The combined therapeutic effects of 131I-iodine-labeled multifunctional copper sulfide-loaded microspheres in treating breast cancer

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1. Introduction

Breast cancer is one of the most common cancers affecting women worldwide.\textsuperscript{1,2} In 2017, about 63,410 cases of female breast carcinoma in situ are predicted to be diagnosed in the United States.\textsuperscript{3} In the clinic, surgery, radiotherapy and adjuvant chemotherapy agents are mostly used to eliminate the primary breast tumor while hormonal therapy and cytotoxic chemotherapy are the main systemic interventions for recurrence or distant metastasis.\textsuperscript{4,5} However, these treatments may still result in limited therapeutic efficacy due to drug resistance or adverse effects. Even worse, insufficient treatment may accelerate the growth of tumors.\textsuperscript{6,7} Therefore, it is imperative to develop more effective therapeutic strategies and combination therapy protocols.

With the development of nanomaterial with intense photothermal coupling effects, nanoparticle-enhanced near-infrared (NIR) laser-induced photothermal therapy (PTT) appears to be a promising strategy for cancer treatment in preclinical studies.\textsuperscript{8-10} Additionally, these nanomaterials can provide multimodalities in cancer diagnosis and therapeutics, including drug delivery,\textsuperscript{11,12} photoacoustic imaging,\textsuperscript{13,14} and positron emission tomography/computed tomography (PET/CT) imaging.\textsuperscript{15,16} It is attractive to design a multifunctional delivery platform that combines all these theranostic modalities in order to synergize the therapeutic efficacy, monitor the therapeutics and achieve accurate therapeutics, eventually be developed as a personalized nanomedicine.

Copper sulfide nanoparticles (CuS), with excellent optical and electrical properties have attracted increasing attention.\textsuperscript{17-19} They serve as a promising platform for controlled drug release,\textsuperscript{20} photoacoustic imaging,\textsuperscript{21,22} PET/CT imaging\textsuperscript{23,24} and radiotherapy.\textsuperscript{25} Compared with most widely used photothermal agents, including metallic nanomaterials such as gold nanoparticles, CuS nanoparticles have several advantages. Firstly, CuS nanoparticles are considered biodegradable inorganic nanomaterials.\textsuperscript{11,26,27} Secondly, the absorption wavelength of CuS nanoparticles is not affected by the solvent or the surrounding environment.\textsuperscript{28} Thirdly, the cost of production for CuS nanoparticles is much lower.

In this study, we synthesized hollow copper sulfide nanoparticle microspheres (HCuSNPs-MS), loaded it with paclitaxel (PTX) and labeled with radioiodine-131 (\textsuperscript{131}I). These microspheres were applied to photothermal therapy, chemotherapy, radiotherapy, photoacoustic imaging, and single photon emission computed tomography/computed tomography (SPECT/CT) imaging in one single setting (Fig. 1). The distribution of the microspheres was imaged via \textsuperscript{131}I- HCU S NPs-MS-PTX-mediated SPECT/CT and photoacoustic imaging. We hypothesized that \textsuperscript{131}I-HCU S NPs-MS-PTX, as a combinatorial regimen, could improve the therapeutic efficacy and provide a real-time distribution of the microspheres in rats bearing an orthotopic breast cancer model.

2. Materials and methods

2.1. Synthesis and characterization of HCU S NPs-MS-PTX

HCuSNPs were prepared by the previously published method.\textsuperscript{27} Non-radioactive iodine-labeled HCU S NPs (I-HCU S NPs) were synthesized by incubating HCU S NPs with sodium iodide (NaI) and sodium perchlorate solution (NaClO\textsubscript{4}) at 32 °C for 30 min. The microspheres containing PTX and HCU S NPs were synthesized as follows: HCU S NPs (200 μL, 2.55 mg) were mixed with dichloromethane (2 mL) containing 100 mg of PLGA (Cupertino, USA, lactide:glycolide = 50:50, viscosity = 0.55–0.75 dL/g) and 5 mg of PTX (Melone Pharmaceutic, Dalian, China) with stirring at room temperature. They were then added into 2% PVA (Cupertino, USA) solution and stirred for 30 s to form a w1/o/w2 multiple emulsion using magnetic stirrer. The w1/o/w2 multiple emulsion was added into 0.5% PVA solution and stirred at 1000 rpm for 3 h by magnetic stirrer. The copper sulfide-loaded microspheres, \textsuperscript{131}I-HCU S NPs-MS-PTX, can serve as a versatile theranostic agent in an orthotopic breast cancer model.
USA) were used to observe the morphology of HCuSNPs, I-HCuSNPs, HCuSNPs-MS, I-HCuSNPs-MS, HCuSNPs-MS-PTX and I-HCuSNPs-MS-PTX, respectively. The optical properties of the HCuSNPs and I-HCuSNPs were recorded with a UV-2401PC UV/Vis spectrophotometer (Shimadzu, Japan). Malvern Matersizer 3000 and Malvern Zetasizer Nano-90 were used to determine the size distribution of HCuSNPs, I-HCuSNPs, HCuSNPs-MS, I-HCuSNPs-MS-PTX, HCuSNPs-MS-PTX, HCuSNPs-MS and I-HCuSNPs-MS, respectively.

2.2. Photothermal effect of HCuSNPs-MS-PTX

To investigate the temperature change mediated by HCuSNPs-MS in vitro, a 915-nm NIR laser (MDL-III-915R, CNI, Changchun, China) was used. NIR laser light (0.5 W/cm², 10 min) was passed through a quartz cuvette containing a HCuSNPs PBS solution (1 mg/mL, 200 µL), HCuSNPs-MS PBS solution (1 mg/mL of HCuSNPs, 200 µL) or a PBS solution (200 µL). During the laser exposure, a thermocouple was inserted into the solution to record the temperature change.

2.3. Drug release of HCuSNPs-MS-PTX

In the study of long-term release of PTX from HCuSNPs-MS-PTX, a solution of HCuSNPs-MS-PTX (200 µL, 0.46 mg/mL, containing 2.285 µg/mL of PTX) containing 0.5% Tween 80 was aspirated and then placed into an EP vial in a 37 °C water bath. At various times three vials were centrifuged at 13,523 × g for 10 min, and the free PTX was quantified by a high performance liquid chromatography (HPLC) system (LC-20AR, Shimadzu, Japan) with an SPD-M20AV photodiode array detector. The two samples were exposed to 915-nm NIR laser light at a power density of 0.5 W/cm² for 3 min immediately after i.t. injection. The temperature change of tumors within 4 min in each group was determined using thin-layer chromatography (TLC). The Whatman No. 1 filter paper was developed with deionized water. A gamma counter (SN-697, Shanghai Institute of nuclear research, Rihuan Instrument Factory, Shanghai, China) was used to quantify the radioactivity. The radiolabeling yield was expressed as percentage of radioactivity of 131I-HCuSNPs-MS-PTX relative to all radiodine activity. For the study of radiolabeling stability, 131I-HCuSNPs-MS-PTX was suspended in saline or 10% fetal bovine serum (FBS) and then the radiolabeling yield was measured at 30 min, 1, 2, 4, 8, 12, 24, 48 and 72 h, respectively.

2.5. Animal preparation

All the animal experimental procedures were in accordance with Ren Ji Institutional Animal Care. Female Sprague–Dawley rats, weight 200–250 g, were purchased from the Ren Ji Hospital Experimental Animal Center, China. Rats were housed in specific pathogen-free conditions at the laboratory animal center. To produce the orthotopic breast cancer model, W256/B cells (100 µL, 5×10⁶ cells) were injected into the second mammary fat pad. When the tumor size reached 5–6 mm, the rats were ready for the experiment.

2.6. Anti-tumor activity and histopathologic evaluation

To evaluate the combinatorial therapeutic efficacy, rats were randomly assigned to 6 groups (A–F, n=4 in each group) as follows and received intra-tumoral (i.t.) injection according to the design (Fig. 2). Group A rats were injected with normal saline (control group, 30 µL). Group B rats were injected with 131I-HCuSNPs-MS-PTX plus laser exposure (10 mg, 30 µL, containing 0.5 mg of PTX, 0.2 mg of HCuSNPs and 30 ± 3 MBq of 131I). Group C rats were injected with 131I-HCuSNPs-MS-PTX (10 mg, 30 µL, containing 0.5 mg of PTX, 0.2 mg of HCuSNPs and 30 ± 3 MBq of 131I). Group D rats were injected with HCuSNPs-MS-PTX plus laser exposure (10 mg, 30 µL, containing 0.5 mg of PTX and 0.2 mg of HCuSNPs). Group E rats were injected with 131I-HCuSNPs-MS plus laser exposure (10 mg, 30 µL, containing 0.5 mg of PTX and 0.2 mg of HCuSNPs). Group F rats were injected with 131I-HCuSNPs-MS plus laser exposure (10 mg, 30 µL, containing 0.2 mg of HCuSNPs and 30 ± 3 MBq of 131I). Group F rats were injected with 131I-HCuSNPs-MS (10 mg, 30 µL, containing 0.2 mg of HCuSNPs and 30 ± 3 MBq of 131I). The groups with laser treatment were exposed to a 915-nm NIR laser at a power density of 0.5 W/cm² for 3 min immediately after i.t. injection. The temperature change of tumors within 4 min in each group was monitored by an infrared thermal imaging camera (DT-980, CEM, Shanghai, China) during laser irradiation.

After an 18F-FDG micro PET/CT scan at 7 day-post-treatment the tumors of each rats were collected and cryosectioned for hematoxylin and eosin (H&E) staining as well as Ki-67 (Novus, USA) and terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL)/DAPI staining (Yeasen, China) according to the manufacturer's protocols. TUNEL-stained slides were observed under a fluorescence microscope (Zeiss Axio Observer.Z1, Germany). The major organs (liver, kidney and

Figure 1  Scheme of 131I-HCuSNPs-MS-PTX.

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Louis, MO, USA) was added with stirring. The mixture was incubated at 32 °C for 30 min. Once the reaction finished, deionized water (1 mL) was added and the sample centrifuged at 67 × g for 10 min to rinse free Na131I. After removing the supernatants, saline (30 µL) was added to yield the final product, 131I-HCuSNPs-MS-PTX. The radiolabeling yield was analyzed using thin-layer chromatography (TLC). The Whatman No. 1 filter paper was developed with deionized water. A gamma counter (SN-697, Shanghai Institute of nuclear research, Rihuan Instrument Factory, Shanghai, China) was used to quantify the radioactivity. The radiolabeling yield was expressed as percentage of radioactivity of 131I-HCuSNPs-MS-PTX relative to all radiodine activity. For the study of radiolabeling stability, 131I-HCuSNPs-MS-PTX was suspended in saline or 10% fetal bovine serum (FBS) and then the radiolabeling yield was measured at 30 min, 1, 2, 4, 8, 12, 24, 48 and 72 h, respectively.
spleen) of rats that treated with $^{131}$I-HCuSNPs-MS-PTX plus laser exposure were collected for H&E staining to investigate the toxicity.

2.7. $^{18}$F-FDG micro PET/CT imaging

$^{18}$F-FDG micro PET/CT imaging was acquired using a PET/CT scanner (Super Nova™ PET/CT, PINGSENG, Shanghai, China), which has a PET spatial resolution of approximately 0.6 mm and a CT resolution of 0.2 mm, before treatment and at 1, 2, 4 and 7 day after i.t. injection. Tumor-bearing rats were anesthetized with isoflurane (2% in oxygen) and placed prone. A 30-min PET/CT scan was performed at 1 h after $^{18}$F-FDG (0.3 mL, 37 MBq) i.t. injection. For data analysis, the region of interest for the tumor was drawn on CT and then copied to PET using Avatar 1.2 software (Pingseng, China). The length ($a$) and width ($b$) of the tumor, maximum standard uptake values ($SUV_{max}$) were determined. Tumor volume ($mm^3$) was presented as $0.5 \times a \times b^2$. 

Figure 2  Scheme of the experimental design of the anti-cancer treatment in rats bearing orthotopic breast tumor. i.t. injection, intra-tumoral injection.

Figure 3  (A) Transmission electron microscopic image of HCuSNPs. (B) Absorption spectra of HCuSNPs. (C) Diameter distribution of HCuSNPs samples determined by dynamic light-scattering measurements. (D) Scanning electron microscopic image of HCuSNPs-MS-PTX. (E) Size distribution of HCuSNPs-MS-PTX. (F) Temperature–time profiles of HCuSNPs-MS in aqueous suspension (1 mg/mL of HCuSNPs), HCuSNPs solution (1 mg/mL of HCuSNPs), or PBS under NIR laser irradiation (915 nm, 0.5 W/cm²). (G) Cumulative release of PTX from HCuSNPs-MS-PTX over time. (H) NIR-light-triggered release of PTX from HCuSNPs-MS-PTX (2.5 mg/mL of PTX). NIR laser light (915 nm, 0.5 W/cm², 3 min). Red arrows indicate laser switched on. Data are presented as Mean ± SD ($n=3$).
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2.8. SPECT/CT and photoacoustic imaging

The tumor-bearing rats that received i.t. injection of $^{131}$I-HCuSNPs-MS-PTX (10 mg, 30 μL, containing 0.5 mg of PTX, 0.2 mg of HCuSNPs and 30±3 MBq of $^{131}$I) plus laser exposure underwent SPECT/CT imaging at 30 min, 24, 48 and 96 h, respectively. To minimize thyroid uptake of free $^{131}$I, all rats were given water containing 0.12% of potassium iodide for 5 days before SPECT/CT imaging. SPECT/CT scans were performed on SPECT/CT system (Precedence 6, Philip, Netherlands). CT scans were performed first (using a 120 kV energy tube at 240 mA and 1 mm per scan), followed by the SPECT acquisition (128 × 128 matrix, 30 frames), which were performed by 6° angular steps in a 25 s per frame.

Another three rats bearing orthotopic breast tumors received i.t. administration of $^{131}$I-HCuSNPs-MS-PTX (10 mg, 30 μL, containing 0.5 mg of PTX, 0.2 mg of HCuSNPs and 30±3 MBq of $^{131}$I) underwent photoacoustic imaging. Bubble-free, clear ultrasound gel was used to facilitate acoustic contact between the transducer and the tumor. Image acquisition was conducted using a Vevo 2100 ultrasound/PA scanner with LZ400 PA probe (30 MHz linear acoustic array transducer, VisualSonics Inc., Toronto, Canada). The photoacoustic scans were conducted before and at 1, 4, and 24 h after i.t. injection, respectively. The rats were anesthetized with isoflurane during the scan. We applied several laser wavelengths, such as 750, 810, 850, 855, 895 and 915 nm for in vivo photoacoustic scan. A spectroscopically separated multiplexer was used to analyze the data with Vevo LAB (Ver 1.7.2) workstation software.

2.9. Statistical analysis

Quantitative data analysis is described as the mean ± standard deviations (SD). Differences in tumor volume and SUVmax values on day 7 between different groups were analyzed using one-way analysis of variance (ANOVA) followed by least-significant difference (LSD) post hoc multiple comparison tests (SPSS 22.0, USA). A P value <0.05 was considered statistically significant. A P<0.001 was considered highly significant.

3. Results

3.1. Characterization of the microsphere

TEM images demonstrated that HCuSNPs were relatively uniform in size, with the average diameter about 191.7 nm (Fig. 3A–B).

| Table 1 | Formulation of the microspheres. |
|---------|----------------------------------|
| Formulation | Microsphere | HCuSNPs loading (%) | EE of PTX loading (%) |
| A        | HCuSNPs-MS-PTX | 2.13 ± 0.20 | 99.34 ± 0.10 |
| B        | PTX-MS       | –            | 98.1 ± 0.11  |
| C        | HCuSNPs-MS   | 2.20 ± 0.15 | –             |

– Not applicable.

Figure 4 (A) In vivo thermal imaging of rats bearing orthotopic breast tumors after i.t. injection with different formulations with or without laser irradiation. (B) Temperature-time profiles of tumors following different treatments (915 nm, 0.5 W/cm², 3 min). Red arrows indicate laser switched on.

The HCuSNPs had a strong absorption band in the NIR region (peaked at ~900 nm, Fig. 3C). SEM images revealed the near-spherical morphology of HCuSNPs-MS-PTX, which had an average size of 9.84 μm (Fig. 3D–E). Additionally, compared with HCuSNPs, I-HCuSNPs had the similar diameter and the UV–vis spectrum of I-HCuSNPs was improved (Supplementary information Fig. S1). From the result, we found that there was no difference between I-HCuSNPs-MS-PTX, HCuSNPs-MS and I-HCuSNPs-MS in size distribution (Supplementary information Fig. S1), indicating that the labeling process did not affect the characteristics of HCuSNPs-MS-PTX. PTX was loaded into PLGA microspheres with the encapsulation efficiency (EE) close to 100%. The HCuSNPs loading efficiency in the microspheres was 2.13 ± 0.2% (Table 1). Exposed to NIR light exposure (0.5 W/cm²) for 10 min, the temperature of HCuSNPs-MS PBS suspension (50 mg/mL of HCuSNPs-MS containing 1 mg/mL of HCuSNPs) increased from 26.4°C to 56.1°C ($\Delta T = 29.7°C$, Fig. 3F). In comparison, no significant temperature change was observed in PBS following laser irradiation. There was no significant difference in temperature elevation between HCuSNPs suspensions and HCuSNPs-MS suspensions containing the same concentration of HCuSNPs ($P=0.982$, $P>0.05$), indicating that encapsulation of HCuSNPs into the PLGA microspheres did not affect the photothermal activity of HCuSNPs.

Fig. 3G showed that HCuSNPs-MS-PTX could provide a sustained and slow release of PTX, with a cumulative release of 7.59±0.15% at day 1 and 41.75±1.02% at day 14, respectively. This result indicated that the microspheres had the property of...
prolonged drug release. NIR laser (0.5 W/cm², 3 min) at 915 nm can trigger the rapid release of PTX from HCuSNPs-MS-PTX (Fig. 3H). After the first laser exposure (0.5 W/cm², 3 min), the cumulative release of PTX increased from 0.6% to 3.29%. During the subsequent 1.5-h interval without laser, only 0.33% of PTX (from 3.29% to 3.62%) was released. The second and third laser irradiation cycles induced 2.62% and 2.7% of PTX release, respectively. In contrast, less than 0.4% of PTX was released over the entire experimental period without laser irradiation (Fig. 3H). The HCuSNPs-MS-PTX (containing 0.2 mg of HCuSNPs) were labeled with ¹³¹I through Cu-I interaction. TLC confirmed the radiolabeling yield of ¹³¹I-HCuSNPs-MS-PTX was as high as 95.8 ± 1.3%. The radiolabeling efficacy of ¹³¹I-HCuSNPs-MS-PTX in saline remained at about 81.04% at 72 h and the serum stability of ¹³¹I-HCuSNPs-MS-PTX decreased to 67.5% due to some deiodination.

Infrared thermography recorded the temperature changes of tumor mediated by the photothermal effects of ¹³¹I-HCuSNPs-MS-PTX in vivo. Fig. 4 indicated that the temperature of tumors increased from 30 to 45 °C within 3 min. In comparison, there was no change of temperature detected in the tumors without laser irradiation.

3.2. Therapeutic effect in vivo

¹⁸F-FDG micro PET/CT was used to monitor the therapeutic effect of ¹³¹I-HCuSNPs-MS-PTX in an orthotopic breast cancer model. Representative micro PET/CT images showed the changes of different groups before and after the treatment (Fig. 5A). We found that the maximum standard uptake value (SUVmax) of ¹⁸F-FDG decreased over time and reached a relatively low SUVmax when compared with other groups at day 7 (Fig. 5A). In other treatment groups, low ¹⁸F-FDG uptake areas were mainly located in the central regions of the tumor, while the peripheral tumor tissues still exhibited high ¹⁸F-FDG uptake during the treatment.

Fig. 5B shows the tumor growth curves of the six groups. Before treatment, tumor volumes were not significantly different between groups ($P > 0.05$). The control group (Group A) showed a time-dependent increase in tumor volume from 95.04 ± 15.90 mm³ (day 0) to 888.64 ± 108.66 mm³ (day 7, $P = 0.0008$, $P < 0.05$). Compared with the control group, tumor growth was delayed for rats that were treated with ¹³¹I-HCuSNPs-MS-PTX plus laser irradiation.
exposure, and the tumor volume decreased from 98.16 ± 7.14 mm³ at day 0 to 36.92 ± 2.3 mm³ at day 7 ($P = 0.005$, $P < 0.05$). Other treatment groups showed intermediate reductions in tumor growth rates in the first several days but there was no significant difference at day 7 when compared with the untreated control group (Fig. 5B). Similar findings were observed in SUVmax changes (Fig. 5C). The SUVmax of tumors that were treated with $^{131}$I-HCuSNPs-MS-PTX plus laser exposure decreased from 7.47 ± 1.24 to 2.02 ± 0.22 ($P = 0.002$, $P < 0.05$), but increased in the control group from 6.82 ± 1.22 to 14.26 ± 0.54 ($P = 0.002$, $P < 0.05$) by 7 days. In other treatment groups, SUVmax decreased within 1 day but increased at 7 days post-treatment.

The results of H&E staining, TUNEL and immunohistochemical staining for Ki-67 were in agreement with the $^{18}$F-FDG PET/CT results, validating that $^{18}$F-FDG PET/CT imaging was an accurate and non-invasive method to monitor tumor therapeutic responses. By H&E staining, tumor cells of control group (group A) displayed pleomorphism, with increases in nucleus/plasma ratios. By contrast, the tumors that were treated with $^{131}$I-HCuSNPs-MS-PTX and laser exposure (group B) demonstrated remarkable degenerative and necrotic changes. The nodule mainly consisted of fibroblasts, histocytes, and lymphoplasmocytes, which surrounded a small focus of tumor cells (Fig. 6). The tumors of the control group (group A) demonstrated much higher expression of Ki-67 than tumors treated with $^{131}$I-HCuSNPs-MS-PTX plus laser exposure. In other treatment groups, the necrosis mainly was located in the center of the tumor while viable tissues remained in periphery. Similarly, Ki-67 was expressed in the periphery of the tumors (Fig. 6). TUNEL staining revealed that there were more apoptotic cells in rats treated with $^{131}$I-HCuSNPs-MS-PTX plus laser exposure than in other groups (Fig. 6). The H&E staining of liver, kidney and spleen did not show any obvious abnormalities, such as inflammation or lesions (Supplementary information Fig. S2).

3.3. SPECT/CT and photoacoustic imaging

SPECT/CT imaging revealed that $^{131}$I-HCuSNPs-MS-PTX was mainly distributed to the tumors with a relatively low distribution

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**Figure 6**  Rats bearing orthotopic breast tumors received i.t. injections of saline or different formulations with or without laser treatment (0.5 W/cm², 3 min, 915 nm). H&E staining, Ki-67 immunostaining and terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) staining of tumor at 7 days post-treatment. Yellow arrow, cells overexpressing of ki-67. Yellow arrow, Ki-67 positive cells. Green fluorescent, TUNEL-positive apoptotic cells; blue fluorescent, 4′,6-diamidino-2-phenylindole (DAPI)-stained nuclei. Bar, 100 μm.
to other organs (Fig. 7A). The intensity of radioactivity that accumulated in tumors decreased substantially within 72 h after injection. Fig. 7B showed representative photoacoustic images of rats that were treated with 131I-HCuSNPs-MS-PTX and laser exposure, before and after i.t. injection, respectively. The contour of the tumor region is clearly seen in the photoacoustic images. Most HCuSNPs-MS-PTX was located in the periphery of the tumors. The photoacoustic signal intensities in treated tumors, before and at 1, 4, and 24 h postinjection were 0.01 $\pm$ 0.05 a.u., 1.41 $\pm$ 0.12 a.u., 0.61 $\pm$ 0.02 a.u. and 0.42 $\pm$ 0.02 a.u., respectively, indicating that the signal intensity decreased over time. In addition, photoacoustic scans also demonstrated that both hemoglobin and HCuSNPs-MS-PTX were detected in the periphery of tumors (Fig. 7B). As hemoglobin is usually purely intravascular, we inferred that 131I-HCuSNPs-MS-PTX was mainly distributed in perivascular tumor tissue.

### 4. Discussion

In this study, we successfully loaded PTX and HCuSNPs into PLGA microspheres, radiolabeled with 131I and tested their efficacy in the inhibition of the growth of tumors in rats bearing an orthotopic breast cancer model. In order to investigate the combined effect of these agents, the loaded dose of PTX and 131I in the 131I-HCuSNPs-MS-PTX microspheres was less than the corresponding effective dose of monotherapy using either PTX or 131I. The result demonstrated that the combinatorial regimen 131I-HCuSNPs-MS-PTX could eliminate W256/B tumors at a relatively low dose. Combinatorial treatment has been reported to provide a more powerful strategy to eliminate tumor tissues. Compared with the widely reported “chemo-photortherapy”32,33, we used a radio-chemo-phototherapy strategy by labeling the nanoparticles with 131I. Since the range in tissue of the 131I-beta rays is approximately 2 mm34, they can kill the peripheral tumor cells that PTX or photothermal therapy may not reach. Moreover, 131I-gamma rays can offer SPECT/CT imaging to detect the distribution of the microspheres.

It was noticed that the photothermal effect in our study (at most 45 $^\circ$C) was mild in comparison with the temperature reported by other studies (e.g., over 50 $^\circ$C) for tumor cell necrosis35,36. Tumor temperatures above 45 $^\circ$C leads to cellular injury or death due to protein denaturation37. The relatively mild photothermal heating (43–45 $^\circ$C) induced by the shorter exposure time and the lower NIR power used here minimized normal cell death. Moreover, hyperthermia could sensitize the tumor cells to the chemotherapeutic agents38,39.

From the $^{18}$F-FDG PET/CT results we found that in groups with one or two therapeutic modals, tumoral $^{18}$F-FDG uptakes decreased when compared with the control group on day 4 post-injection. However, necrotic areas may have developed in the central and relatively high-metabolizing areas remaining in the peripheral tumor tissues where the chemotherapy or photothermal therapy did not reach during the period of early treatment, which induced a recurrence on day 7. By comparison, in rats treated with 131I-HCuSNPs-MS-PTX plus laser irradiation the effects of treatment were most pronounced. A complete inhibition of tumor growth was achieved as a result of the combined effects of chemotherapy, photothermal therapy, and radiotherapy. The combination of three modality treatments in a single setting reduced the dosage of each individual therapy and decreased adverse effects accordingly. Compared with multifunctional radiolabeled nanoparticles, such as CCPM-177Lu40 and 198AuNP-EGCg41, 131I-HCuSNPs-MS-PTX can also serve as SPECT/CT and
photoacoustic contrast agents, allowing real-time detecting of the biodistribution and accumulation of the microspheres in vivo non-invasively. The SPECT/CT imaging offered high sensitivity tracking of $^{131}$I-HCuSNPs-MS-PTX in vivo, allowing precise laser irradiation. It was demonstrated that the sustained radiation therapy was largely confined to tumors, with minimal damage to healthy surrounding tissue or other organs. Moreover, photoacoustic imaging provided useful information regarding the distribution of microspheres inside the tumor.

Due to the size of microspheres, they may not be appropriate for targeted delivery. However, our versatile molecular loading platform, combined with radio- and chemotherapeutic components, appears to be promising for local/regional therapy, such as brachytherapy and transcatheter arterial chemoembolization (TACE). Currently, localized radiotherapy with isotopes is a treatment option for many unresectable solid tumors, for example prostate cancer. However, in the clinic, the implantation of millimeter-size brachytherapy seeds may cause many adverse side effects or severe clinical complications, which greatly limits its applications. Nanomedicine brachytherapy has been applied to breast cancer, prostate cancer, and glioblastoma cancer, with less severe liver, spleen or kidney toxicity. Yook et al. have reported that $^{177}$Lu-labeled gold nanoparticles can serve as a novel neoadjuvant brachytherapy for locally advanced breast cancer. Compared with $^{177}$Lu-labeled gold nanoparticles, our agent has several advantages. Firstly, apart from radiotherapy, $^{131}$I-HCuSNPs-MS-PTX allowed a controlled drug release over a long process is straightforward and the radioisotope $^{131}$I and $^{125}$I are controllable, we can also synthesis the microspheres with an appropriate size for transcatheter arterial chemoembolization to treat hepatocellular carcinoma.

5. Conclusion

Our study confirmed that hollow copper sulfide-loaded microspheres, $^{131}$I-HCuSNPs-MS-PTX, could serve as a versatile theranostic agent in an orthotopic rat breast cancer model. The combination of photothermal, chemotherapeutic, and photoacoustic therapies in a single setting can help to eradicate the tumor completely and reduce the effective dose of monotherapy and decrease side effects accordingly. Simultaneously, SPECT/CT and photoacoustic imaging mediated by $^{131}$I-HCuSNPs-MS-PTX can intuitively monitor the distribution of the injected agents in vivo.

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Appendix A. Supporting information

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