode laser (Fig. 1B).

A cyclic nine-amino acid peptide (Cys-Gly-Asn-Lys-Arg-Thr-Arg-Gly-Cys) , referred to as LyP-1, was chosen as the targeting
ligand based on a screen of several tumor targeting peptides in MDA-MB-435 xenograft tumors, which showed enhanced LyP-1 accumulation in the heated tumors. The LyP-1 peptide has been reported to selectively recognize lymphatics and tumor cells in certain tumor types and subsequently inhibit tumor growth (21, 22). Recently, it was found that the p32 or gC1qR receptor, whose expression is elevated on the surface of tumor-associated cells undergoing stress, is the target molecule for the LyP-1 peptide (23). Thus, we investigated whether the enhanced targeting of LyP-1 relates to upregulation of p32 receptors in the heated tumor.

We first tested the level of p32 expression in MDA-MB-435 xenografts as a function of time post heat treatment. An externally measured temperature of 45 °C was chosen for the laser heat treatment based on a preliminary screen of temperature dependent nanoparticle accumulation. It has been reported that cancer cells are most vulnerable to hyperthermia, chemotherapeutics, or a combined therapy above temperatures of 43 °C (18, 20). Expression of p32 on the MDA-MB-435 tumors was slightly upregulated 6 h after heat treatment, which then returned to almost normal levels 24 h posttreatment (Fig. 1C). Compared with the MDA-MB-435 tumors, less significant changes in the level of heat-mediated p32 expression were observed on C8161 tumors, known as the tumor type that expresses a considerably less amount of p32 compared to MDA-MB-435 tumor (23), over a 24 h period postheating (Fig. S1). Expression of p32 in cultured cells upon heat treatment exhibited a pattern similar to the in vivo xenograft results; the extent of p32 expression on C8161 cells (and cell surfaces) was less than that observed with MDA-MB-435 cells (Fig. S2).
into heated MDA-MB-435 cells relative to unheated cells. In contrast, the C8161 cells displayed lower heat-mediated internalization than the MDA-MB-435 cells (Fig. 1d). The colocalization of p32 receptors and LyP1NW was clearly observed in MDA-MB-435 cells, suggesting that the binding and internalization of LyP1NWs are mediated by p32 receptors on the surface of MDA-MB-435 cells. The lack of interaction of LyP1NWs with C8161 cells is presumed to be due to insufficient availability of p32 receptors on the cell surface (Fig. S2). As expected, control NWs exhibited no interaction in either cell type, regardless of the heat treatment (Fig. S3).

The possibility of selective homing of LyP1NWs to heated xenograft tumors in vivo was then tested. Similar to the in vitro results, targeting of LyP1NWs to heated MDA-MB-435 cells was prominent relative to unheated tumors, since the ability of LyP1NWs to home to heated C8161 tumors was not significantly different relative to the unheated tumors (Figs. 2 and S4). Histological analysis revealed large quantities of LyP1NWs occupying vessel structures that were not colocalized with the blood vessel stain, consistent with the previously reported affinity of LyP-1 for lymphatics (21). In both types of tumors, most of the observed LyP1NWs were either colocalized with p32 receptors or distributed in the extravascular region of the heated tumors. Additionally, the distribution of control NWs in tumors did not correlate with the p32 receptor distribution, even though significant quantities of NWs were observed in the heated tumors. Furthermore, histological images of tumors for which LyP1NWs were administered immediately after heat treatment were similar to those for which LyP1NWs were injected right before heat treatment (Fig. S5), suggesting that prominent targeting of LyP1NWs on the individual cells of heated tumors can be attributed mainly to their binding to the p32 receptors, not the simultaneous hyperthermia.

Having verified temperature-induced amplification of nanoparticle targeting to tumor cells in vitro and to xenograft tumors in vivo, we next evaluated in vitro photothermal-assisted cytotoxicity of targeted therapeutic carriers. Liposomes constructed from lipids that are not thermally sensitive were pre-engineered to a single injection of therapeutic nanoparticles at a

Fig. 2. Temperature-induced amplification of in vivo tumor targeting. (A) Fluorescence intensity from Cy7-labeled LyP-1-conjugated magnetic nanoworms (LyP1NW) and Cy7-labeled control nanoworms (NW) in MDA-MB-435 tumor as a function of externally applied heat (30 min). Heated at (45 °C) and unheated (37 °C) samples indicated with (+) and (−), respectively. The tissues were collected from the mice 24 h postinjection; NIR fluorescence images use Cy7 channel. * indicates P < 0.05 (n = 3 – 4). (B) Fluorescence image of major organs from the mice in (A). T, L, Li, Sp, K, and Br indicate heated tumor, unheated tumor, liver, spleen, kidney, and brain, respectively. (C) Histological analysis of LyP1NW or NW distribution in MDA-MB-435 tumors with (+) or without (−) application of external heat. Green indicates NWs (labeled with Cy 5.5). Cellular stains same as in Fig. 1D, blood vessels stained with CD31 followed by Alexa Fluor® 594 goat antirat IgG. Arrowhead indicates a lymphatic vessel structure displaying a signal from the labeled LyP1NWs. Scale bar is 100 μm. Error bars indicate standard deviations from ≥3 measurements.
relatively low therapeutic dose (3 mgDOX/kg) is able to achieve significant tumor regression or elimination, which has not been observed in this tumor model with previous targeted therapies even with multiple high doses (27, 28). For all the treatments studied in this work, no significant loss of body mass was observed.

Discussion
This study demonstrates that the appropriate combination of nanomaterials currently under investigation in cancer therapy can significantly enhance therapeutic efficacy relative to the individual components. Site-specific photothermal heating of NRs can engineer the local tumor microenvironment to enhance the accumulation of therapeutic targeted liposomes, which increases the overall hyperthermal and chemotherapeutic tumor-destroying effects. This cooperative nanosystem holds clinical relevance because gold salts (for rheumatoid arthritis therapies) (29) and doxorubicin-containing liposomes (Doxil®) have been approved for clinical use, and local hyperthermia is a well-established means of destroying diseased tissues in the human body. Although the liposomes in this study are similar to Doxil®, it should be pointed out that the gold nanorod and iron-oxide nanoworm formulations used in the study are somewhat distinct from clinically approved gold or iron oxide materials. Because they are quite bioinert, much work needs to be done to investigate the long-term fate and biosafety of systemically administered gold nanorods in the human body. Cooperative, synergistic therapies using dual or multiple nanomaterials could significantly reduce the required...
dose of anticancer drugs, mitigating toxic side effects, and more effectively eradicating drug-resistant cancers.

Materials and Methods
Preparation of Gold Nanorod, Magnetic Nanoworm, and Doxorubicin Liposomes. Gold nanorods (NRs) were purchased from Nanopartz with a peak plasmon resonance at 800 nm and coated with polyethylene glycol (PEG) molecules (HS:PEG(5k)). Superparamagnetic, dextran-coated iron oxide nanoparticles (NWs) with a longitudinal size of ~70 nm were synthesized with the published procedure (24), and derivatized with near-infrared (NIR) fluorophore, Cy5.5:Cy7-NHS. For control NWs, partially Cy5.5:Cy7-labeled aminated NWs were coated with a PEG molecule [NHS-PEG(5k)]. For LyP-1-conjugated NWs (LPY1NWs), LyP-1 peptides with extra cysteine were attached to partially Cy5.5:Cy7-labeled aminated NWs via a PEG crosslinker [NHS-PEG(5k)-MAL]. Controls with functional groups were prepared from hydrogenated soy snglycerol-3-phosphocholine (HSPC), cholesterol, and 1,2-di-stearoyl-snglycerol-3-phosphethanolamine-N-polyethylene glycol 2000 [DSPE-PEG(2k)] (75: 50: 6 mol ratio) by lipid film hydration and membrane (100 nm) extrusion (30). Incorporation of DOX was achieved using the pH gradient-driven protocol (31). For LyP-1-conjugated LPs (LYP1LPs), LPs with maleimide groups were prepared from HSPC, cholesterol, DSPE-PEG(2k), and DSPE-PEG(2k)-MAL (75: 50: 6 mol ratio). LyP-1 peptides with extra cysteine were attached to maleimide-terminated LPs in PBS. LPs were intravenously injected in vivo to ensure control LPs and LY1LPs exhibited similar circulation times (blood half-lives for both: ~3 hrs).

In Vitro Cellular Fluorescence Imaging. The cells were treated with 80 ugF/mL of Cy5.5 labeled control NWs or LPY1NWs per well for 20 min at 37 °C or 45 °C in the presence of 10% FBS and incubated for an additional 2 h at 37 °C in the presence of 10% FBS. The cells were then rinsed three times with cell medium, fixed, stained, and imaged by fluorescence microscopy.

In Vivo Temperature-induced Tumor Targeting of Magnetic Nanoworms. Mice bearing bilateral tumors (MDA-MB-435 human carcinoma or C8161 human melanoma) were intravenously injected with Cy7-labeled LyP1NWs or NWs bearing bilateral tumors (MDA-MB-435 human carcinoma or C8161 human melanoma) were intravenously injected with Cy7-labeled LyP1NWs or NWs and one tumor of the mouse was immediately heated at 45 °C for 30 min in a temperature-controlled water bath. At 24 h postinjection, the tissues were harvested and the Cy7 fluorescence in tissues were imaged using NIR fluorescence imaging system (LI-COR Odyssey).

In Vivo Temperature-induced Cytotoxicity of Therapeutic Nanoparticles. Cells were treated with free DOX, control LPs, or LyP1LPs with different concentrations at 37 °C or 45 °C for 20 min (in cell incubator) and then incubated for an additional 4 h at 37 °C. The cells were rinsed with cell medium three times, and then further incubated for 44 h at 37 °C. The cytotoxicity of free DOX, control LPs, or LyP1LPs was evaluated using MTT assay (Invitrogen). Cell viability was expressed as the percentage of viable cells compared to controls (cells treated with PBS). In Vivo Tumor Targeting of Therapeutic Nanoparticles by NR-Mediated Photothermal Heating. Mice bearing bilateral MDA-MB-435 human carcinoma tumors were intravenously injected with NRs (10 mgAu/kg). At 72 h postinjection of NR, control LPs, or LyP1LPs (3 mgDOX/kg) were systemically administered and the tumor in one flank was irradiated with NIR-light (~0.75 W/cm2 and 810 nm) for 30 min, maintaining an average tumor surface temperature at ~45°C under infrared thermographic observation. At 24 h postinjection of liposomes, doxorubicin fluorescence in the homogenized tumors was analyzed.

In Vivo Therapeutic Studies. To study the effect of photothermal treatment on tumor volumes, mice bearing bilateral MDA-MB-435 human carcinoma tumors were intravenously injected with NRs (10 mgAu/kg). At 72 h postinjection of NR, control LPs, or LyP1LPs (3 mgDOX/kg) were systemically administered and the tumor in one flank was irradiated with NIR-light (~0.70 or 0.75 W/cm2 and 810 nm) for 30 min, maintaining average tumor surface temperature at ~45°C. Each therapeutic cohort included 4 – 6 mice. Tumor volume and mouse mass was measured every 3 days after the single treatment for a period of 3-4 weeks by an investigator blinded to the treatments administered. Survival rates (Kaplan Meier analyses) for the photothermal treatments were quantified using mice bearing single MDA-MB-435 human carcinoma tumors, intravenously injected with NRs (10 mgAu/kg). Control LPs or LyP1LPs (3 mgDOX/kg) were systemically administered 72 h postinjection and one of the tumor-bearing flanks was irradiated with NIR-light (~0.75 W/cm2 and 810 nm) for 30 min, maintaining average tumor surface temperature at ~45°C. Each therapeutic cohort included six mice. Tumor volume and mouse mass was measured every 3 days after the single treatment for a period of 9 weeks by an investigator blinded to the treatments administered. Mice were sacrificed when tumors exceeded 500 mm3.

The experimental procedures are described in more detail in SI Materials and Methods.

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Supporting Information

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SI Text

Materials and Methods. Gold nanorod preparation. Gold nanorods (NRs) with a coating of cetyltrimethylammonium bromide (CTAB) were purchased from Nanopartz with a peak plasmon resonance at 800 nm. To replace CTAB-monolayers with polyethylene glycol (PEG) molecules, solutions of CTAB-NRs were brought to 100 μM of 5 kDa thiol-PEG and dialyzed for 24–48 h in a 5 kDa cutoff cellulose ester membrane (SpectraPor). After dialysis, PEG-conjugated NRs were purified further with multiple rounds of centrifugation using molecular weight cutoff centrifugal filters (100 kDa cutoff, Millipore) and stored at 4 °C.

Magnetic nanoworm preparation. Superparamagnetic, dextran-coated iron oxide nanoworms (NWs) with a longitudinal size of ~70 nm were synthesized with the published procedure (1), amminated using 10% vol ammonium hydroxide, and derivatized with near-infrared (NIR) fluorophore, Cy5.5/Cy7-NHS (GE Healthcare). For control NWs, partially Cy5.5/Cy7-labeled amminated NWs were coated with a PEG molecule [NHS-PEG(5k), Nektar]. For LyP-1-conjugated NWs (LyPINWs), LyP-1 peptides with extra cysteine were attached to maleimide-terminated NWs with PEG(2k)-NH2 in the molar ratio of ∼75:50:5:6 by lipid film hydration and membrane (100 nm) extrusion method (2). Encapsulation of DOX into the LPSs was then carried out using the pH gradient-driven loading protocol (3). For LyP-1-conjugated LPSs (LyPILPs), LPSs with maleimide groups were prepared from HSPC, cholesterol, and DSPE-PEG(2k), and DSPE-PEG(2k)-MAL in the molar ratio of 75:50:5:6. LyP-1 peptides with extra cysteine were attached to maleimide-terminated LPSs in PBS. For Alexa Fluor® 488-conjugated LPSs, LPSs with amine groups were prepared from HSPC, cholesterol, and DSPE-PEG(2k)-NH2 in the molar ratio of 75:50:5:6 and conjugated with Alexa Fluor® 488-NHS (Invitrogen). The prepared LPSs were stored in PBS at 4 °C before use. LPSs were characterized via DLS and TEM, and intravenously injected in vivo to ensure control LPSs and LyPILPs exhibited similar circulation times (blood half-lives for both: ~3 h).

Peptide synthesis. LyP-1 peptides, a cyclic nine–amino acid peptide (Cys-Gly-Asn-Lys-Arg-Thr-Arg-Gly-Cys), were synthesized with an automatic microwaveassisted peptide synthesizer (Liberty) using standard solid-phase Fmoc/t-Bu chemistry. During synthesis, the peptides were labeled with 5(6)-carboxyfluorescein (FAM) with a 6-aminohexanoic acid spacer separating the dye from sequence. The LyP-1 peptide was synthesized with a third cysteine on the N terminus of the peptide to facilitate the nanoparticle conjugation. The peptide was chemo-selectively conjugated employing a Michael addition reaction between maleimide functional group on the particle and thiol of the third cysteine placed on the N terminus of the sequence. Details of the synthesis will be published separately.

In vivo photothermal experiments. A custom 30W, variable output 810 nm diode laser source was utilized for thermographic experiments. All experiments were conducted using a 0.8 cm diameter and ~0.75 W/cm² intensity beam. For photothermal tumor heating experiments, athymic (nu/nu) mice bearing a MDA-MB-435 tumor in the left flank were injected intravenously with either PEGylated NRs (10 mgAu/kg) or saline. At 72 h post-injection, infrared thermographic maps of average tumor surface temperature were obtained over 30 min while irradiating left flank area of the mouse with diode laser (810 nm, 0.75 mW/cm²). Photothermal experiments were conducted under the guidance of infrared thermography (FLIR S60 camera).

Detection of protein expression by immunoblotting and immunostaining. For in vitro study, cells were seeded in 2 wells of 6-well culture plate at a density of 50–60% per well in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS and 100 μg/mL penicillin-streptomycin 24 h before heating experiments. The cells were treated for 20 min at 37 °C or 45 °C (in cell incubator) and then incubated for an additional 2 h at 37 °C. For immunoblotting, after washing cells with PBS three times, the cells were harvested, and the cell pellets were resuspended and incubated for 30 min at 4 °C in lysis buffer [20 mmol/L Tris-HCl (pH 7.5) containing 150 mmol/mL NaCl and 1% (vol) Nonidet P-40] supplemented with protease inhibitor mixture (Sigma). The cell debris was removed by centrifugation, and the protein concentration was determined with the bicinchoninic acid protein assay (Pierce). Samples of cell extracts containing 30 μg of protein were analyzed by SDS-PAGE and transferred to polyvinylidene fluoride membrane filters (Invitrogen). p32 protein was detected with anti-p32 antibody (4) and peroxidase-conjugated secondary antibody. Actin was detected with mouse monoclonal anti-actin antibody (Millipore) and peroxidase-conjugated secondary antibody. The antibody complexes on the filters were visualized with the Enhanced Chemiluminescence Plus Western Blotting Detection System (Pierce). For immunostaining, after washing cells with PBS three times, the cells were fixed with 4% paraformaldehyde for 20 min, and blocked with the solution containing 1% BSA in PBS for 30 min, incubated with 5 μg/mL anti-p32 antibody for 1 h, and then with 5 μg/mL Alexa Fluor® 594 goat anti-rabbit IgG antibody for 1 h at room temperature. The nuclei stained with DAPI were observed in blue channel (excitation at 360 nm/emission at 460 nm). The p32 were observed in Cy3.5 channel (excitation at 580 nm/emission at 620 nm).

For in vivo study, MDA-MB-435 or C8161 xenograft tumors (~0.7 cm) implanted on the flank of athymic (nu/nu) mice were heated at different temperature [37 °C (unheated) or 45 °C] for 30 min using temperature-controlled water bath and harvested at different time period after heating treatments. For immunoblotting, the tumors were then homogenized (Tissue Tearor, Biospec Products) and incubated for 30 min at 4 °C in the lysis buffer. The cell debris was removed by centrifugation, and the protein concentration was determined with the bicinchoninic acid protein assay (Pierce). Samples of cell extracts containing...
30 μg of protein were analyzed by SDS-PAGE and transferred to polyvinylidene fluoride membrane filters (Invitrogen). p32 protein was detected with anti-p32 antibody and peroxidase-conjugated secondary antibody. Actin was detected with anti-actin and peroxidase-conjugated secondary antibody. The antibody complexes on the filters were visualized with the Enhanced Chemiluminescence Plus Western Blotting Detection System (Pierce). For immunostaining, the frozen sections of tumors were fixed with acetone for 20 min, and permeabilized and blocked with the solution containing 1% BSA and 0.1% Triton X-100 in PBS for 30 min, incubated with 5 μg/mL anti-p32 antibody overnight at 4°C, and then with 5 μg/mL Alexa Fluor® 594 goat anti-rabbit IgG antibody for 1 h at room temperature. The nuclei stained with DAPI were observed in blue channel (excitation at 360 nm/emission at 460 nm). The p32 were observed in Cy3.5 channel (excitation at 580 nm/emission at 620 nm).

**In vitro cellular fluorescence imaging.** 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) 24 hrs before experiments. The cells were treated with 80 μg/mL of Cy5.5 labeled control NWs or LyP1NWs per well for 20 min at 37°C or 45°C in the presence of 10% FBS and incubated for an additional 2 h at 37°C in the presence of 10% FBS. The cells were then rinsed three times with cell medium, fixed with 4% paraformaldehyde for 20 min, and permeabilized and blocked with the solution containing 1% bovine serum albumin (BSA) and 0.1% Triton X-100 in PBS for 30 min, incubated with 5 μg/mL anti-p32 antibody for 1 h, and then with 5 μg/mL Alexa Fluor® 594 goat anti-rabbit IgG antibody for 1 h at room temperature. The nuclei stained with 4′-6-diamidino-2-phenylindole (DAPI) were observed in blue channel (excitation at 360 nm/emission at 460 nm). The p32 were observed in Cy3.5 channel (excitation at 580 nm/emission at 620 nm). The NWs were observed in Cy5.5 channel (excitation at 680 nm/emission at 720 nm).

**In vivo temperature-induced tumor targeting of magnetic nanoworms.** Mice bearing bilateral MDA-MB-435 human carcinoma or C8161 human melanoma tumors were intravenously injected with Cy7-labeled LyP1NWs or NWs and one flank of the mouse (containing one of the tumors) was immediately heated at 45°C for 30 min in a temperature-controlled water bath. At 24 hrs post-injection, the tissues were harvested and the Cy7 fluorescence in tissues were imaged in Cy7 channel (750 nm excitation/800 nm emission) using NIR fluorescence imaging system (LI-COR Odyssey Infrared Imaging System). For histological analysis, the Cy5.5-labeled LyP1NWs or NWs were injected and the tumors were harvested and sectioned. The frozen sections of tumors were fixed with acetone for 20 min, and permeabilized and blocked with the solution containing 1% bovine serum albumin (BSA) and 0.1% Triton X-100 in PBS for 30 min, incubated with 5 μg/mL anti-p32 antibody or CD31 (BD Pharmingen) overnight at 4°C, and then with 5 μg/mL Alexa Fluor® 594 goat anti-rabbit IgG antibody or Alexa Fluor® 594 goat anti-rat IgG antibody for 1 h at room temperature, respectively. The nuclei stained with DAPI were observed in blue channel (excitation at 360 nm/emission at 460 nm). The p32 or blood vessels were observed in Cy3.5 channel (excitation at 580 nm/emission at 620 nm). The NWs were observed in Cy5.5 channel (excitation at 680 nm/emission at 720 nm).

**In vitro temperature-induced cytotoxicity of therapeutic nanoparticle.** Cells were maintained in Dulbecco’s Modified Eagle’s Medium supplemented with 10% FBS and 100 μg/mL penicillin-streptomycin. The cells were treated with free DOX, control LPs, or LyP1LPs with different concentrations at 37°C or 45°C for 20 min (in cell incubator) and then incubated for an additional 4 h at 37°C. The cells were rinsed with cell medium three times, and then further incubated for 44 h at 37°C. The cytotoxicity of free DOX, control LPs, or LyP1LPs was evaluated using MTT assay (Invitrogen). Cell viability was expressed as the percentage of viable cells compared with controls (cells treated with PBS).

**In vivo tumor targeting of therapeutic nanoparticles by NIR-mediated photothermal heating.** Mice bearing bilateral MDA-MB-435 human carcinoma tumors were intravenously injected with NRs (10 mgAu/kg). At 72 h post-injection of NR, control LPs or LyP1LPs (3 mgDOX/kg) were systemically administered and either right or left flank (area where tumor is located) of mice was immediately irradiated with NIR-light (∼0.75 W/cm² and 810 nm) for 30 min, maintaining average tumor surface temperature at ∼45°C under infrared thermographic observation. At 24 h post-injection of liposomes, the tissues were harvested, weighed, and incubated with 500 μL of 70% EtOH, 0.3 N HCl, and homogenized (Tissue Tearor, Biospec Products) to release doxorubicin from tissues. Following homogenization, another 1 mL of 70% EtOH, 0.3 N HCl was added to samples and they were centrifuged. Supernatants of samples were analyzed for doxorubicin fluorescence using a fluorescence microplate reader (Molecular Devices, SpectraMax GeminiEM) and compared to standard curves. For histological analysis, at 72 h post-injection of NRs, mice bearing bilateral MDA-MB-435 human carcinoma tumors were intravenously injected with Alexa Fluor® 488-labeled control LPs or LyP1LPs (∼0.75 W/cm² and 810 nm) for 30 min, maintaining average tumor surface temperature at ∼45°C under infrared thermographic observation. At 24 h post-injection, the tumors were harvested and sectioned. The frozen sections of tumors were fixed with acetone for 20 min, and stained with DAPI. The nuclei stained with DAPI were observed in blue channel (excitation at 360 nm/emission at 460 nm). The DOX fluorescence was observed in the Cy3 channel (excitation at 540 nm/emission at 580 nm). The (5)-carboxyfluorescein (FAM) and Alexa Fluor® 488 on the LPs were observed in FITC channel (excitation at 490 nm/emission at 520 nm).

**In vivo therapeutic studies.** For tumor volume change studies without photothermal treatments, mice bearing bilateral MDA-MB-435 human carcinoma tumors were intravenously injected with NR, control LPs or LyP1LPs (6 mgDOX/kg) at 0, 6, and 12 days. For tumor volume change studies with photothermal treatments, mice bearing bilateral MDA-MB-435 human carcinoma tumors were intravenously injected with NRs (10 mgAu/kg). At 72 h post-injection of NR, control LPs or LyP1LPs (3 mgDOX/kg) were systemically administered and either right or left flank (area where tumor is located) of mice was immediately irradiated with NIR-light at power densities of ∼0.70 (for 43°C) or ∼0.75 (for 45°C), at a wavelength of 810 nm for 30 min, maintaining average tumor surface temperature at ∼43 or 45°C under infrared thermographic observation. Each therapeutic cohort included 4–6 mice. At regular intervals (every 3 days) after single or multiple treatments, tumor volumes were measured and mice were weighed over 3 or 4 weeks by an investigator blinded to the treatments administered.

For survival rate studies with photothermal treatments by Kaplan Meier analyses, mice bearing single MDA-MB-435 human carcinoma tumors were intravenously injected with NRs (10 mgAu/kg). At 72 h post-injection of NR, control LPs or LyP1LPs (3 mgDOX/kg) were systemically administered and either right or left flank (where a tumor is located) of mice was immediately irradiated with NIR-light (∼0.75 W/cm² and 810 nm) for 30 min, maintaining average tumor surface tempera-
ture at \(\sim 45^\circ C\) under infrared thermographic observation. Each therapeutic cohort included six mice. At regular intervals (every 3 days) after single treatments, tumor volumes were measured and mice were weighed over 9 weeks by an investigator blinded to the treatments administered. Mice were killed when tumors exceeded 500 mm\(^3\).


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.

Fig. S1. Effect of p32 expression in C8161 xenograft tumor on increased temperature and heating time in vivo. A C8161 xenograft tumor on athymic (nu/nu) mouse was heated at 45 °C for 30 min using temperature-controlled water bath. At 6 h after heating treatment, the tumor sections were imaged for analysis of p32 expression by immnostaining (Left). At different time period after heating treatment, the tumors were harvested and processed for analysis of p32 expression by western blot (Right). \(\beta\)-actin was used as a control. Symbols – and + indicate no heating (37 °C) and heating (45 °C), respectively. \(n = 3\).

Fig. S2. Effect of p32 expression in various cultured cells on increased temperature. (A) Immunoblots of p32 expression on various cultured cells at increased temperature. C8161, HeLa, MDA-MB-435, and MDA-MB-231 cells were treated for 20 min at 37 °C or 45 °C (in cell incubator) and then incubated for an additional 2 h at 37 °C. \(\beta\)-actin was used as a control. (B) Fluorescence images of p32 expression on the surface of various cultured cells at increased temperature by immunostaining. The experimental procedure was the same as in (A). For immunostaining, after washing cells with PBS three times, the cells were fixed with 4% paraformaldehyde for 20 min, and blocked with the solution containing 1% BSA in PBS for 30 min, incubated with 5 μg/mL anti-p32 antibody for 1 h, and then with 5 μg/mL Alexa Fluor® 594 goat anti-rabbit IgG antibody for 1 h at room temperature. The nuclei stained with DAPI were observed in blue channel (excitation at 360 nm/emission at 460 nm). The p32 were observed in Cy3.5 channel (excitation at 580 nm/emission at 620 nm). Symbols – and + indicate no heating (37 °C) and heating (45 °C), respectively.
Fig. S3. In vitro cellular binding and internalization of Cy5.5-labeled control magnetic nanoworms (NWs) upon heating. C8161 or MDA-MB-435 cells were treated with NWs (Green) for 20 min at 37 °C or 45 °C (in cell incubator) and then incubated for an additional 2 h at 37 °C. The Cy5.5 fluorescence of NWs was imaged to observe cellular distribution of nanoparticles. The nuclei and p32 were stained with 4′-6-diamidino-2-phenylindole (DAPI, blue), and anti-p32 antibody followed by Alexa Fluor® 594 goat anti-rabbit IgG (Red), respectively. Symbols − and + indicate no heating (37 °C) and heating (45 °C), respectively. Brightness and contrast have been adjusted across the whole image.

Fig. S4. (A) Quantification of C8161 tumor targeting of Cy7-labeled LyP-1-conjugated magnetic nanoworms (LyP1NWs) over Cy7-labeled control magnetic nanoworms (NWs) upon heating. Mice bearing bilateral C8161 tumors were intravenously injected with LyP1NWs or NWs and one tumor was immediately heated at 45 °C for 30 min using temperature-controlled water bath. At 24 h post-injection, the tissues were collected from the mice and the Cy7 fluorescence in tissues was obtained using NIR fluorescence imaging system. (n = 3–4). (B) Histological analysis of LyP1NW and NW distribution in C8161 tumors treated with or without heating. Mice bearing bilateral C8161 tumors were intravenously injected with LyP1NWs or NWs and one tumor was immediately heated at 45 °C for 30 min using temperature-controlled water bath. At 24 h post-injection, the tissues were collected from the mice, stained and imaged using fluorescence microscope. The NWs (Green) was labeled with Cy5.5 to determine nanoparticle distribution with Cy5.5 fluorescence in tumors. The nuclei and p32 were stained with 4′-6-diamidino-2-phenylindole (DAPI, Blue), and anti-p32 antibody followed by Alexa Fluor® 594 goat anti-rabbit IgG (Red), respectively. Symbols − and + indicate no heating (37 °C) and heating (45 °C), respectively. Brightness and contrast have been adjusted across the whole image.
Fig. S5. Histological analysis of MDA-MB-435 tumors injected with LyP1NWs after heat treatment. Mice bearing bilateral MDA-MB-435 tumors were intravenously injected with LyP1NWs immediately after one tumor was heated at 45 °C for 30 min using temperature-controlled water bath. 24 h post-injection, the tissues were collected from the mice, stained and imaged using fluorescence microscope. The NWs (Green) were labeled with Cy5.5 to determine nanoparticle distribution with Cy5.5 fluorescence in tumors. The nuclei and p32 receptor or blood vessels were stained with 4′,6-diamidino-2-phenylindole (DAPI, Blue), and anti-p32 antibody followed by Alexa Fluor® 594 goat anti-rabbit IgG (Red) or CD31 followed by Alexa Fluor® 594 goat anti-rat IgG (Red), respectively. Scale bar is 100 μm.

Fig. S6. Temperature-induced cytotoxicity of various therapeutic molecule or nanoparticle formulations towards C8161 human carcinoma cells by MTT assay. The cells were treated with free DOX, control DOX liposomes (LPs), and LyP-1-conjugated DOX liposomes (LyP1LPs) with different concentrations at 37 °C (A) or 45 °C (B) for 20 min (in cell incubator) and then incubated for an additional 4 h at 37 °C. The cells were rinsed with cell medium three times, incubated for an additional 44 h at 37 °C and then evaluated for cytotoxicity using MTT assay.
Fig. S7.  Histological analysis of DOX distribution in untreated MDA-MB-435 tumors (NR-L) of the mice in fig. 4A. The Alexa Fluor® 488 label (on control LP, Green) or 5(6)-carboxyfluorescein (FAM) label (on LyP-1 of LyP1LP, Green) and DOX (Red) fluorescence of nanoparticles was imaged to determine nanoparticle and DOX distribution in tumors, respectively. The nuclei were stained with 4′-6-diamidino-2-phenylindole (DAPI, Blue). Scale bar is 100 μm.

Fig. S8.  Tumor therapy without photo-thermal heating. Mice bearing bilateral MDA-MB-435 tumors were injected with saline, control LPs or LyP1LPs (6 mgDOX/kg) at 0, 6, and 12 days (arrow head). Tumor volumes were monitored every 3 days after LP injection. Error bars indicate standard deviations from more than three measurements. *P < 0.05 for LyP1LP sample and all other treatment sets (n = 4).

Fig. S9.  NR-mediated photothermal tumor therapy at different temperatures. At 72 h post-injection of NRs (10 mgAu/kg), one tumor of the mice bearing bilateral MDA-MB-435 tumors was irradiated (810 nm, ~0.70 or ~0.75 W/cm² for 43 °C or 45 °C, respectively) for 30 min while maintaining average tumor surface temperature at ~43 or ~45 °C under infrared thermographic surveillance. The control group was not irradiated. Tumor volumes were monitored every 3 days after irradiation. Error bars indicate standard deviations from more than three measurements. −H and +H indicate treatment without and with NR-mediated heating, respectively. *P < 0.05, **P < 0.01 for +H (45 °C) sample and −H sample (n = 4).