ABSTRACT: Breast cancer is one of the major diseases that threaten women’s health. Lymph node (LN) metastasis is the most common metastatic path of breast cancer. Finding a simple, effective, and safe strategy to eliminate metastatic tumors in LNs is highly desired for clinical use. Carbon nanoparticles (CNs), as an LN tracer, have been widely used in the clinical setting. In addition, previous experiments have confirmed that CNs have good photothermal conversion and imaging properties. In this study, we used CNs as a photothermal conversion material and drug carrier, poly(lactic-co-glycolic acid) (PLGA) as a film-forming material, and docetaxel as a chemotherapy drug to prepare multifunctional nanoparticles (DOC-CNPs). The prepared DOC-CNPs present as a black solution, which shows smooth spherical particles under light microscopy and transmission electron microscopy (TEM), and they have a good ability for liquid–gas phase transition, good dispersibility, high drug-loading capacity, and low cytotoxicity. In vitro, they can release drugs and inhibit tumor cells after laser irradiation. The photoacoustic (PA) signal intensity and the photothermal conversion efficiency increased with an increase in the concentration of DOC-CNPs. In vivo, after administration, the DOC-CNPs reached the LNs. After laser irradiation, the DOC-CNPs absorbed laser energy, and the temperature of the LNs increased high enough to achieve photothermal therapy under PA and ultrasound monitoring. Fracture of the DOC-CNPs was caused by the liquid–gas phase transition with the increased temperature, and the ruptured DOC-CNPs released docetaxel to achieve targeted chemotherapy. These findings suggested that DOC-CNPs can achieve precise treatment for metastatic LNs of breast cancer with PA and ultrasound visualization.

INTRODUCTION

Lymphatic metastasis is the primary metastatic pathway for many malignancies, especially breast cancer. Currently, the routine treatment mainly consists of resection of the lymph nodes (LNs) and adjuvant chemotherapy, endocrine therapy, and radiotherapy. However, during the resection process, some LNs cannot be removed due to severe adhesion and tissue invasion, and some complications are caused by surgery, such as upper limb lymphedema, dysfunction, etc. Traditional transvascular chemotherapy leads to insufficient uptake of chemotherapy drugs into the lymphatic system, which results in a poor chemotherapeutic effect. Endocrine therapy with antiestrogen drugs has few side effects, but only hormone receptor-positive patients are suitable for endocrine therapy. Radiotherapy is one of the important treatments for malignant tumors; however, the side effects of radiotherapy are often unbearable for patients, such as radiation pneumonitis, dermatitis, etc. Therefore, a new treatment with higher safety, reliability, and effectiveness for the metastatic LNs of breast cancer is still desired.

In recent years, studies have shown that photothermal therapy (PTT) has enormous potential in tumor treatment. Compared to traditional chemotherapy and radiotherapy, PTT has attracted more attention in tumor treatment because of its minimal invasion, high precision, and little damage to normal tissues. Its principle is to increase the temperature of photothermal conversion materials in tumor tissues after laser irradiation, and the elevated temperature causes degeneration and necrosis of the tumor cells. Therefore, selecting a photothermal conversion material with good biocompatibility, good photothermal conversion capability, and good photothermal stability is the key to improving the therapeutic effect of PTT. However, due to the scattering and absorption of light by shallow tissues, the PTT effect decreases with increasing
Good photothermal conversion materials can effectively increase the efficiency of photothermal conversion; however, it is difficult to inhibit all tumor cells by PTT alone. The combination of PTT and chemotherapy may be a reliable method because sufficient heat can enhance the permeability of cell membranes, which increases the uptake of chemotherapy drugs by the tumor cells.\textsuperscript{16,17} Moreover, to a great extent, the chemotherapeutic effect depends on the concentration of the chemotherapy drug in the tumor tissue. Compared to intravenous administration, direct administration via the lymphatic pathway can greatly increase the concentration of the chemotherapy drug in the lymphatic system and greatly reduce the concentration of the chemotherapy drug in the blood. This strategy can maintain a high concentration of chemotherapy drug in the metastatic LNs, which is beneficial for inhibiting tumor cells.\textsuperscript{18} The key factor of combined photochemotherapy is the good light absorption capacity of the drug carrier.\textsuperscript{30} The carrier should also have the characteristics of strong drug adsorption and a high release rate. In addition, since photothermal conversion materials have very strong light absorption in the near-infrared region, they have great potential in photoacoustic (PA) imaging.\textsuperscript{19,20} which makes it possible to monitor the treatment in real time with PA imaging. The related literature also confirms that PA imaging can be used as a visualization method for monitoring tumor treatment.\textsuperscript{21−23}

Carbon nanoparticles (CNs), as a lymphatic tracer, were approved for clinical use by the Chinese Food and Drug Administration (CFDA), and they are a type of activated carbon, which appears as a black suspension. After the CNs suspension is injected into the tissue near the primary tumor, the CNs can be quickly delivered into the regional LNs through lymph vessels. Since the LNs and lymphatic vessels can be stained black, surgeons can easily distinguish them from the surrounding tissues.\textsuperscript{4,25} Therefore, CNs are widely used in sentinel lymph node biopsy (SLNB) and LN dissection for thyroid cancer, gastric cancer, breast cancer, etc.,\textsuperscript{26−28} and they have been confirmed as a safe and efficient lymphatic tracer. In our previous study, we found that CNs were also a good photothermal conversion material, which exhibited a high photothermal conversion efficiency and good photothermal stability.\textsuperscript{29} Moreover, nanoparticles containing CNs and liquid fluorocarbon (CNPs) were successfully prepared, and it was confirmed that CNPs can provide PA imaging and effective photothermal therapy for tumor cells in vivo and in vitro.\textsuperscript{30} However, during the experiment, we found that the effect of PTT alone was somewhat limited due to the depth of the LNs, scattering and absorption of light by shallow tissues, etc.

How to improve the treatment effect is the direction of our study. We found that some studies reported that nanoscale-activated nanocarbon was used as a chemotherapy drug carrier.\textsuperscript{30−33} In fact, CNs are also a type of nanoscale-activated carbon. Due to their very large surface area, CNs showed very strong adsorption for many chemotherapy drugs, and the physical and chemical properties of the drugs did not change after absorption. Hence, CNs are a perfect chemotherapeutic drug carrier. The key factor of combined photochemotherapy is the good light absorption capacity of the drug carrier, and CNs, with both good photothermal conversion and a high drug-loading capacity, are the right materials to fit this need.

Docetaxel (DOC) is a paclitaxel-based chemotherapy drug, and it is one of the first-choice drugs for the treatment of breast cancer in a clinical setting.\textsuperscript{34,35} However, if DOC is injected intravenously, then the drug concentration in metastatic LNs is low, and thus, the chemotherapeutic effect is poor. Therefore, we intend to encapsulate DOC in CNPs and deliver them into the LNs through the lymphatic system to increase the concentration of DOC in the LNs. Moreover, systemic side effects such as allergic reactions, myelosuppression, and impaired liver and renal function caused by DOC are common via intravenous injection, but if it is incorporated in CNPs and delivered through the lymphatic system, it is theoretically possible to reduce the systemic side effects.

In this study, we used CNs as a photothermal conversion material and drug carrier, poly(lactic-co-glycolic acid) (PLGA) as a film-forming material, and docetaxel as a chemotherapy drug to prepare multifunctional nanoparticles (DOC-CNPs). The novel nanoparticles were injected around the primary tumor, and they were delivered to the LNs through the lymphatic vessels. The aggregation of DOC-CNPs in LNs could enhance PA imaging after laser irradiation.
Sufficient laser irradiation led to an increased temperature of DOC-CNPs, which caused the liquid–gas phase transition of DOC-CNPs, and then, the DOC was released after the DOC-CNPs ruptured. The released DOC and the rising temperature resulted in a combined therapeutic effect. Based on this study, a new treatment model will be created for metastatic LNs through PA image monitoring and combined photothermal chemotherapy.

RESULTS AND DISCUSSION

Synthesis and Characterization of DOC-CNPs. Figure 1 shows a new method of PTT and targeted chemotherapy for...
metastatic LNs. The metastatic LN is irradiated after subcutaneous injection of a type of novel nanoparticle (DOC-CNPs). The prepared DOC-CNPs appear as a black suspension (Figure 2a). It is shown that the DOC-CNPs exhibited a uniform size and high monodispersity with a spherical morphology before laser irradiation (Figure 2c1). After laser irradiation, the volume of parts of DOC-CNPs obviously increased due to PFH liquid–gas phase transition (Figure 2c2). To learn more about the internal structure of DOC-CNPs, high-magnification transmission electron microscopy (TEM) images were acquired. Figure 2b shows the structure of a single DOC-CNP under TEM. The TEM images demonstrated that the black granular CNs and PFH were evenly mixed and distributed in the DOC-CNPs, with white PLGA as the shell. In addition, DOC-CNPs exhibited a narrow size distribution with an average hydrodynamic diameter of 463.7 ± 25.2 nm (mean ± standard deviation) (Figure 2e). This size was far smaller than that of the ultrasonic microbubbles used for LN detection.36,37 Therefore, DOC-CNPs can be easily and promptly absorbed by the lymphatic system and quickly transported to LNs after subcutaneous injection. The average ζ-potential was determined to be −22.6 ± 1.2 mV (mean ± standard deviation) (Figure 2d), indicating the good stability of the DOC-CNPs. After storage for 1 month, it was found that there was no significant change in the morphology of DOC-CNPs under a light microscope. After laser irradiation, the DOC-CNPs also showed a good ability for liquid–gas phase transition. These results indicated the stability of DOC-CNPs.

The standard light absorption curve of CNs was linear with a regression equation of $y = 0.0045 	imes -0.0226$, $R^2 = 0.9840$ (Figure 3a). The encapsulation rate of CNs in the DOC-CNPs was calculated to be 50.74 ± 2.29% (mean ± standard deviation), and the drug loading of DOC-CNPs was 2.76 ± 0.15% (mean ± standard deviation). The standard curve of DOC obtained from high-performance liquid chromatography (HPLC) was linear, with a regression equation $y = 23521 	imes -20060$, $R^2 = 0.9996$ (Figure 3b). The encapsulation of DOC in the DOC-CNPs was calculated to be 63.85 ± 2.41%, and the drug loading of the DOC-CNPs was 10.08 ± 0.39%.

The cumulative drug release rate vs time curve (Figure 3c) shows that the cumulative drug release ratios in the DC + LA group and the DC group were 95 ± 3.15 and 40 ± 1.59%, respectively. In the first 12 h, there was a sudden release of DOC in the DC + LA group; however, the cumulative release rate became stable from 12 to 72 h. In the DC group, there was no sudden release of DOC, and the cumulative release rate gradually and slowly increased. The release of DOC in the DC group was mainly due to the rupture of a portion of the DOC-CNPs at a temperature of 37 °C, and the process of rupture and release was slow. However, the release of DOC in the DC + LA group was mainly due to the rupture of numerous DOC-CNPs caused by laser irradiation, and the process of rupture and release was relatively faster. These results indicate that the laser is a trigger for drug release.

**Photoacoustic Imaging and Photothermal Effect in Vitro.** It was shown that PA imaging was gradually enhanced with increasing DOC-CNPs concentrations in vitro (Figure 4a). A higher concentration of DOC-CNPs contained more CNs, which absorbed more laser energy and converted it to stronger PA signals. The PA signals originating from various concentrations of DOC-CNPs were also quantitatively

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**Figure 4.** (a1) Photoacoustic (PA) signals of DOC-CNPs at different concentrations in vitro. (a2) Quantitative analysis of the PA signal intensity of concentrations in (a1) (**P < 0.05**). (b1) All concentrations of DOC-CNPs reached 50 °C after laser irradiation in vitro. (b2) Time required for different concentrations of DOC, which reached 50 °C after laser irradiation in vitro. (c) Temperature variation curve of DOC-CNPs with/without irradiation for 3 cycles in vitro. The error bars represent mean ± SD for $n = 3$. 

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analyzed, which also confirmed the effect we observed from the images (Figure 4a2).

All concentrations of DOC-CNPs reached 50 °C after laser irradiation (Figure 4b1); however, the curves of the temperature rise were not the same, and the higher concentrations of DOC-CNPs showed faster speeds (Figure 4b2).

In addition, DOC-CNPs were repeatedly irradiated, and they could reach 50 °C after each irradiation (Figure 4c), which showed good photothermal stability of DOC-CNPs. Gold nanomaterials, as extensively studied photothermal materials, have special surface plasmon resonance; however, their near-infrared absorption peaks are closely related to their shapes and their absorption peaks gradually weaken and disappear under extended laser irradiation. In contrast, DOC-CNPs showed the ability to rise to the required temperature under repeated laser irradiation. This stability is beneficial for multiple irradiations during treatment, which not only enhances the PTT effect but also reduces the related adverse reactions caused by excessive irradiation.

Cytotoxicity and Compatibility. After human umbilical vein endothelial cells (HUVECs) were incubated with CNPs at concentrations of 0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL, the cell viabilities were determined to be 90.09, 88.64, 87.08, 86.91, and 86.11%, respectively (Figure 5a). These results neither

Figure 5. (a) Cytotoxicity results of DOC-CNPs on human breast epithelial cells (HUVEC) (mean ± standard deviation for different concentrations of DOC-CNPs). (b) Apoptosis index of different groups after laser exposure (*P < 0.05).

Figure 6. (a1) PA signals of the DOC-CNPs at different concentrations in vivo. (a2) Quantitative analysis of the PA signal intensity of concentrations in (a1) (*P < 0.05). (b1) All concentrations of DOC-CNPs reached different maximum temperatures after laser irradiation in vivo. (b2) Different concentrations of DOC-CNPs reached different maximum temperatures after laser irradiation in vivo. (c) Temperature variation curve of DOC-CNPs with/without irradiation for three cycles in vivo. The error bars represent mean ± SD for n = 3.
showed a definite correlation between CNP concentration and cell viability, nor did they show a significant difference with the control groups (P > 0.05), suggesting that CNPs have low cytotoxicity and good compatibility toward HUVECs cells. It was found that DOC-CNPs without laser irradiation released a little DOC, as shown in Figure 3c; however, no significant incubated cell death was observed. It can be inferred that DOC is a concentration-dependent chemotherapy drug; additionally, the incubated cells are normal breast epithelial cells, and the effect of chemotherapy drugs on normal breast epithelial cells is also small, so DOC-CNPs still showed low cytotoxicity and good compatibility, which is also beneficial to ensure the safety of DOC-CNPs by subcutaneous injection.

**Tumor Therapy Effect in Vitro.** The flow cytometry experiment showed that the apoptosis rates of tumor cells in the DC + LA group were significantly greater than those in the CNP + LA, CT, LA, and DC groups (P < 0.05) (Figure 5b). It can be inferred that DOC-CNPs can damage tumor cells only after laser irradiation because of the photothermal effect of the laser irradiation and the chemotherapeutic effect of the released DOC.

**Photoacoustic Imaging and Photothermal Effect in Vivo.** Similar to the experiment in vitro, it was also shown that the PA images were gradually enhanced with increasing DOC-CNPs concentrations in vivo (Figure 6a1). The experiment also confirmed that more DOC-CNPs entered the LNs after injection in higher concentration groups, which led to stronger PA signals. The PA signals were also quantitatively analyzed (Figure 6a2), and the results also confirmed the effect we observed from the images.

After laser irradiation, all of the LNs injected with different concentrations of DOC-CNPs were detected with an infrared thermal imager, where the image and temperature were collected. This showed that the temperatures of all groups gradually increased and reached the maximum within 80 s (Figure 6b2). However, the maximum temperatures were not the same, and according to the concentration from high to low, the temperatures were 45.0, 42.8, 39.0, 36.8 °C (Figure 6b1). Many studies have confirmed that the key to hyperthermia is temperature control, and 40–45 °C is the proper temperature for killing tumors, while the normal tissues are not or minimally damaged. In this study, the two higher-concentration groups (15 and 20 mg/mL) quickly obtained the required temperature after laser irradiation. In addition, if the laser irradiation continued, the temperature could also be maintained at the maximum, which was beneficial for continuous exposure and achieving a good therapeutic effect. To ensure a good therapeutic effect, we selected the highest concentration for subsequent treatment experiments.

Similar to that in vitro, an experiment of repeated laser irradiation in vivo was also performed. The result obtained was similar, and the maximum temperature reached 45 °C. After repeated irradiation, the maximum temperature could still be maintained (Figure 6c), which confirmed the stability and effectiveness after repeated laser irradiation in vivo.

**Tumor Therapy Effect and Side Effect In Vivo.** After 4 weeks of treatment, the volumes of LNs in the DC + LA group were significantly smaller than those in other groups (P < 0.05) and the LNs were significantly reduced after treatment in the DC + LA group (P < 0.05) (Figure 7a1,a2). The volumes of LNs in the CNP + LA group were also significantly reduced compared to those in other groups (except the DC + LA group) after treatment (P < 0.05), but their reduction was still lower than that in the DC + LA group (P < 0.05) (Figure 7a3,a4), confirming the phototherapy effect of CNP and the chemotherapeutic effect of DOC-CNPs in vivo. The volumes of LNs in the CT group and the LA group were significantly increased after treatment (P < 0.05), while the volume of LNs in the DC group was not significantly changed (P > 0.05) (Figure 7a1,a2). The main reason is that saline and simple laser irradiation had no obvious therapeutic effect on metastatic LNs, while when the DOC-CNPs in the DC group reached the LNs after injection, some DOC-CNPs ruptured and DOC was released in the LNs due to the temperature in vivo, which led to a certain inhibition of tumor cells. However, DOC may only maintain a relatively low concentration in LNs, which was consistent with the experiment of the cumulative release rate in vitro, and DOC is a concentration-dependent chemotherapy drug. Hence, it was difficult to achieve a better therapeutic effect. Therefore, a laser was used as a trigger to cause the DOC-CNPs to rupture in the metastatic LNs, releasing numerous DOC molecules, and the DOC concentration was increased in a short time. A good chemotherapeutic effect was caused by a high concentration of DOC combined with PTT by laser irradiation, and such combination therapy achieved a better therapeutic effect. In addition, in the pathological evaluation of the LNs after treatment, we found that most of the tumor cells in the DC + LA group were apoptotic, and the apoptotic index (AI) was greater than that in other groups (P < 0.05)
Observing the changes in the blood shear wave indicates a lower elasticity value of the medium, and a slower direction of wave propagation. A faster shear wave particle displacement in the elastic medium is perpendicular to the direction of wave propagation. Shear wave is a transverse wave, that is, the direction of wave propagation is perpendicular to the direction of particle displacement. In this study, the images of the blood flow (CDFI, color Doppler flow imaging) in different groups after treatment. (b1) Images of electography after treatment. (b2) Changes of the shear wave velocity before and after treatment in different groups (*P < 0.05).

(Figure 7b1,b2), which further confirmed its good therapeutic effect on metastatic LNs. However, after 4 weeks of treatment, although we observed apoptosis of numerous tumor cells in the LNs, the shrinkage of the LNs is not expected to be obvious, which may be associated with the longer period of time that it takes for the necrotic tumor tissue to be absorbed. Related studies also suggest that the absorption of tumors after thermal ablation may take 3–12 months or even longer for shrinkage of 50% or more. In further studies, we also need to continue observation for a longer period of time to discover the duration required for metastatic LN shrinkage. During the treatment, the skin of the irradiated areas was carefully checked, and no redness or ulceration was found before and after laser irradiation, which confirmed the safety of the laser intensity. In addition, no necrosis or ulcer was found at the injection site of DOC-CNPs, which further indicated the safety of subcutaneous injection.

Shear wave elastography (SWE) is based on shear wave. Shear wave is a transverse wave, that is, the direction of particle displacement in the elastic medium is perpendicular to the direction of wave propagation. A faster shear wave indicates a higher elasticity value of the medium, and a slower shear wave indicates a lower elasticity value of the medium. Observing the changes in the blood flow and elastography in tumors before and after treatment is also beneficial to evaluate the effect of tumor treatment. In this study, the images of the blood flow (CDFI, color Doppler flow imaging) and elastography before and after treatment were recorded. The CDFI images were semiquantitatively analyzed using the Adler grade, and the elastography was quantitatively analyzed using the shear wave velocity. After 4 weeks of treatment, it is observed that the blood flow of LNs after treatment in the DC + LA group was significantly reduced compared to that before treatment (Figure 8a). After semiquantitative analysis by the Adler grade, it was found that the blood flow was rich (grades II and III) before treatment; however, the blood flow was poor after treatment (grades 0 and I). Quantitative analysis was also performed to confirm that the blood flow of the LNs after treatment in the DC + LA group was significantly reduced compared to that before treatment (P < 0.05), and it was also significantly reduced compared to other groups after treatment (P < 0.05) (Table 1). It is shown in the images that the elasticity of LNs in the DC + LA group was reduced after treatment (Figure 8b1). It was also confirmed that the value of the shear wave velocity after treatment in the DC + LA group was significantly lower than that before treatment (P < 0.05), and it was also significantly reduced compared to other groups (P < 0.05) (Figure 8b2), which further indicated a decrease in the elasticity of LNs after treatment. Related studies have shown that a reduction in hardness after tumor treatment is also evidence of an effective treatment.

Furthermore, the side effect of CNPs in vivo was examined after treatment. In general, the side effects of intravenous chemotherapy peaked around the seventh day after chemotherapy drug injection. Therefore, the liver function (ALT and AST) and renal function (BUN and Cr) were tested and routine blood test (WBC, RBC, and Plt) of each group was conducted on the seventh day after treatment in this study. There was no significant difference between the treatment group (DC, LA, CNP + LA, and DC + LA) and the saline group (CT) in the liver and renal functions and the blood.

Table 1. Adler grade of lymph nodes before and after treatment

| group       | Adler grade |
|-------------|-------------|
|             | 0 | 1 | 2 | 3 |
| CT1         | 0 | 0 | 2 | 8 |
| CT2         | 0 | 0 | 1 | 9 |
| LA1         | 0 | 0 | 3 | 7 |
| LA2         | 0 | 0 | 1 | 9 |
| DC1         | 0 | 0 | 4 | 6 |
| DC2         | 0 | 1 | 2 | 7 |
| CNP + LA1   | 0 | 0 | 5 | 5 |
| CNP + LA2   | 1 | 8 | 1 | 0 |
| DC + LA1    | 0 | 0 | 3 | 7 |
| DC + LA2    | 6 | 4 | 0 | 0 |

*Note: (1) Before treatment, (2) after treatment. *There was no significant difference before treatment compared with other groups (P > 0.05). *Compared with other groups, the difference after treatment was statistically significant (P < 0.05).
routine test (Table 2), which further confirmed the safety of CNPs in vivo.

In this study, we did not establish an LN metastasis model for breast cancer, mainly because the skin of the rabbit’s breast area is thin, and the lymphoid adipose tissue is rare, making it inconsistent with the human breast, which has rich lymphoid adipose tissue. Our previous experiment had found that subcutaneous injections of DOC-CNPs in the breast area of rabbits resulted in a small amount of drug reaching the LNs, and the rate was very slow. In contrast, related studies have indicated that with the subcutaneous injection of drugs in human breasts, large amounts of drugs reach the LNs rapidly. Hence, we decided to implant the tumor in the legs to establish a model of popliteal LN metastasis and inject through the footpad with thick adipose tissue, which can easily deliver the DOC-CNPs into LNs through the lymphatic system.

In this study, we still have some shortcomings that should be improved in the future. First, chemotherapy drugs are generally concentration- and time-dependent. Moreover, PTT depends on the concentration of the photothermal conversion material in the tumor. Although it was found that CNs can be retained in LNs for a long period of time in a previous experiment, they gradually decrease over time. Therefore, we can try repeated injections of DOC-CNPs to achieve a better therapeutic effect. Second, the laser power and irradiation time need be optimized for a better therapeutic effect. Combined with the released DOC, an excellent therapeutic effect on metastatic LNs could be acquired. Meanwhile, DOC-CNPs showed good safety and biocompatibility, which provided a new model for the treatment of metastatic LNs.

**CONCLUSIONS**

In this study, we synthesized a novel multifunctional nanoagent with PLGA, CNs, PFH, and DOC as the main materials, which could be delivered into LNs through a highly targeted lymphatic delivery system via subcutaneous injection. After sufficient laser irradiation, DOC-CNPs absorbed light, leading to a liquid–gas phase transition, which can produce a strong photothermal effect. Combined with the released DOC, an excellent therapeutic effect on metastatic LNs could be acquired. Meanwhile, DOC-CNPs showed good safety and biocompatibility, which provided a new model for the treatment of metastatic LNs.

**MATERIALS AND METHODS**

CNs (50 mg/mL) were purchased from Lummy (China). PFH was purchased from Alfa Aesar (U.K.). PLGA (50:50, 12 000 Da MW) was purchased from Daigang (China). Docetaxel (DOC, 20 mg) was purchased from Hengrui Medicine (China). Poly(vinyl alcohol) (PVA 25 000 Da MW) was obtained from Sigma-Aldrich. Methylene chloride (CH2Cl2) was purchased from Chuandong (China). Isopropyl alcohol (C3H8O) was purchased from Yangzi (China). The Cell Counting Kit-8 (CCK-8) was purchased from Dojindo (Japan). Agarose was obtained from Invitrogen (USA). Annexin V-FITC was purchased from BioVision. Deionized water was obtained with a Milli-Q Plus system (Millipore Corporation). Natural saline (NS, 0.9%) was purchased from Tiansheng (China).
Synthesis of DOC-CNPs. First, 200 μL of CNs (50 mg/mL) was mixed with 100 μL of DOC (40 mg/mL) and stirred with an ultrasonic probe (Sonic & Materials, Inc.) at 130 W for 1 min. The mixture was placed on a horizontal thermostat oscillator (NHWW-200F, Aipu, China) for 24 h (37 °C, 120 rpm). Then, 100 μL of PFH was introduced into the mixture and emulsified in an ice bath with an ultrasonic probe at 130 W for 1 min. After that, a black emulsion was obtained. Next, 50 mg of PLGA was dissolved in 1 mL of CH2Cl2. After the dissolution was completed, the previously obtained black emulsion was introduced into the CH2Cl2 mixed with PLGA, and the mixture was emulsified in an ice bath with an ultrasonic probe at 130 W for 1 min. After that, 10 mL of 5% w/v PVA was introduced and homogenized (FJ300-SH, China) in an ice bath for 2 min. Then, an additional 20 mL of 2% w/v CH3OH was introduced and stirred in an ice bath with a magnetic stirrer (HJ-1, Ronghua, China) for 2 h. The solution was centrifuged (Eppendorf AG, Germany) at 8000 rpm for 5 min at 4 °C. After centrifugation, the supernatant was discarded, and the precipitate was washed with deionized water. The process still took place in an ice bath, and both the centrifugation and washing processes were repeated three times. Finally, the washed spheres were diluted in 2.5 mL of degassed deionized water to prepare a stock solution with a concentration of 20 mg/mL and then stored in a centrifuge tube at 4 °C for further use.

To prepare similar nanoparticles without DOC (CNPs), the CNs were not mixed with DOC during the preparation process.

Characterization of DOC-CNPs. The morphological and structural characterization and the size of the DOC-CNPs were determined by TEM (JEM-2100F, Japan). Liquid–gas phase transition was observed using an optical microscope (Olympus CKX41, Canada). The size distribution and ζ-potential of DOC-CNPs were measured by a laser particle size analyzer (Zeta SIZER3000HS, Malvern).

Drug Loading and Laser-Triggered Drug Release. DOC-CNPs (1 mL, 20 mg/mL) were mixed with 1 mL of CH2Cl2 and emulsified for 2 h. Then, the concentration of CNs was detected by an ultraviolet spectrophotometer (UVS, Shanghai Spectroscopy, China), while the concentration of DOC was detected by high-performance liquid chromatography (HPLC, LC-2010A HT, Shimadzu, Kyoto, Japan). The DOC-CNP solution (20 mg/mL) was introduced into a 24-well plate and then irradiated with a laser (808 nm, 2.0 W/cm²) until 50 °C, and the temperature was monitored every 3 s with an infrared thermal imager (FOTRIC, USA).

Drug Loading (Wt) and Drug Release (Wp) were calculated as follows: EE (%) = (Wt/Wp) % and LC = (Wt/Wp) % (Wt: mass of CNs or DOC in DOC-CNPs; Wp: total mass of CNs or DOC used in the prepared sample). DOC-CNPs (5 mL, 20 mg/mL) were irradiated (808 nm, 1.0 W/cm²) for 4 min and then introduced into a dialysis bag. The dialysis bag with DOC-CNPs was immersed in a sealed bottle containing 100 mL of deionized water and placed on a horizontal thermostat oscillator (37 °C, 120 rpm). Then, the bag was immersed in a release medium for dialysis on a horizontal thermostat oscillator (37 °C, 120 r/min). Aliquots of the liquid contained in the sealed bottle were taken at 0.5, 1, 2, 4, 6, 8, 12, 24, 36, 48, 60, and 72 h (1 mL each time, and 1 mL deionized water was added after the removal of each aliquot), and the DOC concentrations of the liquid aliquots were determined by HPLC. The concentration and cumulative release quantity (CRQ) of DOC were calculated with a standard curve, and the cumulative release rate of DOC at different times was also calculated. A drug release curve is plotted between cumulative release ratio and time. DOC-CNPs (5 mL, 20 mg/mL) without laser irradiation were used as the control group.

\[
CRQ (mg) = 100 \times 10^{-3} \times D
\]

where CRQ is the cumulative release quantity; D is the drug concentration measured in the experiment.

\[
CRR = \frac{DR}{TD} \times 100\%
\]

where CRR is the cumulative release ratio; DR is the drug released; TD is the total drug used.

In Vitro Photoacoustic Imaging and Photothermal Conversion Effect. The PA signals of four DOC-CNP solution concentrations (5, 10, 15, and 20 mg/mL) in 2% agarose gel phantom were first measured to investigate the relationship between the concentrations and the PA signal using the VEVO LASR PA imaging system (VevoLAZR, Visual Sonics, Inc., Canada). The PA images were collected, and the PA signals were quantitatively analyzed.

Similarly, the DOC-CNP solutions of the four concentrations (5, 10, 15, and 20 mg/mL) were continuously irradiated with a laser (808 nm, 2.0 W/cm²) until 50 °C, and the temperature was monitored every 3 s with an infrared thermal imager (FOTRIC, USA).

The DOC-CNP solution (20 mg/mL) was introduced into a 24-well plate and then irradiated with a laser (808 nm, 2.0 W/cm²). The temperature of the DOC-CNP solution was monitored by an infrared thermal imager. When the sample temperature reached 50 °C, the laser was shut off. While the sample was restored to room temperature, the laser irradiation was recovered, and the process was repeated three times. The temperature was recorded every 3 s.

Cell Culture. Human breast cancer cells (MDA-MB-231) and human umbilical vein endothelial cells (HUVECs) were obtained from the laboratory of the Ultrasound Engineering Institute of Chongqing Medical University and cultured with RPMI 1640 containing 10% fetal bovine serum at 37 °C with humidified air containing 5% CO2.

Cytotoxicity and Compatibility of DOC-CNPs. The cytotoxicity of the DOC-CNPs was evaluated by determining the viability of HUVECs cells after incubation in media containing DOC-CNPs at concentrations of 0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL. Control experiments were carried out using the complete growth culture media without DOC-CNPs. Cell viability testing was performed through the reduction of the CCK-8 reagent. The cells were first seeded into 96-well plates at a density of 5 \times 10^3 cells per well and incubated at 37 °C in 5% CO2 for 24 h. Then, the former medium was replaced with a new medium containing DOC-CNPs at varying concentrations, and the cells were incubated at 37 °C in 5% CO2 for another 24 h. After that, 150 μL of a new medium and 10 μL of CCK-8 solution were added into each well to replace the former medium, followed by incubation at 37 °C in 5% CO2 for 4 h. Finally, the medium was removed and optical absorbance was then measured at 450 nm on a microplate reader (EL \times 800 Universal Microplate Reader, BioTek Instruments, Inc., USA). The results were expressed as percentages relative to those obtained in the control experiments. The differences in the results obtained from
DOC-CNPs and the controls were analyzed statistically by analysis of variance.

**In Vitro Tumor Therapy Effect.** The cells were divided into five groups, including the DOC-CNPs solution group (DC, 20 μL, 20 mg/mL DOC-CNPs), the laser irradiation group (LA, laser irradiation), the CNP solution with the laser irradiation group (CNP + LA, 20 μL, 20 mg/mL CNPs, with laser irradiation), the DOC-CNPs solution with the laser irradiation group (DC + LA, 20 μL, 20 mg/mL DOC-CNPs, with laser irradiation), and the saline group (CT, 20 μL 0.9% NS), and the CT group was taken as a control. MDA-MB-231 cells were seeded into 24-well plates at a density of 5 × 10^3 cells per well and incubated at 37 °C in 5% CO₂ for 24 h. After incubation, the groups (LA, CNP + LA, and DC + LA) were given the same laser intensity for 2 min (808 nm, 0.5 W/cm²). The cells were resuspended with 250 μL of binding buffer, and the concentration of the cells was modulated to 1 × 10^6/mL. The mixture of 100 μL of cell suspension, 5 μL of Annexin V/ FITC, and 10 μL of PI solution was added into a 5 mL flow tube, and the cells were incubated in a dark place at room temperature for 15 min. Then, 400 μL of PBS was added into the tube, and the apoptosis rate was determined on a flow cytometer (FCM, FACSVantage SE, USA).

**Animal Models.** New Zealand white rabbits (weight range of 2.5–4.0 kg) were purchased from the Animal Center of Chongqing Medical University. Tumor-bearing rabbits with a VX2 liver tumor were obtained from the laboratory of the Chongqing Medical University. Tumor-bearing rabbits with a VX2 liver tumor were obtained from the laboratory of the Chongqing Medical University. Tumor-bearing rabbits were used for experiments when popliteal fossa LNs were randomly divided into four groups according to gender and weight (three in each group), and these rabbits received a percutaneous injection of the DOC-CNPs solution (2 mL, 5, 10, 15, and 20 mg/mL) via the footpad of the hind legs in sterile saline. In addition, the skin of the irradiated areas was observed to determine whether there was redness or ulceration before and after laser irradiation.

**In Vivo Tumor Therapy Effect and Side Effect.** New Zealand white rabbits’ detectable popliteal fossa LNs were divided into five groups in this experiment, including the DOC-CNPs solution group (DC group, 2 mL, 20 mg/mL DOC-CNPs), the laser irradiation group (LA group, laser irradiation), the CNP solution with the laser irradiation group (CNP + LA group, 2 mL, 20 mg/mL CNPs, with laser irradiation), the DOC-CNPs solution with the laser irradiation group (DC + LA group, 2 mL, 20 mg/mL DOC-CNPs, with laser irradiation), and the saline group (CT group, 2 mL 0.9% NS), and the CT group was taken as a control. The drugs of each group (except the LA group) were subcutaneously injected in the footpad of the hind legs. The groups (LA, CNP + LA, and DC + LA groups) were given the same laser intensity (2.0 W/cm²) for 10 min once a day 24 h after injection. The skin temperature in the irradiated area was monitored by an infrared thermal imager during irradiation. In addition, the skin of the irradiated areas was observed to determine whether there was redness or ulceration before and after laser irradiation.

**Metastatic LNs** in each group were examined using an ultrasound instrument in the B-mode (Siemens Acuson S2000, Germany) with a probe (9L4 line array, 7–12 MHz) before treatment and 4 weeks after laser irradiation. In the best ultrasonic section, the long diameter (L) and short diameter (S) were determined, and the volume of LNs was calculated using the following formula:

\[
\text{volume} = \frac{(L \times S^2)}{2}
\]

The blood flow distribution in the LNs was examined using an ultrasound instrument (Siemens Acuson S2000, Germany) in color Doppler flow imaging mode (CDFI, 2 cm/s, 50% gain) with the probe (9L4 line array, 7–12 MHz) after 4 weeks of treatment. The images of CDFI were collected, and the images were classified into four grades with the Adler semiquantitative method, including grade 0 (no significant blood flow signal), grade 1 (a little blood flow signal), grade 2 (more blood flow signal than grade 1, and three to four blood vessels are observed), and grade 3 (very rich blood flow signal, and more than four blood vessels are observed).

Elastic imaging of the LNs was determined using an ultrasound (Siemens Acuson S2000, Germany) with the probe (9L4 line array, 7–12 MHz) 4 weeks after the treatment. In SWE mode, elasticity images could be obtained after the images were stabilized. The area of interest (the hardest areas in the lymph node were shown in dark blue) was selected, and the shear wave velocity values of the areas were measured. Seven consecutive measurements were performed, and the average values were calculated after removing the maximum and minimum values. The elastography values were quantitatively analyzed using the shear wave velocity.
with a higher nuclear staining intensity than the background were classified as positive (apoptotic cells). In each section, five fields of view were randomly selected to count the number of positive cells.

\[
AI = \frac{\text{numbers of positive cells}}{\text{total numbers of cells}} \times 100\%
\]

After 4 weeks of treatment, the blood of rabbits in the four groups was collected to assess the liver function (ALT and AST), renal function (BUN and Cr) and routine blood test (WBC, RBC, and Plt).

**Statistical Analysis.** One-way analysis of variance was carried out to calculate the differences among each group. Data were presented as the mean ± standard deviation. For statistical analysis, each experiment was repeated at least three times.

### Author Contributions

J.C. and Y.L. contributed equally to this study. J.C., Y.L., and L.Y. designed the study and drafted the manuscript. J.C., W.L., and Y.C. performed the experiments. L.H., F.L., and Y.G. analyzed all data. Y.L. and L.Y. revised the manuscript. L.Y. and H.R. approved the final manuscript.

### Notes

The authors declare no competing financial interest. The datasets used and analyzed for the present study are available from the corresponding author upon reasonable request.

All experimental protocols were approved by the Institutional Animal Care and Use Committee of Chongqing Medical University. All of the experimental operations of animals were carried out in accordance with the protocol approved by the Institutional Animal Care and Use Committee of Chongqing Medical University. All animals were treated according to the guidelines of the Care and Use of Laboratory Animals.

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