Cancer Cell Membrane-Coated Gold Nanocages with Hyperthermia-Triggered Drug Release and Homotypic Target Inhibit Growth and Metastasis of Breast Cancer

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The cell-specific targeting drug delivery and controlled release of drug at the cancer cells are still the main challenges for anti-breast cancer metastasis therapy. Herein, the authors first report a biomimetic drug delivery system composed of doxorubicin (DOX)-loaded gold nanocages (AuNs) as the inner cores and 4T1 cancer cell membranes (CMVs) as the outer shells (coated surface of DOX-incorporated AuNs (CDAuNs)). The CDAuNs, perfectly utilizing the natural cancer cell membranes with the homotypic targeting and hyperthermia-responsive ability to cap the DAuNs with the photothermal property, can realize the selective targeting of the homotypic tumor cells, hyperthermia-triggered drug release under the near-infrared laser irradiation, and the combination of chemo/photothermal therapy. The CDAuNs exhibit a stimuli-release of DOX under the hyperthermia and a high cell-specific targeting of the 4T1 cells in vitro. Moreover, the excellent combinational therapy with about 98.9% and 98.5% inhibiting rates of the tumor volume and metastatic nodules is observed in the 4T1 orthotopic mammary tumor models. As a result, CDAuNs can be a promising nanodelivery system for the future therapy of breast cancer.

1. Introduction

Breast cancer is the leading cause of the high mortality in female patients worldwide, and metastasis accounts for over 90% of breast cancer death. In clinic, the chemotherapy remains the standard approach for breast cancer metastasis therapy. However, the severe side effect, low bioavailability, and poor delivery efficiency of the chemotherapeutic agent limit its therapeutic effects. To improve these deficiencies, stimuli-responsive drug delivery systems responding to stimulations including pH, enzyme, magnet, and hyperthermia have gained more and more attentions to achieve the spatiotemporally control of drug release in the specific tumor tissues, increase the drug accumulation in the cancer cells and decrease the side effects.

Among these stimuli, near-infrared (NIR) laser (NIR, 650–900 nm) inducing hyperthermia is generally the perfect choice due to the excellent tissue penetration capability. The hyperthermia can also cause the photothermal therapy (PTT) effects on the cancer cells. Numerous photothermal agents such as gold nanomaterials (e.g., nanocage and nanorod) and carbon nanotubes have been developed. In particular, the gold nanocages (AuNs) with hollow interiors, porous walls, tunable localized surface plasmon resonance (LSPR) band in the NIR region, high efficiency of photothermal conversion and excellent biocompatibility of Au have been explored to load drugs to deal with the malignant tumors. Upon irradiation on AuNs with a NIR laser, the generated hyperthermia could accelerate the release of cargo into the target sites and also preferentially ablate the cancer cells with favorable PTT effects. However, the intrinsic hollow interiors and pores of the AuNs could result in premature drug leakage in vivo. In addition, to date, the nanoparticles with functionalization such as antibodies to improve the targeting efficiency to the specific cells still suffer from the unsatisfied tumor specificity, binding affinity, and the relatively short circulation time in vivo. Therefore, it is imperative to find an optimal strategy to modify AuNs to realize the selective targeting of the cancer cells with high efficiency and stimuli-release of cargo under the hyperthermia away from the premature drug leakage.

Currently, the biomimetic cell membrane-based drug delivery systems have attracted more attention for the development of the intelligent nanoparticles, such as red blood
cell membrane-derived nanoparticles,[22] platelet-mimicking nanoparticles,[23] and stem cell-derived “nanoghosts.”[24] The biomimetic cell membranes-coated nanoparticles could significantly increase the stability of nanoparticles with less leak of drugs in physiological conditions.[23b,25] In addition, a mild hyperthermia at 43 °C could increase the permeability and fluidity of the cell membrane, thereby enhancing the intracellular accumulation of the drugs.[26] Moreover, the cancer cell membranes could bestow the natural ability of targeting the homotypic tumor cells in vitro.[27]

Herein, we designed a cancer cell membrane-coated gold nanocage with hyperthermia-triggered drug release and homotypic target to inhibit metastasis of breast cancer. At first, the anticancer agent doxorubicin (DOX) was incorporated into AuNs as the inner cores (DAuNs). Then, the cancer cell membranes derived from the 4T1 breast cancer cells, a typical metastatic tumor cell line with high metastatic capability,[28] as the shells were coated to the surface of DAuNs (CDAuNs). We hypothesized that the cancer cell membrane-coated AuNs loading drugs could selectively targeted to the tumor cells, photothermal controlled drug release with high spatiotemporal resolution and achieve the chemo/photothermal combination for efficient therapy of metastatic breast cancer (Scheme 1).

2. Results and Discussion

2.1. Preparation and Characterization of CAuNCs-DOX

The cancer cell membrane-coated DAuNs were prepared using the top-down approach. The 4T1 cell membrane vesicles (CMVs) obtained from the 4T1 cells were used as the shells of CDAuNs. The averaged hydrodynamic diameter of CDAuNs was 70.5 ± 0.4 nm, which was a little bigger than that of DAuNs as the core. The zeta-potential of CDAuNs was −18.9 ± 0.4 mV (Figure 1A), which was the same as CMVs (−19.3 ± 0.2 mV), lower than that of DAuNs (−16.5 ± 0.6 mV). The particle size and zeta-potential both indicated that DAuNs were successfully coated by the CMVs. Transmission electron microscope (TEM) was applied to characterize the morphology of DAuNs, CDAuNs, and CDAuNs with NIR irradiation (Figure 1B). The size of DAuNs was about 40 nm as the size of DAuNs detected using the dynamic light scattering (DLS) analyzer. DAuNs exhibited a polyhedron morphology. In addition, it was also further confirmed that CDAuNs were successfully fabricated because of a slight gray shell outside of the DAuNs. After NIR irradiation, the gray shell was damaged, indicating that the thermal destruction generated from AuNs against the lipid bilayer of CMVs, which demonstrated that CDAuNs could be controlled open under the NIR laser irradiation. In addition, the laser irradiation did not change the structure of the AuNs (Figure S1, Supporting Information). The DOX loading ratio and encapsulation efficiency of CDAuNs were 5.5 ± 0.2% and 97.3 ± 0.4%, respectively.

The photothermal conversion experiments were performed to characterize the photothermal effects of CDAuNs (Figure 1C,D). The AuNs, CAuNs, and CDAuNs exhibited similar temperature rises profiles, indicating that the cell membrane coating and the loading drug had negligible effects on the photothermal effects of AuNs. The temperature of CDAuNs could rapidly go up to about 48 °C during the NIR laser irradiation of 808 nm at a power density of 2.5 W cm$^{-2}$ for 5 min. Finally, their temperature went up by about 23 °C during the NIR laser irradiation of 808 nm at a power density of 2.5 W cm$^{-2}$ for 8 min. While the temperature of nanoparticle-free phosphate buffer solution (PBS) only increased by about 3 °C. The superior temperature rise of the AuNs could contribute to destroying the cancer cells because of the hyperthermia (42–47 °C).[29] Moreover, it could also accelerate both the destruction of the membrane shell and release of the encapsulated drugs.[30] UV–vis–NIR spectra were used to investigate the LSPR peak of the formulations (Figure 1E). Compared with the DAuNs, the LSPR peak of CDAuNs at 760 nm has barely altered with a slight blue shift, which could result from the improved dispersion stability of CDAuNs. The characteristic peak of DOX was also found in CDAuNs, indicating the successful encapsulation of DOX into the nanocages. Moreover, after the NIR laser irradiation, the absorption of the AuNs was negligibly changed (Figure S2, Supporting Information). About 75% DOX in DAuNs exhibited a burst release during the first 8 h (Figure 1F), which should be mainly attributed to the premature drug leakage.[18,13] After coating AuNs with cell membranes, the DOX release was obviously sustained with cumulative release of only 38% within 8 h,
indicating the exciting coating efficiency and good stability of CDAuNs during the physiological conditions. Upon irradiation with the 808 nm laser, the DOX release was dramatically accelerated and showed the similar release profile as DAuNs, which could be caused by the fact that the photothermal effects could destroy the cell membranes outer shell of CDAuNs (Figure 1B), simultaneously enhance the diffusion of drug from the hollow interiors and pores of AuNs and increase DOX release into the surrounding medium.[32]

2.2. Cellular Uptake of CAuNCs-DOX

The cellular internalization of CDAuNs was investigated on the 4T1 cancer cells. Only dim red fluorescence was observed in the cells treated with free DOX (Figure 2A). To exclude the premature leakage of DOX in the AuNs and illustrate the major roles of the membrane proteins in increasing the internalization of the drugs, the DAuNs were coated with the synthetic liposomes membrane to obtain the LDAuNs. Compared to the slight fluorescence intensity of the LDAuNs group, CDAuNs showed much higher DOX fluorescence, indicating the cell membranes as the outer shell significantly increased the cellular uptake of the inner core of DAuNs. The retaining cell membrane proteins remained the homotypic targeting ability as observed by Zhang and co-workers.[27] The expression of the related surface molecules on the 4T1 tumor cells such as TF-antigen and E-cadherin has been proved in our previous study.[35] After exposure to NIR laser, the CDAuNs displayed a dramatically increased red fluorescence, which could be due to the fact that the NIR laser irradiation could cause the hyperthermia generated from AuNs followed by destroying the cell membrane shell presented on the nanocages, accelerating the release of DOX from CDAuNs and also increasing the cellular uptake by the enhanced permeability and fluidity of the cancer cell membranes on 4T1 cells.[26a] The flow cytometry assay was applied to quantify the intracellular uptake of the different formulations (Figure 2B). The fluorescence intensity of CDAuNs was time dependent and always stronger than that of the LDAuNs. The CDAuNs with NIR irradiation exhibited the highest fluorescence intensity during all the test time, and its fluorescence intensity was about 2.6-fold higher than that of CDAuNs group after incubation for 4 h. Taken together, the 4T1 cell membranes coating on the AuNs could play major roles in enhancing the uptake of its source cancer cells, and the NIR stimuli inducing hyperthermia could enhance the release of the loading chemotherapeutic drugs, which demonstrated that CDAuNs could achieve the homotypic targeting of 4T1 cells and photothermal-controlled release of drugs at the target points in vitro.

2.3. Phototoxicity Assay In Vitro

The antiproliferative effects of the nanoparticles were detected using sulforhodamine B (SRB) assay. The DOX-free CAuNs showed negligible dark cytotoxicity at the tested concentrations against 4T1 cells, indicating the good biocompatibility of the carriers (Figure 3A). After irradiation, it displayed the cytotoxicity with the IC_{50} value of 2.4 µg mL^{-1}. No obvious influence was observed in the DOX-treated cells with NIR irradiation, indicating the laser had no effects on the cytotoxicity of DOX alone. The cells incubated with CDAuNs exhibited IC_{50} value of 1.1 µg mL^{-1}, 2.8-fold higher than that of LDAuNs, which showed that CDAuNs exhibited a stronger cytotoxicity, which could be due to the accelerated cellular uptake mediated by 4T1 cell membrane proteins. The strongest antiproliferative effects
The biodistribution of CDAuNs was carried out in the 4T1 orthotopic mammary tumor metastasis model (Figure 2A). After i.v. administration at the dose of 5 mg Dox kg\(^{-1}\), in free DOX group, the large fraction of DOX was accumulated into the heart, lung, kidney quickly, and the low concentration of DOX was detected in the tumors, which could contribute to the high systemic toxicity of free DOX to the normal tissues. CDAuNs with or without NIR laser irradiation showed over threefold lower DOX accumulation than that of the free DOX group in the heart tissues. The DOX concentration in CDAuNs was 2.5-fold higher than that of free DOX group in tumors at the first 1 h. During the next 4 to 24 h, the CDAuNs still exhibited high accumulation in tumor, which could be due to the enhanced permeability and retention effect and the cell membrane mediating homotypic targeting potential as demonstrated above. As mentioned previously, the 4T1 breast cancer cells were typically tumor cell lines with high metastatic capability. After two weeks for the tumor implantation, the pulmonary were colocalized by the 4T1 tumor cells as observed in our previous work.\(^{[35]}\) Moreover, the cell membrane-coated nanocages also increase the DOX distribution in the lung tissues, which could be attributed to the homotypic targeting capability. Upon irradiation with NIR laser after injection for 1 h, the DOX accumulation in the tumors enhanced significantly, which was nearly 2.1-fold higher than before. The photothermal effects could be beneficial to accelerate the drug release and diffusion into the deeper tumor areas followed by a higher distribution of DOX in the tumor tissues. Without laser treatment, the DOX could not distribute into the deep tumor tissues because of the high interstitial fluid pressure, reduced transcapillary pressure gradient and dense extracellular matrix.\(^{[36]}\) As a result, the CDAuNs with NIR irradiation could achieve an admirable distribution in the tumor tissues and lungs for the further cancer therapy.

The photothermal treatment was further used to measure the PTT effects of the formulations. The in vivo photothermal imaging was conducted after administration for 1 h of PBS, CAuNs, and CDAuNs into the nude mice. The mice received injection of CAuNs and CDAuNs showed obvious temperature rise (Figure 4B,C). During the irradiation for 8 min in the free DOX group, the temperature only increased to 43 ± 2.1 °C. However, the photothermal effects of the tumors increased with the irradiation time both in the CAuNs and CDAuNs group. The in vivo temperature profile of CDAuNs indicated that the temperature could quickly rise by about 16 °C in 5 min. The tumor temperature in CAuNs group rose to 57 ± 1.5 °C, which was similar to the CAuNs group, indicating the superior heat generating and the tumor targeting accumulation potential in the cell membrane-coated AuNs. It was reported that the reduced heat tolerance of tumor tissues than the normal cells would contribute to the selective destruction of the tumor cells at the temperatures above hyperthermia (42–47 °C).\(^{[29]}\) Therefore, the CDAuNs after laser irradiation would have potential to induce photothermal ablation of tumor cells along with significant PTT effects and the hyperthermia-responsive drug release in vivo.

2.4. In Vivo Biodistribution and PTT Effects

The biodistribution of CDAuNs was carried out in the 4T1 orthotopic mammary tumor metastasis model (Figure 2A). After i.v. administration at the dose of 5 mg Dox kg\(^{-1}\), in free DOX group, the large fraction of DOX was accumulated into the heart, lung, kidney quickly, and the low concentration of DOX was detected in the tumors, which could contribute to the high systemic toxicity of free DOX to the normal tissues. CDAuNs with or without NIR laser irradiation showed over threefold lower DOX accumulation than that of the free DOX group in the heart tissues. The DOX concentration in CDAuNs was 2.5-fold higher than that of free DOX group in tumors at the first 1 h. During the next 4 to 24 h, the CDAuNs still exhibited high accumulation in tumor, which could be due to the enhanced permeability and retention effect and the cell membrane mediating homotypic targeting potential as demonstrated above. As mentioned previously, the 4T1 breast cancer cells were typically tumor cell lines with high metastatic capability. After two weeks for the tumor implantation, the pulmonary were colocalized by the 4T1 tumor cells as observed in our previous work.\(^{[35]}\) Moreover, the cell membrane-coated nanocages also increase the DOX distribution in the lung tissues, which could be attributed to the homotypic targeting capability. Upon irradiation with NIR laser after injection for 1 h, the DOX accumulation in the tumors enhanced significantly, which was nearly 2.1-fold higher than before. The photothermal effects could be beneficial to accelerate the drug release and diffusion into the deeper tumor areas followed by a higher distribution of DOX in the tumor tissues. Without laser treatment, the DOX could not distribute into the deep tumor tissues because of the high interstitial fluid pressure, reduced transcapillary pressure gradient and dense extracellular matrix.\(^{[36]}\) As a result, the CDAuNs with NIR irradiation could achieve an admirable distribution in the tumor tissues and lungs for the further cancer therapy.

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2.5. In Vivo Antitumor and Antimetastasis Efficacy

The in vivo inhibition effects on the primary tumors and lung metastasis of the CDAuNs were evaluated in the 4T1 orthotopic mammary tumor metastasis model. In the group treated with saline, the tumors grew rapidly during the test (Figure 5A). The mice treated with CAuNs without DOX exhibited a similar growth behavior as the saline group without significant antitumor effects and little cell apoptosis (Figure 5D). No obvious difference of the inhibition efficacy was found in the free DOX and CAuNs with NIR irradiation groups. CAuNs showed the low antitumor growth efficiency, which could result from the negligible antiproliferative effects of the AuNs.\(^{[19]}\) The antitumor
effects of CAuNs with NIR irradiation mainly resulted from the PTT effects of the AuNs in the tumor tissues. After loading DOX in CAuNs, the tumor volume was significantly reduced by 61.2%, indicating a relatively higher antitumor efficacy caused by the homotypic targeting in the tumors. When irradiated with a NIR laser after intravenous injection of CDAuNs, the tumor volume was dramatically suppressed by 98.9% compared with the saline group and the strongest cell apoptosis with the highest green fluorescence distributed around the whole tumor tissues, which should attribute to the cell membrane-induced homotypic accumulation of the nanoparticles and significant hyperthermia effect followed by the hyperthermia-triggered release of DOX in the tumor tissues. These antitumor effects of the formulations were consistent with the biodistribution profiles in vivo. The body weight changes of all the mice were detected as an indicator of systemic toxicity (Figure 5B). The mice treated with free DOX exhibited obvious weight loss during the test, but no significant weight loss was found in other groups, demonstrating the absence of severe systemic toxicity.

To evaluate the antimetastasis efficacy of the CDAuNs, the lungs were imaged and the numbers of pulmonary metastatic nodules were quantified (Figure 5C,E). Tremendous metastatic nodules were observed on the lungs of the saline and CAuNs groups, indicating that the nanocarriers without drug showed negligible effects on inhibiting the tumor metastasis. Compared to the saline group, the number of metastatic nodules in the free DOX group was reduced by 34.7%. After irradiation with NIR laser, the CAuNs exhibited the PTT effects in the tumors, thereby decreasing the lung metastatic nodules. The CDAuNs further increased the antimetastasis effects with fewer metastatic nodules after exposed under the NIR laser. The metastases were dramatically decreased by 98.5% along with the decreasing metastatic areas in H&E staining of the lung tissues (Figure 5F), indicating that the systemic administration of CDAuNs in combination of homotypic targeting of tumors with hyperthermia-triggered release of DOX under the NIR irradiation could induce an exciting effects in inhibiting tumor growth and metastasis in vivo. Taken together, the CDAuNs could be utilized as efficient agents to deal with cancer metastasis.

2.6. In Vivo Toxicity Assessment

The in vivo toxicity was detected using the histopathological analysis. The obvious accumulation of neutrophils was
observed in the heart of free DOX group (Figure 6), suggesting the evident cardiotoxicity of free DOX. In contrast, the CDAuNs with or without NIR laser irradiation groups did not show neutrophils accumulation, which could be due to the decreased distribution of DOX in heart as shown above. No obvious pathological change was found in the other detected organs of the CDAuNs with NIR group, indicating the good biocompatibility of CDAuNs with NIR irradiation.

3. Conclusion

In summary, we fabricated a biomimetic nanodrug delivery system to fight against the breast cancer metastasis. The CDAuNs were prepared from the cancer cell membrane-derived shells and the drug-loaded AuNCs cores. The high photothermal conversion ability of AuNCs and the rapid release of DOX under the NIR laser irradiation indicated the successful hyperthermia-responsive drug release behavior of the nanoparticles. The superior targeting efficiency of the 4T1 cells and the higher accumulation of the CDAuNs in tumor tissues demonstrated that the nanoparticles achieved selectively targeting of the 4T1 tumor cells. Finally, the exciting antitumor growth and metastasis effects suggested that the combination of photothermal therapy and chemotherapy rather than any of them alone showed great potential to the breast cancer treatment. As a result, the CDAuNs could serve as a promising nanodrug delivery system with tumor-directed and hyperthermia-responsive drug release ability for the future breast cancer therapy.

4. Experimental Section

Materials: AuNs were obtained from XFNano (Nanjing, China). Doxorubicin hydrochloride (DOX), SRB, and Hoechst 33342 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Soybean phosphatidylcholine (S100, SPC) was obtained from Lipoid GmbH (Ludwigshafen, Germany). Trypsin-ethylenediamine tetraacetic acid (EDTA), fetal bovine serum (FBS), and PBS were obtained from Gibco-BRL (Burlington, Canada). The Roswell Park Memorial Institute (RPMI) 1640 medium and antibiotics were purchased from Invitrogen (Oregon, USA). Protease Inhibitor Cocktail (EDTA-Free, 100 × in dimethylsulfoxide (DMSO)) was purchased from SelleckChem (Houston, USA). TrypanBlue and bicinchoninic acid (BCA) protein assay kit was obtained from Beyotime (Shanghai, China). Radio-immunoprecipitation assay (RIPA) lysis buffer was obtained from Roche (Basel, Switzerland). All other chemical reagents were of analytical grade and used without further purification.

Cell Culture: 4T1 mammary breast cancer cell line was obtained from cell bank of Shanghai, CAS (Shanghai, China) and were grown in RPMI 1640 (Gibico, USA) with 10% FBS, 100 U mL⁻¹ penicillin G sodium, 100 µg mL⁻¹ streptomycin sulfate, 2.5 g L⁻¹ glucose, and 0.11 g L⁻¹ sodium pyruvate. The cells were incubated at 37 °C in a humidified incubator with 5% CO₂.

Animals: Female BALB/c nude mice, female BALB/c mice (4–5 week old, 18–22 g), and Sprague-Dawley rats were purchased from Shanghai Experimental Animal Center (Shanghai). All the animal procedures were carried out under the guideline approved by the Institutional Animal Care and Use Committee (IACUC) of the Shanghai Institute of Materia Medica, CAS.

Preparation and Characterization of CDAuNs: To prepare CDAuNs, extrusion approach was used.[34] First, the 4T1 cell membrane vesicles (CMVs) as the outer shells were prepared. Briefly, the harvested 4T1 cells were treated with the hypertonic Tris buffer (pH = 7.4) at 4 °C for 1 h, homogenized at 22 000 rpm for 1 min (Precellys 24, Bertin technology, France) and centrifuged at 500 × g for 10 min to remove...
the intracellular contents. Then the supernatants were centrifuged at 10 000 \( \times g \) for 10 min and 100 000 \( \times g \) for 1 h (BiofugeStratos, Thermo) to get the cell pellets. Afterward, the pellets were washed with PBS. The resulting pellets were sonicated for 5 s using an ultrasonicator (JYD-650L, Zhixin Inc., China) and extruded through 400 nm and then 200 nm polycarbonate membranes (LiposoFast LF1, Avestin, Canada). Second, the DAuNs as the inner cores were prepared by incubating the AuNs (0.1 mg mL\(^{-1}\)) with DOX (0.05 mg mL\(^{-1}\)) under stirring at room temperature for 24 h. The final mixture was centrifuged and washed with PBS to remove the residual free DOX. Finally, DAuNs were then mixed with 4T1 CMs (1 mg) which were quantified by lyophilization and extruded through 100 nm polycarbonate membranes. Then the particle size and zeta potential of CMVs, DAuNs, and CDAuNs with NIR irradiation were characterized using a TEM (JEOL JEM-1230, Tokyo, Japan). The thermal profiles of AuNs, CAuNs, and CDAuNs in PBS were measured upon irradiation of an 808 nm laser (MDL-N-10W, Changchun New Industries, China) at 2.5 W cm\(^{-2}\) for 8 min, the thermographic images were determined by an infrared thermal camera (A150-15-M, Irtech Ltd.), and the temperatures at different time points were recorded by the accompanied software. The PBS was also determined as negative control. The UV–vis–NIR absorption spectra of DOX, DAuNs, and CDAuNs in PBS were measured using a UV–vis spectrometer. The DOX release from the different formulations including the DAuNs, CDAuNs, and CDAuNs with NIR irradiation at 2.5 W cm\(^{-2}\) for 5 min was determined in PBS or 1640 media at 37 °C. At predetermined time intervals, 100 µL of the medium was taken out from the suspension and replaced with an equal volume of the fresh medium. The released DOX were obtained by centrifugation, and the DOX concentration were measured using fluorescence spectrophotometry.

**Figure 5.** In vivo antitumor and antimetastasis effects of the nanoparticles in the 4T1 tumor-bearing nude mice: A) tumor growth profiles, B) body weight changes, and C) quantitative analysis of pulmonary metastatic nodules for every group. D) Terminal-deoxynucleoitidyl transferase mediated nick end labeling (TUNEL) staining (200x) of the tumor tissues, E) the collected lungs, and F) H&E staining (Scale bar = 100 µm) of the lung tissues from the mice. White arrows indicated the metastatic models. Data were given as the mean ± SD (\( n = 5 \)). Statistical significance: *\( p < 0.05 \) and **\( p < 0.01 \).

**Cellular Internalization Experiment:** To investigate the intracellular uptake of the DOX-loaded formulations, 4T1 cells seeded in the 24-well plates were treated with the free DOX, LDAuNs, which were obtained by extruding the DAuNs and the synthetic liposomes prepared with soybean-phosphatidylcholine (PC) and cholesterol using weight ratio of 75:25, CAuNs, and CDAuNs with NIR irradiation of 5 min for 4 h at the equivalent DOX concentration of 5.0 µg mL\(^{-1}\), respectively. Then, the cells were stained with Hoechst 33342, washed with PBS, and imaged using a fluorescence microscope (Olympus, Japan). To further quantitatively determine the cellular internalization, the 4T1 cells treated with the free DOX, LDAuNs, CDAuNs, and CDAuNs with NIR irradiation of 5 min were incubated for 1, 2, and 4 h at the equivalent DOX concentration of 5.0 µg mL\(^{-1}\), respectively. Afterward, the cells were collected, washed, and suspended in PBS to detect the fluorescence intensity of DOX using a flow cytometry, and the data were analyzed with a Cell Quest software.

**In Vitro Cytotoxicity and Photothermal Ablation Assays:** 4T1 cells were seeded in 96-well plates at a density of 5 \times 10^5 cells per well and...
incubated for 24 h. Afterward, the cells were treated with DOX, CAuNs, and CDAuNs at DOX concentrations from 0.01 to 10 µg mL⁻¹ or CAuNs concentrations from 0.18 to 181 µg mL⁻¹ for 24 h. The plates used to investigate the dark toxicity of the formulations were kept on incubation in the dark for another 24 h. The other treated plates with the same formulations prepared to investigate the phototoxicity were exposed to the 808 nm laser irradiation at 2.5 W cm⁻² for 5 min and incubated for another 24 h. SRB assay was applied to measure the cell viability.

For the photothermal ablation study, 4T1 cells were seeded in 96-well plates at a density of 5 × 10⁴ cells per well and incubated for 24 h. Then the cells were incubated with DOX, CAuNs, and CDAuNs at DOX concentration of 2 µg mL⁻¹ for 12 h. Afterward, the cells were irradiated at 2.5 W cm⁻² for 5 min and incubated for another 24 h, and cells were treated with LIVE/DEAD Viability/Cytotoxicity Kit according to the protocol and detected with a fluorescent microscope (IX81, Olympus, Japan).

In Vivo Biodistribution: The in vivo biodistribution assay was determined in the orthotopic breast tumor model. The female nude mice were subcutaneously injected with 100 µL of cell suspension (9 × 10⁵ 4T1 cells) to initiate the breast tumor. After two weeks, the nude mice were randomly assigned to three groups and intravenously injected with DOX, CAuNs, and CDAuNs with NIR irradiation of 5 min receiving injection for 1 h, respectively (5 mg kg⁻¹ of DOX). For each group, the mice were sacrificed at 1, 4, 8, and 24 h after administration. The main organs including heart, liver, spleen, lung, kidney, and tumor were collected and washed with PBS. Then the typical organs were weighed, and about 100 mg of each organ was homogenized in DMSO, followed by centrifugation at 20 000 × g for 10 min. The collected supernatants were measured for the DOX content by fluorescence spectrophotometry and expressed as DOX weight in per gram of tissue.

In Vivo Temperature Measurement: The in vivo photothermal effects were also determined in the orthotopic breast tumor model as described above. After two weeks, the tumor bearing mice were intravenously injected with PBS, CAuNs, and CDAuNs at the CAuNs dose of 91 mg kg⁻¹ per mouse, respectively. After injection for 1 h, the mice were anesthetized and the tumors were exposed to 808 nm laser at 2.5 W cm⁻² for 5 min. The thermographic images were determined by an infrared thermal camera (A150-15-M, Irtech Ltd.) and the photothermal temperatures at different time points were recorded with the accompanied software.

In Vivo Antitumor and Antimetastasis Effect: The in vivo orthotopic breast tumor model was established by subcutaneously injecting 9 × 10⁵ 4T1 cells into the right mammary gland of mice. When the tumor volume grew to about 100–200 mm³ after injection, the mice were randomly assigned to six groups (n = 5) and intravenously injected with saline, DOX, CAuNs, CDAuNs, and CAuNs with NIR laser and CDAuNs with NIR laser irradiation at 2.5 W cm⁻² for 5 min (5 mg kg⁻¹ of DOX and 91 mg kg⁻¹ of CAuNs) every 3 d for four times, respectively. The animal weight and tumor volume were measured every 3 d until the test end. Tumor volume (V) was calculated using the formula: \[ V = \frac{L \times W \times W}{2} \] (L: the longest diameter of tumor and W: the shortest diameter perpendicular to length). After about 3 weeks, the mice were sacrificed, and the lungs of each group were collected, rinsed with PBS, and imaged with a camera. The metastatic nodules on the pulmonary tissues were counted, and the lung sections were stained by hematoxylin and eosin (H&E) to further evaluate the metastatic foci. The obtained tumors were used for TUNEL detection.

Preliminary In Vivo Toxicity Assessment: The female BALB/c nude mice (18–22 g) were intravenously injected with saline, DOX, CAuNs, CDAuNs, and CAuNs with NIR laser and CDAuNs with NIR laser irradiation at 2.5 W cm⁻² for 5 min (5 mg kg⁻¹ of DOX and 91 mg kg⁻¹ of CAuNs) every 3 d for four times, respectively. After 22 d from the first injection, the mice were sacrificed, and the heart, liver, spleen, lung, and kidney were harvested. The obtained tissues were fixed in formalin for the next paraffin sectioning and hematoxylin and eosin (H&E) assays to evaluate the histopathologic toxicity of the typical tissues.

Figure 6. Histopathologic examination of the tissues including heart, liver, spleen, lung, and kidney from BALB/c nude mice after intravenous administration of saline, DOX, CAuNs, and CDAuNs with NIR irradiation for 22 d. Black arrows indicated the occurrence of inflammation.
Statistical Analysis: The mean ± SD were determined for all the treatment groups. Statistical analysis was performed by Student’s t-test (two-tailed). The difference between two groups was considered statistically significant for *p < 0.05 and very significant for **p < 0.01.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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