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 many systems have not demonstrated clinical success. Here, we present a cooperative nanosystem consisting of two discrete nanomaterials. The first component is gold nanorods (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) that 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-231 tumors that are treated with the combined NR/LyP-1LP therapeutic system display significant reductions in tumor volume compared with individual nanoparticles or untreated targeted cooperate system.

cancer therapy | gold nanorods | liposomes | magnetic nanoworms | protein expression

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 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 nanosystem, 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-231 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).


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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 postheat 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-medièated 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 tumors 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 xenografted tumors in vivo, we next evaluated in vitro photothermal-assisted particle targeting to tumor cells in vitro and to xenografted tumors in vivo. We next evaluated in vitro photothermal-assisted particle targeting to tumor cells in vitro and to xenografted tumors in vivo. We next evaluated in vitro photothermal-assisted particle targeting to tumor cells in vitro and to xenografted tumors in vivo. We next evaluated in vitro photothermal-assisted particle targeting to tumor cells in vitro and to xenografted tumors in vivo.
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...

![Fig. 4](image-url)

**Fig. 4.** Successful antitumor therapy using cooperative nanosystem, demonstrated in mice bearing MDA-MB-435 tumors. (A) Quantification of in vivo accumulation of DOX in tumors as a function of NR-mediated laser heating of LyP-1-conjugated liposomes (LyP1LP) or control liposomes that contain no targeting peptide (LP). NR + L and NR – L indicate mice containing gold nanorods that were or were not subjected to laser treatment, respectively. Amount of DOX present quantified by fluorescence microscopy to yield a percentage of injected dose per tissue mass. * indicates P < 0.05 (n = 3 – 4). (B) Histological analysis of DOX distribution in tumors from the mice in (A) who were subjected to NR-mediated thermal therapy showing the distribution of nanoparticles (Alexa Fluor® 488 label on control liposome and 5(6)-carboxyfluorescein (FAM) label on LyP-1, Green) and DOX (Red). Nuclei stained with DAPI (Blue). Scale bar is 100 μm. (C) Change in tumor volume of different treatment groups containing bilateral MDA-MB-435 xenograft tumors. 72 h postinjection of gold nanorods (NR, 10 mgAu/kg), mice were injected with a single dose of saline, control liposomes (LP), and LyP-1-conjugated liposomes (LyP1LP). “+ H (Hyperthermia)” denotes one of the two tumors in the animal that was irradiated with the NIR laser. The tumor not irradiated is indicated as “− H”. Tumor volumes monitored every 3 d postirradiation. Error bars indicate standard deviations from ≥3 measurements. * indicates P < 0.05 and ** indicates P < 0.02 for + H + LyP1LP sample and all other treatment sets (n = 4 – 6). (D) Survival rate in different treatment groups after a single dose (3 mgDOX/kg) into mice (n = 6) containing single MDA-MB-435 xenograft tumors. Error bars indicate standard deviations from ≥3 measurements.
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 nanorods (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 attached to partially Cy5.5/Cy7-labeled aminated NWs via a PEG crosslinker for a period of 9 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 NPs (10 mgAu/kg). At 72 h post-injection 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 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 NPs (10 mgAu/kg). At 72 h post-injection 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 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 NPs (10 mgAu/kg). Control LPS or LyP1LPs (3 mgDOX/kg) were systemically administered 72 h post-injection 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 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. Student's t test was used for statistical analysis of the results. The experimental procedures are described in more detail in SI Materials and Methods.

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18. Медицинские науки


