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Combined Cancer Therapy with Hyaluronan-Decorated Fullerene-Silica Multifunctional Nanoparticles to Target Cancer Stem-Like Cells

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Abstract

Cancer stem-like cells (CSCs) are resistant to chemotherapy and highly tumorigenic, which contributes to tumor occurrence and post-treatment relapse. We developed a novel C60 fullerene-silica nanoparticle system surface-decorated with hyaluronan (HA) to target the variant CD44 overexpressed on breast CSCs. Furthermore, doxorubicin hydrochloride (DOX) and indocyanine green (ICG) can be encapsulated in the nanoparticles with ultrahigh encapsulation efficiency (> 90%) and loading content (e.g., 48.5% at a drug-to-nanoparticle feeding ratio of 1:1, compared to the commonly used drug-to-nanoparticle feeding ratio of 1:20 with a drug loading content of less than 5%). As a result, the DOX and ICG-laden nanoparticles can be used as a single nanoplatform to achieve combined chemo, photodynamic, and photothermal therapy under near infrared laser irradiation for effective destruction of the breast CSCs both *in vitro* and *in vivo*, with no evident systemic toxicity. Moreover, we found the nanoparticles with a higher drug loading content (e.g., 48.5 versus 4.6%) also have significantly higher antitumor efficacy, given the same total drug dose. These results demonstrate the great potential of the multifunctional hybrid nanoparticle system for augmenting cancer therapy by eliminating the CSCs.

Keywords

Combination therapy; CSC; CD44; chemotherapy; photothermal therapy; photodynamic therapy

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1. Introduction

Cancer stem-like cells (CSCs) are highly tumorigenic [1,2]. Moreover, the CSCs are highly resistant to the commonly used anticancer drugs, causing cancer recurrence after the conventional chemotherapy [3–5]. As a result, CSCs have attracted a great deal of attention lately in the field of cancer research [6]. To overcome the drug resistance of CSCs, nanoparticle-based drug delivery systems have been developed to target the CSCs. However, it is still difficult to achieve complete tumor destruction *in vivo* by chemotherapy alone at present, even with targeted drug delivery [7–12]. This is probably due to the complexity of the CSC drug resistance induced by both the intrinsic properties (e.g., quiescence/dormancy and high resistance to apoptosis) and the extrinsic factors in their microenvironment or niche [13–18].

One strategy to overcome this challenge is to combine chemotherapy with other treatment modalities such as photothermal therapy (PTT) and/or photodynamic therapy (PDT) [19–22]. The unique physicochemical properties of nanoparticles offer the opportunity to integrate different theranostic modalities within a single nanoplatform for combination therapy together with imaging. The enhanced antitumor capacity of combination therapy has been demonstrated with non-stem cancer cells [19,23–27]. However, no study was reported to investigate the combination of chemotherapy, PDT, and PTT for killing the CSCs.

In this study, we developed a novel nanoplatform of C60 fullerene and mesoporous silica to achieve combined chemotherapy, PDT, and PTT of the breast CSCs, by decorating the nanoplatform with hyaluronan (HA) to target the malignant cells via the variant CD44 overexpressed on their plasma membrane [7–10]. Two breast cancer cell lines (MDA-MB-231 and MCF-7) were used in this study. Under the conventional 2D culture, the triple-negative MDA-MB-231 cells are CD44 positive while the MCF-7 cells are CD44 negative. We used 3D-cultured MDA-MB-231 and MCF-7 mammary tumor spheroids (i.e., mammospheres) enriched with CSCs to check the anti-CSC capability of the nanoparticles *in vitro*. Lastly, we tested the efficacy and safety of the combination therapy enabled by the hybrid nanoparticles to destroy tumor and the CSCs *in vivo* using orthotopic triple-negative human breast tumor grown in mice using the MDA-MB-231 mammosphere cells that are enriched with the CSCs. Our data show that the novel nanoplatform can be utilized to effectively kill the breast CSCs both *in vitro* and *in vivo*, probably due to its small size (~60 nm) that is excellent for cellular uptake and passive targeting of tumor, its capability of actively targeting the CD44 overexpressed on the breast CSCs, its high drug loading content to efficiently carry drugs into the CSCs, and its capacity of achieving combined chemotherapy, PDT, and PTT simultaneously.

2. Materials and methods

2.1 Materials

Triton X-100, hexanol, cyclohexane, toluene, fullerene (C60), tetraethyl orthosilicate (TEOS), (3-aminopropyl) trimethoxysilane (APTMS), doxorubicin hydrochloride (DOX) and indocyanine green (ICG) were purchased from Sigma (St. Louis, MO, USA). Hyaluronan (HA) was purchased from Lifecore Biomedical (Chaska, MN, USA). Fetal

bovine serum (FBS), penicillin, and streptomycin were purchased from Invitrogen (Carlsbad, CA, USA). The DMEM, EMEM and DMEM/F-12K cell culture media was purchased from ATCC (Manassas, VA, USA). The cell counting kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies (Rockville, MD, USA). All other chemicals were purchased from Sigma unless specifically mentioned otherwise.

2.2 Synthesis of nanoparticles

The fullerene-silica hybrid nanoparticles were prepared by a reverse microemulsion method. First, hexanol (2 ml), Triton X-100 (2 ml), cyclohexane (5 ml), and deionized water (0.5 ml) were mixed together. A total of 2 ml of fullerene in toluene (2 mg/ml) was then added into the mixture. Afterward, ammonium hydroxide (28 wt%, 60 μ l) and TEOS (100 μ l) were consecutively added and the sample was stirred at 800 rpm using a mini-stir bar for 24 h at room temperature. Next, the sample was added into ethanol (30 ml) to stop the reaction. The final sample was centrifuged at 13,800g for 10 min to obtain fullerene embedded silica nanoparticles (i.e., C60S). After washing with deionized water and ethanol twice, the nanoparticles were dispersed in 3 ml of ethanol and 20 ml of APTMS was added. The mixture was stirred at 200 rpm using a mini-stir bar for 12 h at room temperature to obtain the APTMS-coated C60S (i.e., C60S-A) nanoparticles. Finally, the sample of C60S-A nanoparticles was mixed with HA solution (1 mg/ml, 10 ml) and stirred for 5 h at room temperature to produce the HA decorated C60S (i.e., HC60S) nanoparticles. The HC60S nanoparticles were collected by centrifuging at 13,800g for 10 min.

2.3 Encapsulation and release of DOX and ICG

Encapsulation of DOX and ICG was conducted by mixing the agents with the nanoparticles at two different feeding ratios: 1:1:20 (DOX:ICG:empty nanoparticles in weight) for low drug loading content and 1:1:1 for high drug loading content. Of note, unless specifically mentioned to be different (for high loading content), the feeding ratio was 1:1:20 by default.

The encapsulation efficiency (EE) of DOX and/or ICG in the nanoparticles (i.e., HC60S-DI) was calculated using the following equation:

$$EE = M_{\text{Encapsulated}} / M_{\text{Fed}} \times 100\% \quad (1)$$

where $M_{\text{Encapsulated}}$ represents the amount (in weight) of agents encapsulated in the nanoparticles and M_{Fed} is the total amount of agents fed for encapsulation. The amount of DOX and ICG was determined spectrophotometrically using a Beckman Coulter (Indianapolis, IN, USA) DU 800 UV-Vis spectrophotometer based on their absorbance at 483.5 and 778 nm, respectively.

The loading content (LC) of DOX and ICG was calculated using the following equation:

$$LC = M_{\text{Agent}} / M_{\text{Total}} \times 100\% \quad (2)$$

where M_{Agent} represents the amount of DOX and/or ICG in nanoparticles and M_{Total} is the total amount of agent-laden nanoparticles including both the encapsulated agents and the non-agent materials for making the empty nanoparticles.

To determine the drug release *in vitro*, the HC60S-DI nanoparticles (20–30 mg) were reconstituted in PBS (5 ml, at pH 5 or 7.4) and transferred into dialysis bags (MWCO: 20 kDa) that were placed in 30 ml of the same PBS solution at 37 °C and stirred at 110 rpm using a mini-stir bar. At various times, 100 μ l of the dialysate was collected and the remaining dialysate replenished with the same amount of fresh PBS. The concentration of the released DOX in the removed dialysate was determined using UV-Vis spectrophotometry based on the absorbance at 483.5 nm. For NIR laser irradiation-triggered drug release, the supernatant of the nanoparticle solutions was obtained by centrifuging at 13,800g and analyzed in the same way using UV-Vis spectrophotometry.

2.4 Characterization of nanoparticles

The nanoparticle size and surface zeta potential were assessed using a Brookhaven (Holtsville, NY) 90 Plus/BI-MAS dynamic light scattering (DLS) instrument by dispersing the nanoparticles (1 mg/ml) in deionized water.

The Fourier Transform Infrared (FTIR) analysis of the nanoparticles was conducted using a Perkin Elmer (Waltham, MA, USA) Spectrum 100 FTIR spectrometer, for which samples were dried, ground in an agate mortar, thoroughly mixed with KBr at a ratio of ~1:80 (nanoparticle:KBr) in weight, and pressed into small discs at 10 tons for 5 min. The data were collected over 400–4000 cm^{-1} .

The nanoparticle morphology was characterized using both transmission (TEM) and scanning (SEM) electron microscopy. For TEM studies, the nanoparticles were examined either directly (for C60S nanoparticles) or after negatively stained with uranyl acetate solution (2% w/w, for HC60S nanoparticles) using an FEI (Moorestown, NJ, USA) Tecnai G2 Spirit transmission electron microscope. The SEM experiments were conducted by depositing 10 μ l of aqueous solutions of the nanoparticles on a freshly cleaved mica grid and allowing them to dry for 60 minutes in air. A thin film of Au was then sputtered onto the nanoparticles on the substrate. Samples were imaged with an FEI NOVA Nano400 scanning electron microscope.

2.5 Cell and mammosphere culture

The MCF-7 and MDA-MB-231 human breast cancer cells (ATCC, Manassas, VA, USA) were cultured in EMEM and DMEM medium, respectively, supplemented with 10% FBS, 100 U/ml penicillin, and 100 μ g/ml streptomycin at 37 °C in a humidified 5% CO_2 incubator. Medium was changed every other day. Cells between passage 5 and 20 at ~70% confluence were detached for passaging and/or further experimental use.

The commonly used 3D suspension culture was utilized to obtain mammospheres enriched with mammary CSCs [7,12,28,29]. Briefly, MDA-MB-231 and MCF-7 cells were cultured in 24-well ultralow attachment plates (Corning, Lowell, MA) at a density of 20,000 cells/ml in serum-free DMEM/F12-K supplemented with 5 μ g/ml insulin, 20 ng/ml epidermal growth

factor, 20 ng/ml basic fibroblast growth factor, 1x B27 (Invitrogen, Carlsbad, CA, USA), 0.4% (w/v) bovine serum albumin, 100 U/ml penicillin, and 100 µg/ml streptomycin. After 10 days of culture, the mammospheres were collected for further experimental use.

2.6 In vitro cell viability

For cell viability studies, the 2D cultured MDA-MB-231 and MCF-7 cells were seeded and incubated with various drug formulations at different concentrations in culture medium in 96-well plates for 12 h, irradiated with NIR laser at 1.5 W/cm² for 3 min if needed, and further cultured for 12 h. For samples with the NIR laser irradiation, the medium with various drug formulations was temporarily removed (i.e., put back after the NIR laser irradiation) and the cells washed with PBS for three times before irradiation. The cell number in the samples with the various treatments was then quantified using the cell counting kit-8 (CCK-8) based on sensitive colorimetric assay, according to the instructions given by the manufacturer (Dojindo Molecular Technologies). The cell number in control samples prepared in the same way without any treatments (i.e., cultured in pure medium) was also determined using the CCK-8. The cell viability was calculated by normalizing the cell number in each sample to the average cell number in the control samples. The viability of cells in the 3D mammospheres with the various treatments was determined in the same way as that for the 2D cultured cells, except that the 3D mammospheres were further cultured for 60 h after the first 12 h incubation and (if necessary) NIR laser irradiation.

2.7 Detection of singlet oxygen production

For this study, deionized water, solution of HC60S nanoparticles, solution of HC60S nanoparticles irradiated with NIR laser were mixed with Singlet Oxygen Sensor Green reagent (Life Technologies, Carlsbad, CA, USA). The fluorescence intensity in the mixtures was measured using a Jasco (Easton, MD, USA) FP-6200 spectrofluorometer with excitation and emission at 488 and 525 nm, respectively.

2.8 Detection of reactive oxygen species (ROS) in cells

To detect ROS in MDA-MB-231 cells, they were cultured with different nanoparticles for 24 h. After NIR laser irradiation (if necessary), the cells were mixed with 25 µM dichlorodihydro-fluorescein diacetate (DCFH-DA, Cell Bio Labs Inc., San Diego, CA, USA) for 45 min. ROS production was then detected quantitatively using a PerkinElmer (Waltham, MA, USA) VICTORX3 Multilabel microplate reader and qualitatively by fluorescence microscopy.

2.9 Specific binding between HC60S-DI and CD44

To study the specific binding between HC60S-DI nanoparticles and 2D cultured MDA-MB-231 and MCF-7 cells, the cells were seeded onto collagen-coated cover glasses (Nunc, Thermo Fisher Scientific Inc., Waltham, USA) at a density of 2×10^5 cells/well in 6-well plate and grown at 37 °C for 12 h. The cells were then cultured with 2 ml of fresh cold medium containing different drug (10 µg/ml) formulations for 3 h at 4 °C. For the mammospheres, they were put on ice for 2 h to minimize the metabolic activity before incubating with different drug (10 µg/ml) formulations for 3 h at 4 °C. The cells and

mammospheres were then washed with 1x PBS, fixed in 4% paraformaldehyde, washed and further stained for nuclei with Hoechst 33342 (5 μ M) for examination using an Olympus FluoView™ FV1000 confocal microscope.

2.10 In vitro imaging

For *in vitro* imaging, the 2D cultured cells were seeded onto collagen-coated cover glasses (Nunc, Thermo Fisher Scientific Inc., Waltham, USA) at a density of 2×10^5 cells/well in 6-well plate and grown at 37 °C for 12 h. The medium was then replaced with 2 ml of fresh medium containing different drug (10 μ g/ml) formulations. The mammospheres were incubated with medium containing different drug (10 μ g/ml) formulations. After incubation at 37 °C for 3 h, the mammospheres and cover glass attached with cells was mounted onto a glass slide with anti-fade mounting medium (Vector Laboratories Burlingame, CA, USA) for examination using an Olympus FluoView™ FV1000 confocal microscope.

2.11 Animals and xenograft tumors

The 6-week-old athymic female NU/NU nude mice were purchased from Charles River (Wilmington, MA, USA) and maintained on a 16:8 h light-dark cycle. All procedures for animal use were approved by the Institutional Animal Care and Use Committee (IACUC) at The Ohio State University and all efforts were made to minimize animal suffering. To obtain xenograft of human breast tumor in the nude mice, detached MDA-MB-231 mammosphere cells were suspended at 200,000 cells/ml in a mixture (1:1) of 1x PBS and matrigel. A total of 20,000 cells in 100 μ l of the mixture were injected into the fat pad of each 7-week-old female mouse.

2.12 In vivo imaging and biodistribution

For *in vivo* imaging, after the tumor reached ~5 mm in long diameter, the mice were injected with 100 μ l saline, 100 μ g ICG in 100 μ l saline, HC60S-DI nanoparticles (DOX: 100 μ g and ICG: 100 μ g for both low and high drug loading content nanoparticles) in 100 μ l saline. *In vivo* fluorescence images were taken at 6 h after intravenous injection via the tail vein using a PerkinElmer (Waltham, MA, USA) IVIS instrument with excitation at 780 nm and an 831 nm filter to collect the fluorescence emission of ICG. After *in vivo* imaging, the mice were sacrificed and the tumor, liver, kidney, lung, spleen, and heart were removed and collected for further *ex vivo* fluorescence imaging of ICG using the same IVIS instrument.

2.13 In vivo antitumor efficacy and safety

When tumors reached ~5 mm in long diameter, mice were treated with 100 μ l saline or blank HC60S nanoparticles, DOX&ICG with NIR laser irradiation (0.7 W/cm² for 3 min, DOX: 1.5 mg/kg, ICG: 1.5 mg/kg body weight), C60S-DI nanoparticles with NIR laser irradiation (0.7 W/cm² for 3 min, DOX: 1.5 mg/kg, ICG: 1.5 mg/kg body weight), HC60S-DI nanoparticles (DOX: 1.5 mg/kg, ICG: 1.5 mg/kg body weight), HC60S-DI nanoparticles with NIR laser irradiation (0.7 W/cm² for 3 min, DOX: 1.5 mg/kg body weight), and HC60S-DI nanoparticles (feeding ratio of DOX:ICG:nanoparticles, 1:1:1) with NIR laser irradiation (0.7 W/cm² for 3 min, DOX: 1.5 mg/kg, ICG: 1.5 mg/kg body weight). A total of 100 μ l of saline was used as the carrier for all drug formulations. The intravenous drug

injection was performed on day 1 and the NIR laser irradiation was conducted at 12 h after the intravenous drug injection. During the NIR laser irradiation process, after every 1 min of irradiation, the tumor was allowed to spontaneously cool at room temperature for 30 s before the next irradiation to ensure that the overall tumor temperature was lower than ~43 °C.

Tumor growth was monitored every other day. The tumor volume (V) was calculated as: $V = (L \times W^2)/2$, where L is long diameter and W is short diameter of tumor determined using a caliper. The mice were euthanized on day 29 after the drug injection. Tumors, livers, lungs, hearts, spleens, and kidneys were collected, formalin fixed, paraffin embedded, and haematoxylin&eosin (H&E) stained for further histological analysis.

2.14 CD44, CD133, Annexin V, and PI Staining

For immunohistochemical staining of CD44 and CD133 (two CSC surface markers), tumors were collected and put in frozen with the Tissue-Tek (Sakura Finetek, Torrance, CA, USA) O.C.T. Compound and Cryomold at -80 °C for 24 h. The tumors were then cut into slices of 10 µm thick using a cryo-microtome and transferred onto microscope slides. For CD133 staining, the slides were washed with FcR blocking reagent and incubated with PE labeled CD133 (1:11 dilution, Miltenyi Biotec, Auburn, CA, USA) for 30 min in the dark at room temperature. For CD44 staining, the slides were incubated in 3% BSA in 1× PBST (1× PBS and 0.05% Tween 20) at room temperature for 1 h to block potential non-specific binding, followed by overnight incubation at 4 °C with primary antibodies CD44 (1:40 dilution, Abcam, Cambridge, MA, USA). The samples were then washed 3 times and incubated in dark at room temperature for 1 h with secondary antibodies (Abcam, FITC labeled) diluted in 3% BSA in 1× PBST (1:50 dilution). Afterward, the samples were washed and further stained for nuclei using Hoechst 33342 (5 µM) for further examination using an Olympus FV1000 confocal microscope.

For staining with Annexin V and PI, the tumors were cut into slices of 20 µm thick using the same method mentioned above. The slides were washed with PBS for three times, and transferred to 1 × binding buffer (0.1 M HEPES, pH 7.4, 1.4 M NaCl, 25 mM CaCl₂, diluted to 1 × prior to use) with Annexin V (BD, San Jose, CA, USA) and PI (BD, San Jose, CA, USA). Then, the slides were incubated with the binding buffer for 15 min at room temperature in dark. The samples were then washed and further stained for nuclei using Hoechst 33342 (5 µM) for further examination using an Olympus FV1000 confocal microscope.

2.15 Side population analysis

To analyze the side population, fresh tumors were gashed into small squares (~0.5 mm³) and washed with HBSS containing calcium and magnesium for 3 times. Then, collagenase (100 U/ml, Life Technologies, NY, USA) were added and incubated with the tumor tissues for 4 h at 37 °C. Approximately every half an hour, gently agitate the tissue pieces carefully so that no tissue pieces stick to the side of the tube above the collagenase. The dispersed cells were collected by passing through a sterile nylon mesh and washed with HBSS. Afterward, the cells were stained with Hoechst 33342 dye (5 µg/mL) in dark for 90 min at 37 °C with shaking every 15 min. At the end of incubation, cells were spun down and re-suspended in ice-cold PBS with 5% FBS. The side population was analyzed with a BD LSR-II Flow

Cytometer using a dual wavelength analysis (blue at 450 nm and red at 660 nm) after excitation with 405 nm UV light.

2.16 Statistical analysis

All data are reported as mean $\pm$ standard deviation (SD) from at least three independent runs. The Kruskal–Wallis H -test and the Mann–Whitney U -test were used to assess the overall among-group and two-group differences, respectively. All statistical analyses were carried out with the IBM (Armonk, NY, USA) SPSS software (version 22). The difference is considered statistically significant when the p value is less than 0.05.

3. Results and discussion

3.1. Characterization of nanoparticles and drug encapsulation

As illustrated in Fig. 1A, the C60S nanoparticles were generated by embedding fullerene in the center of a mesoporous silica matrix. APTMS was then modified on the C60S nanoparticles to form the C60S-A nanoparticles. Lastly, HA was fused on the surface of the C60S-A nanoparticles to produce the HA decorated C60S (HC60S) nanoparticles. The HC60S nanoparticles are further loaded with doxorubicin hydrochloride (DOX or D) for chemotherapy and indocyanine green (ICG or I) for near infrared (NIR) imaging to form the HC60S-DI nanoparticles. As illustrated in Fig. 1B, the goal is to achieve effective destruction of tumor including breast CSCs by targeted delivery of the agents to tumor through the ligand-receptor binding mechanism between HA on the nanoparticle and the variant CD44 overexpressed on breast cancer cells and particularly their CSCs [7,8], and by combined chemotherapy, PTT, and PDT via the photothermal and photodynamic effects of ICG and fullerene under NIR (800 nm) laser irradiation [19].

The structure of the C60S and HC60S nanoparticle was visualized by transmission electron microscopy (TEM). As shown in Fig. 2A, the C60S nanoparticles have a core-shell structure and the HA coating (arrows) is observable on the surface of the HC60S nanoparticles after negative staining. Scanning electron microscopy (SEM) images indicate that both the C60S and HC60S nanoparticles have a monodisperse size distribution with a diameter of ~ 60 nm (Fig. 2B). Successful decoration of HA is further confirmed by the surface zeta potential of C60S, C60S-A, and HC60S nanoparticles in deionized water. The zeta potential of C60S nanoparticles is highly negative (Fig. 2C). Because of the primary amine groups in APTMS, the zeta potential of the C60S-A nanoparticles is positive. While the zeta potential of the resultant HC60S nanoparticles returns to negative due to the coating with HA. Moreover, the HC60S nanoparticles are smaller and have a narrower size distribution than the C60S nanoparticles, suggesting the HA coating could prevent the nanoparticles from forming aggregates in aqueous solution (Fig. 2D). The existence of fullerene, silica, and HA in the HC60S nanoparticles is further confirmed with Fourier transform infrared spectroscopy (FTIR) (Fig. S1). These data indicate the successful preparation of the HA-coated fullerene-silica hybrid nanoparticles.

Previous studies have shown that the C60S nanoparticles is excellent for encapsulating both DOX and ICG [19]. Similarly, the HC60S nanoparticles can also be used to encapsulate

DOX with high efficiency (> 95%) by simply mixing them together for 30 min at the feeding ratio of DOX:HC60S nanoparticles = 1:20 (HC60S+D, Fig. 2E). The encapsulation efficiency (EE) of ICG is low if it is simply mixed with the HC60S nanoparticles at ICG:HC60S nanoparticles = 1:20 (HC60S+I, Fig. 2E). In contrast, when ICG is mixed with DOX and HC60S nanoparticles together, the EE of ICG can be greatly improved to approximately 100% with 30 min mixing (DOX:ICG:HC60S nanoparticles = 1:1:20, Fig. 2E). At a high feeding ratio of 1:1:1, the EE of DOX and ICG is still very high ($94.0 \pm 1.9\%$ for DOX, and $91.1 \pm 1.2\%$ for ICG) with only 30 min of mixing (Fig. S2A). As a result, the drug (DOX) loading content (LC) is $4.6 \pm 0.2\%$ for the low drug feeding ratio of 1:20 and it is as high as $48.5 \pm 0.1\%$ for the high drug feeding ratio of 1:1 (Fig. S2B). The wavelength of the absorbance peak of ICG in the HC60S-DI nanoparticles increases with a slight redshift to 805 nm from 777 nm for free ICG (Fig. 2F). Interestingly, the major absorbance peak undergoes blueshift if ICG and DOX are mixed directly (Fig. 2F).

3.2. Photothermal effect of HC60S-DI nanoparticles and NIR irradiation-controlled release

To confirm the NIR irradiation-controlled drug release, we checked the photothermal effects of HC60S-DI nanoparticles first as the release of DOX from the nanoparticles is mainly dependent on the temperature to break the interaction between DOX and fullerene-silica according to previous studies [19,30]. ICG has been shown to absorb and convert the energy of NIR light into heat [31,32]. Indeed, as shown in Fig. S3, the temperature of the aqueous HC60S-DI nanoparticle solution could increase significantly under the NIR laser irradiation and the heat can quickly dissipate into the environment when the laser is off. We next investigated the release of DOX at 37 °C from the HC60S-DI nanoparticles. The drug release at pH 7.4 is very slow and it increases slightly at pH 5.0 (Fig. S4). Importantly, the drug release can be triggered and precisely controlled by NIR laser irradiation under both the neutral and acid conditions (Fig. S4), which can be attributed to the NIR laser irradiation-induced heating.

The photothermal effect shown in Fig. S3 suggests that the HC60S-DI nanoparticles can be used to achieve PTT for the destruction of cancer cells. To test this hypothesis, we incubated the MDA-MB-231 cells with HC60S or HC60S-I (HC60S encapsulated with ICG only) nanoparticles and irradiated with the NIR laser for three minutes. As shown in Fig. S5, the live/dead (green/red) staining clearly shows that the HC60S-I nanoparticles can destroy the cancer cells under NIR laser irradiation in a dose-dependent manner while the HC60S nanoparticles without ICG cannot even at the highest dose tested (0.4 mg/ml).

3.3. Photodynamic effect of HC60S and HC60S-DI nanoparticles

Besides chemotherapy (DOX) and PTT, we also found that the HC60S nanoparticles can be used for PDT. As shown in Fig. 3A, the photodynamic effect of HC60S nanoparticles was determined by checking the production of singlet oxygen in aqueous solutions. A commercial singlet oxygen sensor with fluorescence was used for this detection. The data show that the fluorescence intensity of the HC60S nanoparticles solution with NIR laser irradiation (HC60S+L) was greatly increased compared that of the nanoparticle solution without laser irradiation, indicating the laser irradiation-triggered production of singlet

oxygen. Furthermore, the production of singlet oxygen increases continuously with time (Fig. 3B).

To show the capability of the HC60S nanoparticles in generating ROS in cancer cells for PDT, we incubated MDA-MB-231 cells with different types of nanoparticles and treated with laser (if necessary). The results are shown in Fig. 3C. First, neither of the nanoparticles could induce ROS production in the cancer cells without laser irradiation. In addition, both the blank HC60S nanoparticles and HC60S-D nanoparticles (HC60S nanoparticles encapsulated with DOX only) could induce ROS production in the cancer cells with laser irradiation. More importantly, the HC60S-DI group show the most intense fluorescence after laser irradiation, which should be due to the combined effect on the cancer cells in the presence of DOX (for chemotherapy) and ICG (for PTT) (Fig. 3C). The quantitative data measured by 96-well plate are shown in Fig. S6. The fluorescence intensity of ROS in the cancer cells is consistent with the confocal microscopy data shown in Fig. 3C.

3.4. Capability of targeting breast CSCs with the HC60S nanoparticles

First, we characterized the expression of CD44 and CD133 on MCF-7 and MDA-MB-231 cells under the conventional 2D adherent culture in regular culture medium and in their mammospheres obtained by the commonly used 3D suspension culture in cancer stem cell medium. As shown in Figures S7–S8, the 2D cultured MDA-MB-231 cells are CD44+ and largely CD133+ (Figure S7) while the 2D cultured MCF-7 cells are CD44– and CD133– (Figure S8). Moreover, the expression of both CD44 and CD133 is higher in the 3D cultured MDA-MB-231 and MCF-7 mammosphere cells (enriched with their CSCs) than the corresponding 2D cultured cells. To study the CSC targeting capability of the HC60S nanoparticles, we incubated the C60S-DI and HC60S-DI nanoparticles with 2D cultured MDA-MB-231 and MCF-7 cancer cells and 3D cultured MDA-MB-231 and MCF-7 mammospheres enriched with CSCs at both 4 °C and 37 °C for 3 h. The use of 4 °C is to minimize cellular metabolism and uptake of nanoparticles by endocytosis for investigating the binding between the nanoparticles and cells. Typical confocal images showing the intracellular DOX together with the stains of nuclei and ICG in the cells incubated with the nanoparticles are shown in Figs. 4–5 (for high magnification) and Figs. S9–S10 (for low magnification). As shown in Fig. 4A and S9A, no fluorescence can be detected on the 2D cultured MDA-MB-231 and MCF-7 cells after incubation with free DOX&ICG and C60S-DI nanoparticles at 4 °C for 3 h. Importantly, strong fluorescence of both DOX and ICG is observable on (not in) the MDA-MB-231 cells treated with HC60S-DI nanoparticles at 4 °C for 3 h, indicating that the HA on the HC60S-DI nanoparticles could bind and target the CD44 overexpressed on the MDA-MB-231 cells. By contrast, the fluorescence in the MCF-7 cells treated HC60S-DI nanoparticles at 4 °C for 3 h is barely detectable. This is not surprising as the 2D cultured MCF-7 cells are CD44 negative with no specific binding to HA on the nanoparticles.

We next incubated the HC60S-DI nanoparticles with the 2D cultured MDA-MB-231 and MCF-7 cells at 37 °C for 3h to check cellular uptake of the nanoparticles and the laser controlled drug release from the nanoparticles in the cells *in vitro*. As shown in Fig. 4B and S7B, the fluorescence intensity of both DOX and ICG in the HC60S-DI nanoparticles

treated MDA-MB-231 cells is much higher than the free DOX&ICG and C60S-DI nanoparticles treated cells, further confirming the targeting capability of the HC60S-DI nanoparticles. The fluorescence in the HC60S-DI nanoparticle treated MCF-7 cells is similar to that in the cells treated with C60S-DI nanoparticles and lower than that in the free DOX&ICG treated cells (Fig. 4B). More importantly, the HC60S-DI nanoparticle treated MDA-MB-231 and MCF-7 cells with laser irradiation show the strongest fluorescence intensity, confirming the laser controlled drug release from the nanoparticles inside the cells.

To further check the capability of targeting CSCs, we incubated the HC60S-DI nanoparticles with both MDA-MB-231 and MCF-7 mammospheres enriched with CSCs. According to Fig. S7 and previous study [12], the MCF-7 mammosphere cells are CD44 positive. Indeed, unlike the 2D cultured MCF-7 cells, the fluorescence of DOX and ICG is observable on both the MDA-MB-231 and MCF-7 mammosphere cells after incubating with the HC60S-DI nanoparticles at 4 °C for 3 h (Fig. 5A and S10A). In order to confirm the specific targeting capability of the HC60S nanoparticles, we further pre-blocked the 2D and 3D cultured MDA-MB-231 and MCF-7 cells with HA, and then incubated with the HC60S-DI nanoparticles. As shown in Fig. S11, pre-blocking the cells with HA indeed minimizes the binding between the HC60S nanoparticles and the 2D and 3D cultured cells. Moreover, the HC60S-DI nanoparticle treated MDA-MB-231 and MCF-7 mammospheres also have higher fluorescence intensity of DOX and ICG than the free DOX&ICG and C60S-DI nanoparticles treated mammospheres at 37 °C for 3h (Fig. 5B and S10B). Similar to the 2D cultured cells, the HC60S-DI nanoparticles treated mammospheres with laser irradiation also have the strongest fluorescence intensity, indicating that the NIR laser controlled drug release can be achieved in the mammospheres enriched with CSCs.

The aforementioned data indicate that we successfully prepared a hybrid nanoplatform capable of targeting the CSCs via the variant CD44 overexpressed on the cells for combined chemotherapy, PTT, and PDT. Therefore, we further investigated the anti-CSC capability of the HC60S-DI nanoparticles under NIR laser irradiation both *in vitro* and *in vivo*.

3.5. Enhanced destruction of breast cancer cells and CSCs *in vitro*

To investigate the anticancer capacity of HC60S-DI nanoparticles *in vitro*, both the 2D cultured MDA-MB-231 and MCF-7 cancer cells and their 3D cultured mammospheres enriched with CSCs were treated with free DOX, blank HC60S nanoparticles, and various agent (DOX and/or ICG) laden nanoparticles either without or with NIR laser irradiation at 1.5 W/cm² for 3 min. The 2D cultured MDA-MB-231 and MCF-7 cancer cells were incubated with the various formulations for a total of 24 h and either with or without the NIR laser irradiation at 12 h of incubation. The cell viability was calculated by normalizing the cell number in the samples with the various treatments to the average cell number in control samples without any treatment (i.e., cultured in pure medium all the time). As shown in Fig. 6A, the HC60S nanoparticles without any drug were not harmful to the cells and even slightly promote the cell growth for conditions with a viability greater than 100%, suggesting the minimal cytotoxicity of the blank nanoparticles. The cytotoxicity of HC60S-D and HC60S-DI nanoparticles to the MDA-MB-231 and MCF-7 cancer cells is similar to that of free DOX. Furthermore, the HC60S-DI nanoparticles with laser irradiation are more

cytotoxic than free DOX, probably due to the combined effect of chemo, PDT and PTT. Under NIR laser irradiation, the HC60S-DI nanoparticles exhibit higher toxicity than the C60S-DI nanoparticles, suggesting the targeting effects of HA could enhance the anticancer capacity *in vitro*. Moreover, we found that given the same total dose of drug and NIR laser irradiation, the HC60S-DI nanoparticles with a high loading content (drug feeding ratio of DOX:ICG:HC60S nanoparticles = 1:1:1) exhibit significantly higher cytotoxicity than the same nanoparticles made with a lower drug feeding ratio (DOX:ICG:HC60S nanoparticles = 1:1:20) (Fig. 6A). This is because the bonding between DOX and the fullerene-silica matrix in the nanoparticles with a higher loading content can be more efficiently destroyed to release more DOX, under the same dose of ICG and NIR laser irradiation [19].

We further investigated the capability of the HC60S-DI nanoparticles either with or without the NIR laser irradiation at 12 h in destroying the mammospheres enriched with CSCs for a total of 72 h of incubation. As shown in Fig. 6B, the blank HC60S nanoparticles without any drug are not harmful to the mammospheres and even slightly promote the cell growth for conditions with a viability greater than 100%. Similarly, the HC60S-DI nanoparticles with higher loading content (DOX:ICG:HC60S nanoparticles = 1:1:1) exhibit significantly higher cytotoxicity to the mammospheres under NIR laser irradiation. The mammospheres enriched with CSCs can be effectively destroyed by the HC60S-DI nanoparticles with NIR laser irradiation, presumably due to the combined effect of chemotherapy, PDT, and PTT, although chemotherapy alone with either free DOX or the HC60S-DI nanoparticles without laser irradiation is not as effective. It is worth noting that the total incubation time with the various drug formulations for the 2D cultured cells is much shorter (24 h versus 72 h) than the 3D cultured mammosphere cells, in order to demonstrate the effect of the high drug loading content with NIR laser irradiation on enhancing the anticancer capacity *in vitro*. Nonetheless, the viability of the 3D cultured mammosphere cells is still higher than that of the 2D cultured cells at low dose of free DOX (e.g., 0.1 $\mu\text{g}/\text{ml}$), indicating the highly drug resistant nature of the 3D cultured mammosphere cells.

3.6. Enhanced targeting of breast tumor and antitumor capacity in vivo

We next investigated the tumor targeting capability of the HC60S-DI nanoparticles in mice. As shown in Fig. 7A, highly enhanced fluorescence of ICG is observable in tumor at 6 h after intravenous injection of the HC60S-DI nanoparticles, compared to free ICG. Interestingly, we found that the fluorescence of ICG in MDA-MB-231 tumors (orthotopic xenograft) from mice treated with the HC60S-DI nanoparticles with a high drug loading content (DOX:ICG:HC60S nanoparticles = 1:1:1) is much higher than that in the tumors from mice treated by the nanoparticles with a low drug loading content (DOX:ICG:HC60S nanoparticles = 1:1:20). To confirm the observation from whole animal imaging, various organs were harvested for *ex vivo* imaging to check the biodistribution of ICG after sacrificing the mice at 6 h. Indeed, only tumors from the HC60S-DI nanoparticles treated groups show strong fluorescence of ICG (Fig. S12), and the fluorescence in tumors from mice treated with the HC60S-DI nanoparticles with a high drug loading content is stronger.

We further investigated the antitumor efficacy and safety of the HC60S-DI nanoparticles *in vivo* by treating orthotopic MDA-MB-231 human mammary tumor-bearing mice. The

tumors were produced by injecting 20,000 MDA-MB-231 mammosphere cells per animal into the fat pad of 6–8 week-old female nude mice. The tumor-bearing mice were divided randomly into seven groups: saline, blank nanoparticles (HC60S), free DOX&ICG with NIR laser irradiation (DOX&ICG+L), C60S-DI nanoparticles with NIR laser irradiation (C60S-DI+L), HC60S-DI nanoparticles, HC60S-DI nanoparticles with NIR laser irradiation (HC60S-DI+L), and high drug loading content HC60S-DI nanoparticles with NIR laser irradiation (HC60S-DI+L (1:1:1)). Mice were treated with the various formations at a total dose of 1.5 mg/kg body weight of DOX via intravenous injection when the tumor reached a volume of $\sim 100 \text{ mm}^3$. As shown in Fig. 7B, tumor growth for treatments with blank HC60S nanoparticles is similar to that of saline control. The change in tumor volume for the treatment with free DOX&ICG and C60S-DI nanoparticles with laser irradiation is not significantly different from that of the saline control either. For the treatment with HC60S-DI nanoparticles, tumor growth was slightly inhibited compared to the saline control. After laser irradiation, the tumor growth was further slowed down, probably due to the combined effect of chemotherapy, PDT and PTT. More importantly, the HC60S-DI nanoparticles of high drug loading content with NIR laser irradiation exhibited the best anti-tumor capacity. Complete tumor destruction was observed in three out of five mice and the remaining two tumors are quite small compared with the tumor from other groups (see the images in Fig. 7B). The weight of the tumors harvested from the high drug loading content HC60S-DI group is significantly less than that from the other groups (Fig. S13).

Representative images showing the orthotopic breast tumors grown in the mammary fat pad of mice and the difference in their sizes after the various treatments on day 29 are given in Fig. 7C (top row). Histological examination (H&E stain) revealed extensive necrosis in the tumors from the high drug loading content HC60S-DI+L (1:1:1) group, while tumors from all the other groups were more viable (Fig. 7C, bottom row). Furthermore, extensive apoptosis (Annexin V staining) and primary/secondary necrosis (PI staining) was observable in tumors from the high drug loading content HC60S-DI+L (1:1:1) group (Fig. 7D). These data demonstrate the excellent *in vivo* antitumor efficacy of the high drug loading content HC60S-DI nanoparticles with NIR laser irradiation, probably due to the combined effect of chemotherapy, PDT and PTT.

Moreover, we did not notice any obvious side effect of the HC60S nanoparticles. Neither death nor significant drop of body weight was noted for the mice treated with saline, blank HC60S nanoparticles, and all the five drug formulations (Fig. S14). Major organs in mice of the high drug loading content HC60S-DI+L (1:1:1) group were collected for histology analysis on day 29 after the treatment. No evident damage to the critical organs was noticeable in the H&E stained tissue slices (Fig. S15). These results suggest the excellent safety of the HC60S nanoparticles for targeted delivery of DOX and ICG *in vivo*.

3.7. Enhanced destruction of breast CSCs in vivo

To understand the *in vivo* anti-CSC capacity of the HC60S-DI nanoparticles, we performed flow cytometry analysis of the subpopulation of cancer cells (often called the side population) that are capable of excluding Hoechst 33342 in the tumors from the various treatment groups. This rare side population has been taken as one of the CSC subpopulations

that are drug resistant. Typical images of two-channel flow cytometry data showing distinct side populations in the boxed region and the percentage data from three independent runs are given in Fig. 8A and 8B, respectively. A significantly lower percentage of the side population was consistently observed in tumors from the high drug loading content HC60S-DI+L (1:1:1) group on day 29. Both the variant CD44 and CD133 have been widely used as the surface markers of CSCs. We further checked the expression of the two CSC markers in the *in vivo* tumors from mice with the various treatments by immunohistochemical staining. As shown in Fig. 8C and S16 (for high and low magnifications, respectively), the expression of the two CSC markers in tumors from the HC60S, DOX&ICG+L, C60S-DI+L and HC60S-DI treatment groups was high and similar to that of saline control. These data together with that shown in Fig. 8A–B suggest that only a small portion of the cells with the CSC markers exclude the nuclear dye Hoechst, which is consistent with the literature [7,8,28,33,34]. Importantly, reduced expression of the variant CD44 and CD133 was evident in the tumors from the HC60S-DI+L treatment group. More importantly, minimal expression of CD44 and CD133 in tumors from the high drug loading content HC60S-DI+L (1:1:1) treatment group is observable. These data indicate the extraordinary capability of the HC60S-DI nanoparticles in combination with NIR laser irradiation in targeting and destroying the breast CSCs *in vivo*. However, it is worth noting that CD44 may not be a universal marker for CSCs of all types of cancers and it is by no means the only cellular surface molecule that could interact with hyaluronan [35–38]. Therefore, caution should be taken to extend the findings obtained in this study using breast cancer to the therapy of many other types of cancers.

4. Conclusions

In summary, we successfully prepared a novel hybrid nanoplatfrom that is capable of targeting the CSCs and achieving combined chemotherapy, PDT and PTT with NIR laser irradiation. HA was decorated on the nanoparticles for targeting the breast CSCs via its interaction (or binding) with the variant CD44 overexpressed on breast cancer cells (and particularly CSCs). Fullerene in the nanoparticles was used as the agent for PDT. DOX and ICG encapsulated in the nanoparticles were used for chemotherapy and PTT, respectively. Moreover, DOX and ICG could be efficiently encapsulated in the nanoparticles even with high drug feeding ratio, resulting in a high drug loading content. The controlled drug release can be achieved with NIR laser irradiation. Both the *in vitro* and *in vivo* studies confirmed that the high drug loading content HC60S-DI nanoparticles could target and inhibit the breast CSCs more efficiently than the low loading content nanoparticles. Lastly, our extensive *in vitro* studies with two types of breast cancer cells (i.e., MDA-MB-231 and MCF-7) and their CSCs together with *in vivo* studies using orthotopic triple negative human breast tumors (i.e., MDA-MB-231) grown in mice, demonstrate the great potential of this multifunctional (chemotherapy, PDT, and PTT) HC60S-DI nanoparticles for targeting and destroying the CSCs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://>

Fig. 1.

A schematic illustration of the nanoparticle synthesis and the capability of targeting cancer stem-like cells (CSCs) and achieving combined chemotherapy, photodynamic therapy (PDT), and photothermal therapy (PTT) under near infrared (NIR) laser irradiation. (A) A schematic illustration of the procedure for preparing fullerene- (C60) embedded silica (C60S) nanoparticles, modifying the C60S nanoparticles with APTMS to form C60S-A nanoparticles, and further coating the C60S-A nanoparticles with hyaluronan (HA) to form HC60S nanoparticles, and loading the nanoparticles with the doxorubicin hydrochloride (DOX) and indocyanine green (ICG) to give the HC60S-DI nanoparticles. (B) The capacity of the HC60S-DI nanoparticles in targeting tumor via the enhanced permeability and retention (EPR) effect of tumor vasculature compared to normal vasculature (boxout on the left of the animal) and specific binding between CD44 overexpressed on CSCs and HA decorated on the nanoparticle surface (boxout at the bottom). Under NIR laser irradiation, the combined effect of chemotherapy, PDT, and PTT of the HC60S-DI nanoparticles could efficiently destroy the CSCs *in vivo* (boxout at the bottom).

Fig. 2.

Characterization of nanoparticles. (A) Transmission electron microscopy (TEM) images of the C60S and HC60S nanoparticles. Also shown are the zoom-in views showing the core-shell structure of C60S nanoparticles and the HA layer on the surface of HC60S nanoparticles. (B) Scanning electron microscopy (SEM) images of the C60S and HC60S nanoparticles showing their homogeneous size distribution. (C) The difference in surface zeta potential of C60S, C60SA and HC60S nanoparticles indicating the successful coating of first positively charged APTMS and then negatively charged HA on the surface of C60S nanoparticles. (D) Size distribution of C60S and HC60S nanoparticles in DI water (1 mg/ml). (E) Encapsulation efficiency (EE) of DOX and ICG by mixing the agents with HC60S nanoparticles for 30 min. The HC60S nanoparticles could encapsulate DOX efficiently but not ICG by simply mixing. However, by mixing DOX and ICG together with HC60S nanoparticles, both DOX and ICG could be encapsulated in the nanoparticles with an EE of ~100% by simply mixing for only 30 min. (F) UV-Vis absorbance of free DOX, free ICG, simple mixture of free DOX and ICG (DOX&ICG), and DOX and ICG laden HC60S nanoparticles (HC60S-DI).

Fig. 3.

Photodynamic effect of HC60S nanoparticles. (A) The production of singlet oxygen by the HC60S nanoparticles with and without NIR laser irradiation (1.5 W/cm² for 3 min) at 22 °C at different concentrations. (B) The time dependent production of singlet oxygen of HC60S nanoparticles with and without NIR laser irradiation (1.5 W/cm²) at 22 °C in 10 min. (C) The photodynamic effect of HC60S, HC60S-D, and HC60S-DI nanoparticles examined by the production of reactive oxygen species (ROS) in cancer cells. Cells were cultured with the different nanoparticles for 24 h. After NIR laser irradiation (if necessary, at 1.5 W/cm² for 1 min), the cells were mixed with 25 μM dichloro-dihydro-fluorescein diacetate (DCFH-DA) for 45 min.

Fig. 4.

Confocal images showing the binding affinity of the HC60S-DI nanoparticles with 2D cultured MDA-MB-231 cells and MCF-7 cells at 4 °C (to minimize endocytosis for uptake) and the cellular uptake of the HC60S-DI nanoparticles at 37 °C with or without NIR laser irradiation (1.5 W/cm² for 3 min). (A) MDA-MB-231 and MCF-7 cells were incubated with free DOX&ICG, C60S-DI, and HC60S-DI nanoparticles at 4 °C for 3 h. The HC60S-DI could bind more efficiently on the MDA-MB-231 than MCF-7 cells, because the MDA-MB-231 cells are CD44 positive while MCF-7 cells are CD44 negative. (B) MDA-MB-231 and MCF-7 cells were incubated with free DOX&ICG, C60S-DI, and HC60S-DI nanoparticles at 37 °C for 3 h with or without laser irradiation. The cellular uptake of HC60S-DI in MDA-MB-231 cells is higher than that in MCF-7 cells, which further confirm the capability of the HC60S-DI nanoparticle in targeting CD44+ positive cells. The images also demonstrate the NIR laser irradiation controlled DOX release inside cells.

Fig. 5.

Confocal images showing the binding affinity of the HC60S-DI nanoparticles with 3D cultured MDA-MB-231 cells and MCF-7 mammospheres enriched with CSCs at 4 °C and the cellular uptake of the HC60S-DI nanoparticles at 37 °C with or without NIR laser irradiation (1.5 W/cm² for 3 min). (A) MDA-MB-231 and MCF-7 mammospheres were incubated with free DOX&ICG, C60S-DI, and HC60S-DI nanoparticles at 4 °C for 3 h. The HC60S-DI nanoparticles could bind on both the MDA-MB-231 and MCF-7 mammospheres that are CD44 positive. (B) MDA-MB-231 and MCF-7 mammospheres were incubated with free DOX&ICG, C60S-DI nanoparticles, and HC60S-DI nanoparticles at 37 °C for 3 h with or without laser irradiation. The NIR laser irradiation controlled drug release can be achieved in both the mammospheres.

Fig. 6.

Viability of 2D cultured MDA-MB-231 and MCF-7 cells and their mammosphere cells enriched with CSCs after various treatments. (A) Viability of 2D cultured MDA-MB-231 and MCF-7 cells after various treatments. The HC60S-DI nanoparticles (DOX:ICG:HC60S nanoparticles = 1:1:1) with high drug loading content and NIR laser irradiation (1.5 W/cm² for 3 min) are the most effective in destroying the cancer cells. (B) Viability of the 3D cultured MDAMB- 231 and MCF-7 mammosphere cells after various treatments. The results also show that the high drug loading content HC60S-DI nanoparticles with laser irradiation are the best in terms of killing the CSCs. **p* < 0.05 (n=3), compared to the other seven groups. The 2D cultured MDAMB- 231 and MCF-7 cells were seeded and incubated with the various drug formulations at different concentrations in 96-well plates for 12 h, irradiated with NIR laser if needed, and further cultured for 12 h. The cell viability was then evaluated using the CCK-8 according to the manufacturer's instructions. Mammospheres were cultured and incubated with various drug formulations at different concentrations in 24-well ultralow attachment plates for 12 h, irradiated with NIR laser if needed, and further cultured for 60 h. The condition with pure medium was conducted in parallel for each DOX concentration to serve as control. The total incubation time for the 2D cultured cells is much shorter (24 h versus 72 h) than 3D cultured mammosphere cells, in order to demonstrate the effect of the high drug loading content with NIR laser irradiation in enhancing the anticancer capacity *in vitro*. Nonetheless, the viability of the 3D cultured mammosphere cells is still higher than that of the 2D cultured cells at low dose of free DOX (e.g., 0.1 µg/ml), indicating the highly drug resistant nature of the 3D cultured mammosphere cells.

Fig. 7.

In vivo tumor targeting and antitumor capacity of HC60S-DI nanoparticles with NIR laser irradiation. (A) *In vivo* whole animal imaging of ICG fluorescence at 6 h after intravenous injection via the tail vein of free ICG and HC60S-DI nanoparticles with low and high drug loading content (DOX:ICG:HC60S nanoparticles = 1:1:20 and 1:1:1). The arrows indicate the locations of tumor in mice. (B) Tumor growth curves for the different treatments (five mice per group) and images of the tumors collected after sacrificing the mice on day 29. $*p < 0.05$ ($n=5$). When tumors were established and reached ~5 mm in long diameter, mice were treated with the various drug formulations with or without NIR laser irradiation. The tumor volume (V) was calculated as: $V = (L \times W^2)/2$, where L is long diameter and W is short diameter of tumor determined using a caliper. (C) Representative images of tumor and histology (H&E) images of the tumors collected on day 29. (D) Staining of early and late apoptosis of cells in the tumors collected on day 29 with Annexin V and PI, respectively. The NIR laser irradiation was at 0.7 W/cm^2 for a total of 3 min at 12 h after the intravenous drug administration.

Fig. 8.

The HC60S-DI nanoparticles with NIR laser irradiation (0.7 W/cm^2 for 3 min at 12 h after the intravenous drug administration) are capable of effectively destroying CSCs *in vivo*. (A) Typical two-channel flow cytometry data showing the distinct side population of cells that exclude Hoechst 33342 stain of nuclei in tumors obtained after sacrificing the mice with the different treatments on day 29. The side population was analyzed with a BD LSR-II flow cytometer using a dual-wavelength analysis (blue at 450 nm and red at 660 nm) after excitation with the 405 nm UV light. (B) The corresponding quantitative percentage data of the side population from the flow cytometry analyses. $*p < 0.05$ ($n=3$). (C) Immunohistochemical staining of the variant CD44 and CD133 in tumors from mice with the different treatments showing diminished expression of both CD44 and CD133 after the treatment with the high drug loading content HC60S-DI nanoparticles and NIR laser irradiation.