Multifunctional Nanobubbles Carrying Indocyanine Green and Paclitaxel for Molecular Imaging and Treatment of Prostate Cancer

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Abstract

**Background:** Combining ultrasound imaging with photoacoustic imaging provides tissue imaging with high contrast and high resolution, thereby enabling rapid and direct measurement and tracking of tumor growth and metastasis. At the same time, ultrasound targeted nanobubble destruction (UTND) provides an effective way to drug accumulation; effectively increasing the content of the drug in the tumor area and reducing potential side effects, thereby contributing effectively to the treatment of the tumors.

**Result:** In this study, we prepared multifunctional nanobubbles (NBs) carrying indocyanine green (ICG) and paclitaxel (PTX) (ICG-PTX NBs), and study their application in ultrasound imaging of prostate cancer as well as their therapeutic effect on prostate cancer when combined with ultrasound targeted nanobubble destruction (UTND). ICG-PTX NBs were prepared by mechanical oscillation method. Their particle size and Zeta potential of ICG-PTX NBs were 469.5±32.87 nm and -21.70±1.222 mV, respectively. The encapsulation efficiency and drug loading efficiency of ICG were 68% and 6.2%, respectively. In vitro imaging experiments showed that ICG-PTX NBs were highly amenable to multimodal imaging including ultrasound, photoacoustic and fluorescence imaging, and the imaging effect is positively correlated with their concentration. The imaging effect of tumor xenografts also indicated that ICG-PTX NBs were of good use for multimodal imaging. In experiments testing the growth of PC-3 cells in vitro and tumor xenografts in vivo, the ICG-PTX NBs+US group showed more significant inhibition of cell proliferation and promotion of cell apoptosis compared to the other groups (P < 0.05). Blood biochemical analysis of the six groups showed that the levels of aspartate aminotransferase (AST), phenylalanine aminotransferase (ALT), serum creatinine (CRE) and blood urea nitrogen (BUN) in the ICG-PTX NBs group and the ICG-PTX NBs+US group were significantly lower than those in the PTX group (P < 0.05). Moreover, H&E staining of
tissue sections from vital organ showed no obvious abnormalities in the ICG-PTX NBs group and the ICG-PTX NBs+US group.

**Conclusions:** ICG-PTX NBs can be used as a non-invasive, pro-apoptotic contrast agent for both diagnosis and treatment that can achieve enhanced imaging, effective inhibition and killing of prostate cancer under the guidance of multimodal imaging including ultrasound, fluorescence and photoacoustic imaging.

**Background**

Prostate cancer is one of the most common malignancies in the male genitourinary system. Over the recent years, the incidence and mortality of prostate cancer in China has been increasing. Early diagnosis and effective treatment are the basis for ensuring a good prognosis in patients with prostate cancer [1, 2]. Previous studies have shown that ultrasound molecular imaging provides new ideas for early diagnosis, treatment and efficient monitoring of prostate cancer because of its real-time dynamic imaging, therapeutic controllability and easy operation [3, 4]. However, due to the poor sensitivity and specificity of conventional ultrasound diagnosis of prostate cancer, the development of a high-sensitivity contrast-enhanced ultrasound contrast agent is pertinent in ultrasound molecular imaging of prostate cancer [5]. Near-infrared fluorescence imaging has the advantages of real-time imaging, high sensitivity, no use of ionizing radiations and less spending. It has been extensively used for early diagnosis and targeted therapy of diseases, however, due to its strong optical scattering and limited penetration depth which leads to poor spatial positioning, it has significant limitations in clinical applications [6]. Combining the high penetration depth of pure ultrasound imaging with the high contrast of pure optical imaging, photoacoustic imaging provides tissue imaging with high contrast and high resolution, thereby enabling rapid and direct measurement and tracking of tumor growth and metastasis, as well as real-time imaging and analysis of the obtained
data. It has great application potential in tumor diagnosis and treatment monitoring [7]. Therefore, constructing a contrast agent suitable for multimodal imaging of tumors such as ultrasound, fluorescence and photoacoustic imaging can overcome the limitations of a single imaging technique and is garnering attention.

Indocyanine Green (ICG) is a near-infrared (Near Infrared, NIR) fluorescent dye that is approved by the US Food and Drug Administration (FDA). Current clinical practice shows that ICG can not only be used as a fluorescence probe for retinal angiography, cardiovascular and hepatic imaging, but also for the accurate localization of certain carcinomas in situ as well as metastatic tumors. However, the shortcomings of ICG when used singly in photoacoustic imaging, such as rapid clearance, poor stability, and lack of specificity in distribution form major obstacles in the diagnosis of tumors [8-12]. Certain evidences have proposed the combination of ICG with carriers in order to prolong their duration in circulation and increase their efficacy. In this regard, studies have confirmed that liposomes have an appreciable affinity, so they can effectively encase ICG, improve its stability in circulation, and thus enhance the photoacoustic and fluorescent imaging for tumor tissues [13-16].

The construction of multifunctional nano-drugs that can simultaneously integrate diagnosis and treatment is one of the current research directions for cancer treatment. Multifunctional nano-drugs are targeted to the diseased tissues by integrating therapeutic and imaging agents into nanoparticles, which can significantly improve the therapeutic effect and reduce the toxic side effects on normal tissues [17]. Paclitaxel (PTX) is a diterpene found in the bark and branches of taxane plants. It is the first natural phytochemical that has been approved by the FDA and is considered to be the most effective natural anticancer drug. Studies have found that paclitaxel can promote microtubule assembly and maintain microtubule stability, leading to the inability of cells
to form spindles and spindle fibers during mitosis, thereby inhibiting cell division, proliferation and the subsequent growth of cancer cells [18]. Liposomes have the advantages of good biocompatibility, simple preparation process, fewer toxic and side effects, and are widely used in gene transfection, drug delivery and in vivo imaging [19]. Our previous findings and other related studies have shown that nanobubbles with a lipid shell have the characteristics of entering tumor tissues through tumor blood vessels and concentrating therein for a long time [20]. At the same time, ultrasound targeted nanobubble destruction (UTND) provides an effective way to direct drugs to specific parts of the body; effectively increasing the content of the drug in the tumor area and reducing potential side effects, thereby contributing effectively to the treatment of the tumors. The underlying mechanisms may be associated with the cavitation effect of the ultrasound [21-25]. Cavitation effect can cause reversible pores to appear on the cell membrane, resulting in increased permeability of the cell membrane, and enhance anticancer drugs to enter tumor cells. Based on these observations, along with the advantage of the strong affinity of lipid shell for ICG and paclitaxel, multifunctional liposome nanobubbles (ICG-PTX NBs) containing both ICG and the chemotherapeutic drug paclitaxel were constructed to study their application in ultrasound (US), fluorescence (FL) and photoacoustic (PA) imaging of prostate cancer xenografts, and to simultaneously analyze their effects on inhibiting prostate cancer growth under ultrasound irradiation. This would offer avenues for the development of safe and stable nanobubbles with functions of multimodal imaging as well as integrated diagnosis and treatment for prostate cancer.

Materials And Methods

Reagents

Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM) medium, and trypsin were purchased from BI, Dutch. 1,2-dipalmitoyl-glycero-3 phosphate (DPPA), 1,2-
Dipalmitoyl-sn-glycero-3-phosphoglycerol (DPPG), 1,2-Dipalmitoyl-sn-glycero-3-hosphatidic ethanolamine (DPPE) and 1,2-Dipalmitoul-sn-glycero-3-phosphocholine (DPPC) were purchased from Corden Pharma, Switzerland. 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-PEG-2000 (DSPE-PEG2000) was purchased from NANOCs, USA. CCK-8 reagents and TUNNEL kit were purchased from Shanghai Biyuntian Biotechnology Co., Ltd. Paclitaxel was procured from Beijing Suolaibao Co., Ltd. Indocyanine Green was purchased from Sigma, USA. Perfluoropropane was purchased from Tianjin Institute of Physics and Chemistry. Aspartate aminotransferase test kit, phenylalanine aminotransferase test kit, serum creatinine test kit and blood urea nitrogen test kit were procured from Jiancheng Bioengineering Institute, Nanjing, China. Male nude mice were purchased from Beijing Huakangfu Biotechnology Co., Ltd.

**Cell culture and establishment of xenograft models**

The human prostate cancer cell line PC-3 was were kindly provided by Stem Cell Bank, Chinese Academy of Sciences. Cells were cultured in DMEM containing 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C with 5% CO₂. The cells in the exponential growth phase were digested with 0.25% trypsin and passaged or used for experiments. A total of 200 uL of 5×10⁶/mL PC-3 cell suspension was injected into the dorsal side of 4-5 weeks old male nude mice. Animal experiments were performed when the tumor volume reached 100 mm³ (tumor volume = a × b² / 2, a is the long diameter, b is the short diameter). All the animal experimental protocols were approved by the Animal Ethics Committee of the Third Military Medical University.

**Preparation and characterization of ICG-PTX NBs**

According to the ratio 3: 3: 3: 1: 1, a total of 11 mg of DPPC, DPPE, DPPG, DPPA, and DSPE-PEG2000 as well as 2 mg of paclitaxel, 500 ug of ICG were dissolved in 1000 μL of
PBS/glycerol solution and heated for 20 min at 45 °C. The contents were transferred to another vial, air in the vial was replaced using perfluoropropane, the vial was then shaken in an ST amalgam capsule blender (AT&M, China) for 90 s, and allowed to stand overnight at 4 °C. All the liquid in the vial was then transferred to a 10 mL centrifuge tube to be centrifuged at 300 rpm for 3 min, and the intermediate emulsion was collected into an eppendorf tube to finish the preparation of ICG-PTX NBs. Another 11 mg of lipids were used to prepare blank nanobubbles following the above steps (Blank NBs).

The concentrations of ICG-PTX NBs and Blank NBs were calculated using hemocytometer. The particle size, particle size distribution, and surface zeta potential were measured by the particle size detector Zetasizer nano ZS90 (Malvern, UK). The measurements were repeated three times. The shape, size and distribution of the nanobubbles were observed by optical microscopy (Olympus, Japan) and transmission electron microscopy (JEOL, Japan). The nanobubbles were stored at 4 °C, the changes in particle size and fluorescence intensity of ICG-PTX NBs were measured at 0, 1, 3, 5, and 7 days to analyze the stability of ICG-PTX NBs. The ultraviolet-visible spectrophotometer (Thermo Fisher, USA) was used to obtain the UV absorption spectra of ICG-PTX NBs, Blank NBs, ICG and double distilled water. The standard curves of ICG and PTX were plotted and the encapsulation efficiency (EE) and drug loading efficiency (LE) of ICG and PTX in ICG-PTX NBs were calculated.

Calculation of EE and LE:

\[
EE = \left( \frac{\text{amount of ICG encapsulated in nanobubbles}}{\text{total amount of ICG added}} \right) \times 100\%
\]

\[
LE = \left( \frac{\text{amount of ICG encapsulated in nanobubbles}}{\text{the total amount of lipids used for the preparation of nanobubbles}} \right) \times 100\%
\]

**Hemolytic action of ICG-PTX NBs**

After the eyeballs of the nude mice were removed, fresh blood was collected in an
anticoagulant tube, mixed and transferred to a 10 mL centrifuge tube. 3× volume of PBS was added and the blood samples were centrifuged at 2000 rpm for 10 min to remove the supernatant. The washing was repeated 3 times until the supernatant was colorless and transparent. An appropriate amount of red blood cells (RBCs) and PBS were used to prepare 2% RBC suspension, and incubated with 1.0×10⁸/mL ICG-PTX NBs and Blank NBs for 1 h. Double distilled water was used as positive control and PBS as negative control. The absorbance at 545 nm was measured by an ultraviolet-visible spectrophotometer, and the hemolysis rate was calculated using the following formula:

\[
\text{[(sample absorbance value - absorbance value of the negative control group) / (absorbance value of the positive control group - absorbance value of the negative control group)]} \times 100\%
\]

**In vitro ultrasound, photoacoustic and fluorescence imaging using ICG-PTX NBs**

Different concentrations of ICG-PTX NBs were placed in a cavity model made of 1% gel (1.0×10⁸/mL, 5.0×10⁷/mL, 1.0×10⁷/mL, 5.0×10⁶/mL, 1.0×10⁶/mL), and the corresponding ultrasound images in B-mode were acquired using the Vevo 2100 small animal ultrasound imaging system (VisualSonics, Canada). The 1.0×10⁸/mL ICG-PTX NBs were mechanically blasted using ultrasonic waves of high mechanical index and the ultrasonic images before and after blasting were analyzed. The DFY-type diagnostic instrument for quantitative analysis of ultrasonic images (Chongqing Institute of Ultrasound Molecular Imaging, China) was used to quantitatively assess the images and calculate the gray value for statistical analysis. Different concentrations of ICG-PTX NBs (1.0×10⁸/mL, 5.0×10⁷/mL, 1.0×10⁷/mL, 5.0×10⁶/mL, 1.0×10⁶/mL) were placed in a cavity model made of 1% gel for photoacoustic imaging at a wavelength of 825 nm, and a Vevo Laser photoacoustic imager (VisualSonics, Canada) was used to collect photoacoustic
images and quantitatively analyze photoacoustic signals. The $1.0 \times 10^8$/mL ICG-PTX NBs, ICG solution (0.35 µg/mL) with equal concentrations of ICG and double distilled water were respectively added to the eppendorf tubes, and then placed in the IVIS Spectrum living animal imaging system (PerkinElmer, USA). Under the irradiation conditions of excitation wavelength of 740 nm and emission wavelength of 820 nm, the eppendorf tubes were scanned and their fluorescence intensities were quantified.

**In vivo ultrasound, photoacoustic and fluorescence imaging using ICG-PTX NBs**

When the volume of the subcutaneous tumor reached approximately $1\text{cm}^3$, tumor blood flow was observed using ultrasound examination and tumors with rich blood flow were selected. After anesthetizing with isoflurane, the nude mice were fixed in prone position and scanned using the Vevo 2100 small animal ultrasound imaging system and MS250 high frequency probe. The sections with optimal imaging effect were selected and the optimal values of imaging parameters were determined (center frequency of the probe $18$ MHz,). After injecting 200 µL ICG-PTX NBs ($1.0 \times 10^8$/mL) through the orbital vein, the ultrasound imager was used to continuously acquire images at different time points after injection of the contrast agent, and dedicated software (Vevo 2100 onboard software, VisualSonics, Canada) was used to analyze the time-intensity curve. After the contrast-enhanced echo had subsided, the “burst” button was hit to blast the residual contrast agent. The 200 µL Blank NBs ($1.0 \times 10^8$/mL) were injected in the same way after the echo had completely subsided.

Chloral hydrate (4%; 0.20 mL/20 g) was intraperitoneally injected into nude mice as an anesthesia. The nude mice were fixed in prone position and the values of imaging parameters were adjusted (laser wavelength 825 nm, central frequency of the probe $21$ MHz). The Vevo LAZR photoacoustic imager was used to collect the photoacoustic images
of the tumor before the injection of the contrast agent. A total of 200 µL ICG-PTX NBs (1.0×10^8/mL) and ICG solution (0.35 µg/mL) of equal concentration were injected into the orbital vein. The photoacoustic imager was used to continuously observe and store dynamic images. Quantitative analysis of the photoacoustic signal in the region of interest was performed, and the time-photoacoustic signal intensity curve was plotted.

After anesthetizing with isoflurane, the nude mice were fixed in lateral position, and the parameters of the IVIS Spectrum living animal imaging system (with excitation and emission wavelengths set to 740 nm and 820 nm, respectively) were set up to collect fluorescence images of the nude mice before injection of contrast agents. A total of 200 µL of ICG-PTX NBs (1.0×10^8/mL) and ICG solution (0.35 µg/mL) of equal concentration were injected into the nude mice through the orbital vein respectively, and fluorescence images were collected at different time points (3 min, 5 min, 10 min, 15 min, 30 min, 60 min) for quantitative analysis of the metabolism of ICG-PTX NBs and ICG in tumor-bearing mice. Two hours after the injection of contrast agents, the animals were sacrificed, and heart, liver, spleen, lung, kidney and tumor tissues were isolated for fluorescence imaging. The fluorescence intensity of each organ was quantitatively analyzed by IVIS fluorescence analysis software to determine the level of residual ICG-PTX NBs in different tissues and organs.

**Effect of indocyanine green on cavitation of nanobubbles**

After the cells in the logarithmic growth phase were inoculated in a 96-well plate and cultured for 24 h, they were divided into 8 groups, namely, blank nanobubbles group (Blank NBs), indocyanine green nanobubbles group (ICG NBs), paclitaxel nanobubbles group (PTX NBs), indocyanine green and paclitaxel nanobubbles group (ICG-PTX NBs), blank nanobubbles plus ultrasound irradiation group (Blank NBs+US), and indocyanine
green nanobubbles plus ultrasound irradiation group (ICG NBs+US), paclitaxel nanobubbles plus ultrasound irradiation group (PTX NBs+US), indocyanine green and paclitaxel nanobubbles plus ultrasound irradiation group (ICG-PTX NBs+US). The preparation method of ICG NBs and PTX NBs was the same as that of the ICG-PTX NBs. An appropriate amount of Blank NBs, ICG NBs, PTX NBs, and ICG-PTX NBs at concentrations of $1.0 \times 10^8$/mL, $5.0 \times 10^7$/mL, $1.0 \times 10^7$/mL, $5.0 \times 10^6$/mL were added to the cells respectively.

After the ultrasound irradiation intervention ($1 \text{ W/cm}^2$ irradiation for 20 s), cells were cultured for another 24 h, the optical density value (OD value) of each group was determined by CCK-8 assay, and the inhibition rate of cell proliferation of each group was calculated following the formula:

\[
\text{inhibition rate of cell proliferation} \quad (\%) = \frac{[(\text{OD experimental group} - \text{OD blank group}) / (\text{OD control group} - \text{OD blank group})]}{100}\% 
\]

After the cells in the logarithmic growth phase were inoculated in a 6-well plate and cultured for 24 h, treatments were given following the above grouping, and the cells were cultured for another 24 h. The cells were then collected to prepare cell suspension with the staining buffer, and stained with fluorescein isothiocyanate (FITC) labelled Annexin V (Annexin V-FITC) and propidium idide (PI) for 5 to 15 min at room temperature in dark. Flow cytometry (ACEA, USA) was used to analyze the apoptotic effect in each group, and for the further analysis of the effect of ICG on the cavitation of nanobubbles.

**In vitro antitumor activity of ICG-PTX NBs**

PC-3 cells in logarithmic growth phase were resuspended to prepare single-cell suspension after digestion, inoculated into a 96-well plate at $1 \times 10^4$ cells/well, and cultured at 37 °C in an incubator with 5% CO$_2$ and saturated humidity for 24 h. The cells were then divided into six groups: blank control group (PBS), ultrasound irradiation group (US), paclitaxel
group (PTX), paclitaxel plus ultrasound irradiation group (PTX+US), ICG-PTX NBs group and ICG-PTX NBs plus ultrasound irradiation group (ICG-PTX NBs+US). Reagents were administered to the PTX group, PTX+US group, ICG-PTX NBs group, and ICG-PTX NBs+US group at an IC$_{50}$ concentration. After drug administration, ultrasound irradiation at 1 W/cm$^2$ for 20 s was administered in the US group, PTX+US group and ICG-PTX NBs+US group. After the stipulated intervention for each group, cells were cultured for another 24 h; the OD value of each group was determined by CCK-8 assay, and the rate of inhibition of cell proliferation was calculated. PC-3 cells in logarithmic growth phase were inoculated into 6-well plates at 5×10$^4$ cells/well and cultured for 24 h. After the intervention for each group, cells were cultured for another 24 h. After digestion and centrifugation, 50,000-100,000 cells were collected, and resuspended in Annexin V-FITC solution, followed by staining with Annexin V-FITC and PI for 5 to 15 min at room temperature in dark. The apoptotic effect of each group was analyzed by flow cytometry.

**Inhibition effect of ICG-PTX NBs on prostate tumor growth and their in vivo safety**

When the tumor volume reached 100 mm$^3$, the tumor-bearing nude mice were randomly divided into six groups (n = 5): PBS, US, PTX, PTX+US, ICG-PTX NBs, and ICG-PTX NBs+US groups, all treatment groups were administered drugs through orbital vein injection every 3 days. Nude mice in PTX, PTX+US, ICG-PTX NBs and ICG-PTX NBs+US groups received paclitaxel at a total dose of 20 mg/kg, and US group, PTX+US group and ICG-PTX NBs+US group were subjected to ultrasound irradiation at 1 W/cm$^2$ for 60 s. Drugs were administered for over a period of 18 days. The tumor volumes and body weights of mice were measured before each administration, and the tumor growth curve and changes in mouse body weight were plotted till the last administration. Before the initiation of
treatment and before the last tumor treatment, the maximum sections of the tumors in each group was scanned by contrast-enhanced ultrasound, and the tumor growth of each experimental group before and after treatment was recorded. Fresh blood was collected in anticoagulated tubes after the eyeballs of each experimental group were harvested, and blood biochemical indicators such as aspartate aminotransferase (AST), phenylalanine aminotransferase (ALT), serum creatinine (CRE) and blood urea nitrogen (BUN) were analyzed to assess the toxic and side effects of the drugs on liver and kidney function of tumor-bearing mice. At the end of the experiment, mice were sacrificed by spinal dislocation, and the tumor tissues were routinely fixed, embedded, sectioned and subjected to Hematoxylin-eosin (H&E) and TUNNEL immunohistochemical staining to observe the morphological changes and apoptosis in each group. In addition, H&E staining was performed in the tissues of heart, liver, spleen, lung, kidney, etc. obtained from mice in each experimental group, and the biosafety of the drugs was analyzed.

**Statistical analysis**

One-way ANOVA or Student’s t-test were performed using the Social Pack for Social Sciences 22.0, and the analyzed data were expressed as mean ± standard deviation. P < 0.05 indicates significant difference in statistical analysis; * indicates P < 0.05 and ** indicates P < 0.01.

**Results**

**Characterization of ICG-PTX NBs**

The appearance of the ICG-PTX NBs and Blank NBs was observed (Fig. 1a), and ICG-PTX NBs presented as a pale green emulsion, while Blank NBs presented as a white emulsion, indicating that ICG was effectively encased in ICG-PTX NBs. Under light microscopy, ICG-PTX NBs were found to be evenly distributed in size and shape without aggregation (Fig. 1b).
1b). The concentration of ICG-PTX NBs was about $(12.04\pm2.02)\times10^8$/mL, the concentration of Blank NBs was about $(14.05\pm2.79)\times10^8$/mL, and there was no significant difference between them ($P > 0.05$). Under transmission electron microscopy, ICG-PTX NBs had a regular spherical shape with a clear and smooth surface (Fig. 1c). Further, data from Zetasizer nano ZS90 particle size detector suggested ICG-PTX NBs particle size to be $469.5 \pm 32.87$ nm with a polydispersity coefficient of 0.209 (Fig. 1e); and Blank NBs had a particle size of $427.9 \pm 35.39$ nm and a polydispersity factor of 0.067 (Fig. 1d), and there was no significant difference between them ($P > 0.05$). The potentials of ICG-PTX NBs and Blank NBs were $-21.70\pm1.222$ mV and $-23.63\pm0.7965$ mV, respectively. The strong negative Zeta potential of the two kind of nanobubbles is an important factor for the nanobubbles to avoid aggregation and remain stable. The particle sizes of ICG-PTX NBs measured at different time points (1, 3, 5, 7 days) since preparation were $484.7\pm34.54$ nm, $544.6\pm29.75$ nm, $623.3\pm34.09$ nm, $968.2\pm94.11$ nm, respectively. On the 5th day of storage, the particle size of ICG-PTX NBs was significantly different from the particle size on the day of preparation, indicating that ICG-PTX NBs have good stability within 5 days of preparation (Fig. 1f). The UV absorption spectrum showed that ICG-PTX NBs had a major absorption peak at 780 nm, indicating that ICG was successfully loaded into ICG-PTX NBs (Fig. 1g). In addition, by plotting ICG and PTX standard curves, it could be found that the encapsulation efficiency of ICG in ICG-PTX NBs was 68% and the drug loading efficiency was 6.2%; while the encapsulation efficiency of PTX was 78.2%, and the corresponding drug loading efficiency was 5.82%.

Hemolysis is an important criterion for evaluating the biosafety of nanobubbles in drug delivery. The hemolytic action is evaluated by determining the content of hemoglobin released during the contact of nanobubbles with red blood cells. After red blood cells were
incubated for 1 h with ICG-PTX NBs and Blank NBs, all the contents settled at the bottom of the tube, the supernatant was colorless and transparent, and the measured hemolysis rates were less than 5% for both. In the positive control group, the solution was red and clear, and there were no RBCs at the bottom of the tube (Fig. 1h). The experimental results showed that ICG-PTX NBs do not cause hemolysis.

**In vitro ultrasound, photoacoustic and fluorescence imaging using ICG-PTX NBs**

In the in vitro agarose model, the signal intensity of ultrasound imaging of ICG-PTX NBs was found to be positively correlated with their concentration (Fig. 2a-e). After ultrasound irradiation of high mechanical index, the ultrasound imaging intensity of ICG-PTX NBs was significantly reduced, indicating that ICG-PTX NBs can be destroyed by ultrasound of high mechanical index (Fig. 2c-d). In vitro photoacoustic imaging showed that ICG-PTX NBs had photoacoustic imaging capabilities, and the photoacoustic image contrast was enhanced with increasing ICG-PTX NBs concentration (Fig. 2a-f). When IVIS Spectrum living animal imaging system was used to scan ICG-PTX NBs, ICG solution and double distilled water; ICG-PTX NBs and ICG solution groups showed obvious fluorescence signals, and the fluorescence signal of ICG-PTX NBs was markedly stronger than that of ICG solution, and no significant fluorescence signal was seen in the double distilled water group (Fig. 2b-g). Together, results of ultrasound, photoacoustic, fluorescence imaging in in vivo model indicates that ICG-PTX NBs have multimodal imaging capabilities.

**In vivo ultrasound, photoacoustic and fluorescence imaging using ICG-PTX NBs**

Analysis and comparison of ultrasound molecular imaging effects of ICG-PTX NBs and Blank NBs in PC-3 xenografts

The time taken to reach peak intensity for ICG-PTX NBs was 18.10 ± 0.8227 s, the peak intensity was 17.45 ± 0.7765 dB, the duration was 22.70 ± 0.4821 s and the area under the curve was 13124 ± 209.3 dB.s, which was not found to be significantly different from
those of Blank NBs (P > 0.05) (Table 1). The time-intensity curve showed no significant difference in ultrasound molecular imaging effect between ICG-PTX NBs and Blank NBs in PC-3 xenografts (Fig. 3b).

Analysis and comparison of photoacoustic imaging effects of ICG-PTX NBs and ICG solution in PC-3 xenografts

After injection of ICG-PTX NBs, the photoacoustic (PA) signal in the xenograft area gradually increased, and the photoacoustic imaging effect was observed to be the best after 5 min of injection. The PA signal in the tumor area was still enhanced 15 min after the injection. There was no significant increase in photoacoustic signals at each time point in the xenograft area of the nude mice injected with ICG solution (Fig. 3a). The time-intensity curve indicates that ICG-PTX NBs have significant photoacoustic imaging capabilities (Fig. 3c).

**Table 1. Ultrasound parameters of ICG-PTX NBs and Blank NBs in nude mice bearing PC-3 tumors**

| Contrast agent | Peak time/s | Peak intensity/dB | Duration time/min | AUC / dB.s |
|----------------|-------------|------------------|------------------|------------|
| Blank NBs      | 15.89 ± 1.991 | 16.04 ± 2.231    | 22.56 ± 0.4812   | 12626 ±     |
| ICG-PTX NBs    | 15.66 ± 1.468  | 16.21 ± 2.585    | 22.7 ± 0.4821    | 12273 ±     |

**In vivo fluorescence imaging effects of ICG-PTX NBs and ICG solution in PC-3 xenografts**

Three minutes after ICG-PTX NBs injection, obvious fluorescence signals were observed in the liver and tumor sites. After 5 min, the fluorescence signal of the tumor sites began to decay gradually, and after 60 min, only a small amount of fluorescence signal remained. Within 60 min of ICG solution injection, obvious fluorescence signals were observed in the liver, but no obvious fluorescence signal was observed at tumor sites (Fig. 4a). The time-
fluorescence intensity curve showed a significant difference in the fluorescence imaging effect between ICG-PTX NBs and ICG solution in PC-3 xenografts ($P < 0.05$) (Fig. 4c). In addition, the vital organs (heart, liver, spleen, lung, kidney) and tumor tissues of tumor-bearing nude mice were collected for ex vivo fluorescence imaging at 2 h after injection. The results showed that the vital organs and tumor tissues of both ICG-PTX NBs group and ICG solution group had different intensities of fluorescence signals, with the liver and kidney being the most marked. In addition, the fluorescence signal of tumor tissues in the ICG-PTX NBs group was significantly stronger than that in the ICG solution group ($P < 0.05$) (Fig. 4b-d).

**Effect of ICG on nanobubble cavitation**

CCK-8 assay was utilized to assess the status of PC-3 cell proliferation after treatments with four different concentrations of Blank NBs, ICG NBs, PTX NBs and ICG-PTX NBs with or without ultrasound irradiation. Before ultrasound irradiation, there was no significant difference in cell viability between the different concentrations of Blank NBs group and ICG NBs group ($P > 0.05$); or PTX NBs group and ICG-PTX NBs group ($P > 0.05$). After ultrasound irradiation also, no significant difference in cell viability was observed between Blank NBs+US group and ICG NBs+US group ($P > 0.05$); or PTX NBs+US group and ICG-PTX NBs+US group. There were significant differences in cell viability before and after ultrasound irradiation in the PTX NBs and ICG-PTX NBs groups ($P < 0.05$) (Fig. 5b-c). The results of CCK-8 assay showed that ICG had no significant effect on the cytotoxicity of nanobubbles before or after ultrasound irradiation. Flow cytometry was used to analyze cell apoptosis status of PC-3 cells after treatments of Blank NBs, ICG NBs, PTX NBs and ICG-PTX NBs with or without ultrasound irradiation. The results were consistent with those of CCK-8 assay. (Fig. 5a-d). These observations indicate that loading ICG has no significant effect on the therapeutic functions of nanobubbles, and under the action of ultrasonic
irradiation, ICG does not have a differential effect on the cavitation of nanobubbles.

**Antitumor activity of ICG-PTX NBs in vitro**

CCK-8 assay was used to evaluate the inhibitory effect on PC-3 cell proliferation in different treatment groups. The results showed that the US group, PTX group, PTX+US group, ICG-PTX NBs group, ICG-PTX NBs+US group had different degrees of inhibitory effect on cell proliferation. PC-3 cells in the ICG-PTX NBs+US group had the lowest survival rate that was significantly different from that of the ICG-PTX NBs group (P < 0.05). There was no statistical difference in cell survival rate between the PTX group and the ICG-PTX NBs group (P > 0.05), but the cell survival rate of the ICG-PTX NBs+US group was significantly lower than that of the PTX+US group (P < 0.05) (Fig. 6b). Annexin V-FITC/PI double staining kit was used to evaluate the apoptosis of PC-3 cells after different treatments. The total apoptotic effects of the experimental groups were ranked as follows: ICG-PTX NBs+US > PTX+US > ICG-PTX NBs > PTX > US > PBS. These results are consistent with those of the CCK-8 assay (Fig 6a-c). The total apoptosis rate of ICG-PTX NBs+US group was the highest, which was significantly different from that of the ICG-PTX NBs group (P < 0.05), indicating that ultrasound irradiation can promote apoptosis. There was no obvious difference between ICG-PTX NBs group and PTX group, and the apoptosis rates of ICG-PTX NBs+US group and PTX+US group were found to be significantly higher than that of US group; indicating that ICG-PTX NBs stably carry PTX and have an anti-tumor effect.

**Inhibitory effect of ICG-PTX NBs on the growth of PC-3 xenografts in nude mice**

The tumor volume changes in tumor-bearing nude mice (six groups) were observed after an 18 day-treatment period with PBS, US, PTX, PTX+US, ICG-PTX NBs and ICG-PTX NBs+US. Among these, the ICG-PTX NBs+US group showed no significant tumor growth, while the remaining five groups had tumors with varying degrees of growth. The tumor
growth curve showed that the volume of tumors in the control group increased by 3.5 times after the end of treatment; ICG-PTX NBs group increased by 2.5 times; ICG-PTX NBs+US group grew the slowest (1.5 times). The tumor volume was significantly smaller in the ICG-PTX NBs group and the ICG-PTX NBs+US group compared with that of the PBS and PTX groups (P < 0.05), and there was also a significantly difference in tumor volume between ICG-PTX NBs group and ICG-PTX NBs+US group (P < 0.05) (Fig 7d). Contrast-enhanced ultrasound images showed that there were unfilled anechoic areas in the center of xenograft tumors in nude mice of the PTX group, PTX+US group, ICG-PTX NBs group, and ICG-PTX NBs+US group, indicating necrosis inside the xenograft tumors, which was the most severe in the ICG-PTX NBs+US group (Fig. 7b). Our observations indicate that tumor necrosis was promoted by ultrasound irradiation, producing a significant therapeutic effect. Further, TUNNEL staining was used to assess the number of apoptotic cells in each treatment group. The number of apoptotic cells in the ICG-PTX NBs+US group was the highest. H&E staining showed cell necrosis in each treatment group, which was found to be most significant in the ICG-PTX NBs+US group (Fig. 8). In addition, H&E staining of vital organs such as heart, liver, spleen, lung and kidney showed that the concerned organs from each treatment group had normal cellular morphology, clear structure and no apparent histological changes (Fig. 9). The results of blood biochemical analysis showed that the PTX group had the highest levels of ALT, AST, BUN and CER compared with the PBS control group, followed by the PTX+US group, the ICG-PTX NBs group and the ICG-PTX NBs+US group. The ALT, AST, BUN and CER values of the PTX group were significantly different from those of the PBS group (P <0.05). Only the ALT and AST values of the ICG-PTX NBs group and the ICG-PTX NBs+US group were significantly different from the PBS group ( P <0.05), and the ALT, AST, BUN and CER values of the ICG-PTX NBs group and the ICG-PTX NBs+US group were lower than those of the PTX group and
were statistically significant (P < 0.05). These data show that liver function (ALT, AST) in ICG-PTX NBs group and ICG-PTX NBs+US group are different from normal control group (P < 0.05). Moreover, the liver function and kidney function of ICG-PTX NBs group and ICG-PTX NBs+US group were also significantly different from those of PTX group (P < 0.05) (Fig. 10), indicating that ICG-PTX NBs and ICG-PTX NBs+US are less toxic than free PTX.

Discussion

Over the recent years, prostate cancer has become one of the tumors with the highest growth rate of incidence. It is characterized by insidious onset, easy bone metastasis and hormone dependence. Finding a method for early diagnosis and effective treatment of prostate cancer has always been the focus and challenge in cancer research. Related treatment studies have shown that although endocrine therapy is effective in the initial treatment of prostate cancer by effectively controlling the disease development in patients; however, the patient always enters the hormone-independent stage as time progresses. At this stage, treatment is based on chemotherapy. Paclitaxel chemotherapy combined with prednisone can not only effectively improve the clinical symptoms of prostate cancer, but also relieve pain, thereby prolonging the survival time of patients. For these reasons, it become the first choice for the treatment of hormone-independent prostate cancer [26]. However, akin to chemotherapy for other cancers, prostate cancer chemotherapy also pose problems such as toxicity and side effects on liver, heart and kidney, unamenable to monitoring in real time if the drug reaches the target organ, and difficulty in locally concentrating the chemotherapeutic agents. Resolving these issues forms the key link in improving chemotherapy for prostate cancer [27].

Liposomes have the advantages of good biocompatibility and biodegradability, and efficient loading of lipophilic and hydrophilic drugs. Therapeutic drugs can be loaded inside the double-layer lipid shells or lipid cores, or they can be attached to the surface of
liposomes, showing great advantages in drug delivery [28]. In our previous study, a lipid-shell nanobubble carrying AR dsRNA was constructed for androgen-independent prostate cancer, which was confirmed to accumulate in prostate cancer lesions and release AR dsRNA under ultrasound irradiation, thereby inhibiting the growth of prostate cancer. This shows that nanobubbles can be used as a drug or gene carrier with strong penetrability and high drug loading efficiency [29]. However, since ultrasound imaging generates grayscale images by acoustic reflection, contrast-enhanced ultrasonography alone cannot sensitively display and monitor the aggregation and disappearance of drug-loaded nanobubbles in local lesions. Photoacoustic imaging is a new type of non-invasive and non-radiative imaging method that has been rapidly developed in the recent years. It combines the high spatial resolution of ultrasonography and the high contrast of optical imaging to achieve visual dynamic imaging of the target tissue in a damage-free, real-time, multi-level and multi-contrast manner, providing a novel approach for the early detection and therapeutic monitoring of tumors. Multi-modal imaging technology, combining ultrasound and photoacoustic methods, can overcome the shortcomings of single imaging method discussed above. The combination of multiple imaging technologies can complement each other and is expected to solve the problems encountered in tumor diagnosis and post-treatment evaluation. The near-infrared fluorescent dye ICG can simultaneously achieve photoacoustic and fluorescence imaging, but it has drawbacks such as rapid elimination in vivo and poor stability in aqueous solution when used alone. Related studies have shown that encapsulation of ICG into liposome carriers can improve its stability, prolongs its duration in circulation, and increases its fluorescence and photoacoustic signal intensity [30]. Based on the advantages of lipid-shell nanobubbles that they can encapsulate therapeutic drugs and be used for ultrasound/photoacoustic/fluorescent multimodal imaging, as well as the important role of
paclitaxel in prostate cancer chemotherapy, this study integrated the superiority of lipid nanobubbles, multi-modal imaging and the chemotherapeutic drug paclitaxel. We encapsulated both ICG and paclitaxel into the lipid shell to construct nanobubbles that can be used for ultrasound/photoacoustic/fluorescent multimodal imaging and integrated diagnosis and treatment, thereby generating new avenues for the diagnosis and treatment of prostate cancer.

In vitro fluorescence imaging experiments showed that ICG-PTX NBs had slightly stronger fluorescence intensity than ICG at the same ICG concentration, because ICG binds to lipid membrane and is completely and stably integrated in ICG-PTX NBs, indicating that the presence of lipids in ICG-PTX NBs is effective in stabilizing and increasing the fluorescence intensity of ICG, which is consistent with the findings of Kraft, John C et al. [31]. The fluorescence measurement illustrated that the fluorescence intensity of ICG in ICG-PTX NBs was still significantly higher than that of free ICG after 7 days, indicating that ICG was loaded in liposomes and its fluorescence stability was significantly improved. In addition, in vitro photoacoustic and ultrasound imaging also confirmed that with the increase of ICG-PTX NBs concentration, the signal intensity of photoacoustic and ultrasonic imaging also increased, indicating that ICG-PTX NBs are of good use for ultrasound, fluorescence and photoacoustic imaging. In vivo imaging of prostate cancer xenografts in nude mice demonstrated that ICG-PTX NBs have the advantages of stable metabolism and safety in vivo and can accumulate rapidly at tumor sites in a short period of time and achieve ultrasound/fluorescence/photoacoustic multimodal imaging, supporting high contrast, real-time and non-invasive imaging. Related studies have confirmed that the size of nanomedicine is a key factor in passive targeting and drug accumulation during drug delivery and has an important impact on the therapeutic effect of drugs. Therefore, generally large-sized nanobubbles accumulate only near the vasculature, and small-sized
nanobubbles can rapidly diffuse throughout the tumor matrix and provide better penetration. Our previous research results confirmed that the nanobubble particle size of 500 nm can rely on the EPR effect to smoothly enter the tumor tissue through the tumor neovascular wall and accumulate in the tumor tissue for a long time. However, drug delivery systems that rely on tumor EPR effects have limitations such as low drug utilization [32]. Under the action of ultrasound irradiation, the nanobubbles can undergo unstable expansion and intense collapse, resulting in temporary and permanent pores in the neovascular basement membrane of the tumor, which facilitates the entry of more nanobubbles from the tumor vessels into the tumor tissue [14, 33]. Studies by Rajeet Chandan et al. have shown that ultrasound irradiation can completely disintegrate the nanobubble core and the spherical structure constructed by the bound liposomes into lipid fragments, resulting in complete loss of liposome double-layer integrity, hence triggering drug release [24]. The ICG-PTX NBs designed and constructed in this experiment are also lipid-shell nanobubbles with a particle size of 427.9±35.39 nm. In vivo experiments have confirmed that they can enter the tumor tissue through the tumor neovascular wall, enhance their infiltration and accumulation in prostate cancer and achieve multimodal imaging and effective treatment of prostate cancer. At the same time, under the action of ultrasound irradiation, ICG and PTX in the liposome of the nanobubble shell can be effectively released into the tumor tissue in a controlled manner to achieve the killing of prostate cancer cells in a close range with high efficiency. Further results showed that under the action of ultrasound radiation, the tumor volume inhibition and necrosis of tumor tissue in the ICG-PTX NBs group were enhanced compared with the groups that were not subjected to ultrasound radiation. This is majorly because cavitation mediates the enhancement of cell permeability and triggers the rupture of nanobubbles to release paclitaxel and the synergy of the two further improve the anti-tumor effect of ICG-PTX
NBs.

Compared to free drugs, nano drug delivery systems based on liposome-encapsulation are effective in enhancing bioavailability and reducing the side effects of anti-tumor drugs [34]. The results of this study show that compared to the direct treatment with PTX alone, ICG-PTX NBs not only have significant anti-tumor effects in vitro and in vivo, but also do not cause any significant damage to vital organs such as liver and kidney. At the same time, the release of drugs at tumor sites caused by cavitation effects reduces the systemic toxicity of paclitaxel. H&E staining confirmed that ICG-PTX NBs and ICG-PTX NBs+US cause no significant toxic and side effects on vital organs such as heart, liver, spleen, lung and kidney. Blood biochemical analysis showed that ICG-PTX NBs and ICG-PTX NBs+US with liposome-encapsulation had lower toxic effects than PTX alone, further confirming that the ICG-PTX NBs constructed in this study can not only effectively kill tumor cells under ultrasound irradiation and inhibit tumor growth, but are also safe without causing significant damage to important organs of the human body.

Conclusions

As a multimodal contrast agent and carrier for chemotherapeutic drugs, the ICG-PTX NBs constructed in this study, while effectively achieving ultrasound/photoacoustic/fluorescence multimodal imaging, can significantly enhance PTX release and apoptosis of prostate cancer cells under the synergistic effect of low intensity ultrasound irradiation, inhibit tumor growth, and display appreciable biosafety. The successful preparation and experimental study with ICG-PTX NBs provides new strategies and methods for multimodal imaging diagnosis and close-range local chemotherapy of prostate cancer. However, ICG-PTX NBs are incapable of active targeting. Therefore, our future improvisations would involve strategies to construct ICG-PTX NBs that can target prostate cancer, by actively and specifically binding to prostate cancer cells. This would
allow targeted multimodal imaging of tumors, and further enhance the killing effect in
tumor cells under guided ultrasound irradiation.

Abbreviations

UTND: ultrasound targeted nanobubble destruction; NBs: nanobubbles; AST: aspartate
aminotransferase; ALT: phenylalanine aminotransferase; CRE: serum creatinine; BUN:
blood urea nitrogen; ICG: indocyanine green; NIR: near infrared; PTX: paclitaxel; US:
ultrasound; FL: fluorescence; PA: photoacoustic; PBS: phosphate-buffered saline; DMEM:
Dulbecco’s modified eagle’s medium; H&E: hematoxylin and eosin; EPR: enhanced
permeability and retention.

Declarations

Authors’ contributions

YLG, LHZ and MML conceived and designed the experiments. MML, YXW, and BQ performed
the experiments. MML, KJF and DJS analyzed the data. MML and YLP prepared all the
figures. LHZ, YLG and YL prepared the manuscript. All authors read and approved the final
manuscript. Conceptualization.

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Availability of data and materials

All data generated or analyzed during this study are included in the article and additional
file.

Ethics approval and consent to participate

All animal experiments were approved by the Laboratory Animal Welfare and Ethics
Committee of the Army Medical University and was performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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Figures
Basic characteristics of ICG-PTX NBs. a The photographs of Blank NBs and ICG-PTX NBs. b The distribution of ICG-PTX NBs under an optical microscope. c The morphology of ICG-PTX NBs under a transmission electron microscope. d The particle size of Blank NBs. e The particle size of ICG-PTX NBs. f Histogram of the particle size of ICG-PTX NBs changes over time. *P<0.05, **P<0.01. g The UV-vis absorption spectra of Blank NBs, ICG-PTX NBs, ICG and H2O. (H) The photograph of hemolysis of erythrocytes incubated with Blank NBs and ICG-PTX NBs for 1 h and hemolytic ratio measured by UV–vis spectrophotometry at 545 nm. Deionized water and PBS are used as positive and negative control, respectively.
In vitro imaging of ICG-PTX NBs. a Ultrasound images and photoacoustic images of ICG-PTX NBs at different concentrations in vitro. b Fluorescence images of H2O·ICG-PTX NBs and ICG. c Ultrasound images of ICG-PTX NBs before and after destruction. d Quantification of imaging intensity of ICG-PTX NBs before and after destruction. e Quantification of ultrasound imaging of ICG-PTX NBs in vitro. f Quantification of photoacoustic imaging of ICG-PTX NBs in vitro. g Quantification of fluorescence imaging of H2O·ICG-PTX NBs and ICG in vitro. *P<0.05, **P<0.01.
In vivo imaging characteristics of nude mice bearing PC-3 tumors after orbital vein injected of ICG-PTX NBs. a Ultrasound images of Blank NBs and ICG-PTX NBs and photoacoustic images of ICG-PTX NBs and ICG in transplanted tumor tissues. b Time-ultrasound intensity curves of nanobubbles in transplanted tumor tissues (n = 3). c Time-photoacoustic intensity curves of ICG-PTX NBs and ICG in transplanted tumor tissues (n = 3).
Figure 4

In vivo fluorescence imaging characteristics of nude mice bearing PC-3 tumors after orbital vein injected of ICG and ICG-PTX NBs. a The near-infrared fluorescence images of tumor-bearing nude mice. b NIR fluorescence images of major organs and tumors after 2 h post-injection of ICG and ICG-PTX NBs. c Time-fluorescence intensity curve in tumor region at different time points. d Semiquantitative biodistribution of ICG and ICG-PTX NBs in nude mice determined by the averaged fluorescence intensity of each organ. The data are shown as mean ± SD (n = 3), *P<0.05, **P<0.01.
Figure 5

The effect of ICG on nanobubbles cavitation in vitro. a FCM images in each group.

b Cell viability of PC-3 cells treated with Blank NBs, ICG NBs with or without US irradiation. c Cell viability of PC-3 cells treated with PTX NBs, ICG-PTX NBs with or without US irradiation. d Apoptosis of PC-3 cells treated with Blank NBs, ICG NBs, PTX NBs and ICG-PTX NBs with or without US irradiation. The data are shown as mean ± SD (n = 3), *P<0.05, **P<0.01.
Figure 6

In vitro cytotoxicity and apoptosis. a FCM images of PC-3 cells treated with PBS, US, PTX, PTX+US, ICG-PTX NBs and ICG-PTX NBs+US. b Cell viability of PC-3 cells treated with PBS, PTX, ICG-PTX NBs with or without US irradiation. c Apoptosis of PC-3 cells treated with PBS, PTX, ICG-PTX NBs with or without US irradiation, EA: early apoptosis, LA: late apoptosis, TA: total apoptosis. Each bar represents the mean ± SD of three experiments. *P < 0.05, **P < 0.01.
Figure 7

In vivo antitumor efficacy in xenograft mice model with PC-3. a Tumor volume in the various treatment groups. The red circles represent the xenograft areas. Scale, 1 cm. b Contrast-enhanced imaging of the largest section of the transplanted tumor before and after treatment in the various treatment groups, in which the red circle indicates the area of the transplanted tumor. c Changes in
the body weight in the various treatment groups. d Tumor growth curve in the various treatment groups. e Changes in the ultrasonic signal intensity of the maximum sections of xenografts in the six groups. The data are shown as mean ± SD (n = 3), compared to PBS,*P < 0.05, **P < 0.01; compared to PTX,#P<0.05,# #P<0.01; compared to ICG-PTX NBs,△P<0.05, △△P<0.01.

Figure 8

H&E and tunnel stained images of tumor tissue section from xenograft-bearing mice receiving different treatment after 18 days treatment. Analysis of H&E assays, blue staining the cell nuclei, while red staining the cytoplasmic and extracellular matrix. Analysis of TUNEL assays, brown staining the cell nuclei indicates the apoptosis- and proliferation-positive tumor cells, whereas blue staining the cell nuclei indicates the apoptosis- and proliferation-negative tumor cells. Scale, 100 μm.
Figure 9

H&E stained images of sliced major organs including the heart, liver, spleen, lung, and kidney collected from mice sacrificed after injection of ICG-PTX NBs with or without US irradiation. Scale, 100 μm.
Figure 10

Blood biochemistry measurements of tumor-bearing mice after injection of ICG-PTX NBs with or without US irradiation. Each bar represents the mean ± SD of three experiments. *P < 0.05, **P < 0.01